

Metabolic Signatures of Sepsis: Clinical Phenotypes and Mapping Patient's Journey in the ICU

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Statement of Originality

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Abstract

Sepsis is a complex syndrome which originates from a dysregulated host response to an infection causing organ dysfunction. It affects 48.9 million people annually, with 11 million deaths on yearly basis. Sepsis associated mortality in the intensive care units (ICUs) is still one of the major health concerns. The heterogeneity of this life-threatening condition makes it challenging to understand and manage. Finding better ways to monitor patients with sepsis over the course of their ICU will allow better prognostication, assessment of treatment response may aid understanding of sepsis pathology.

In this study, metabolic phenotyping, a technique with a great potential for identifying changes in metabolism was employed. Serum samples collected from patients with septic shock enrolled into two clinical trials were profiled using analytical platforms, nuclear magnetic resonance spectroscopy (NMR) and ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS). Both untargeted data and targeted panels of small molecules, lipids and lipoproteins were obtained using NMR and UHPLC-MS to investigate how the metabolic profiles of patients with septic shock differ based on outcome, organ failure and treatment at enrolment and over the course of the ICU stay. Various bioinformatic techniques including univariate, multivariate, and linear mixed effect modelling were used.

Overall, the study revealed that there are baseline differences in the small molecule and lipid profiles of survivors and non-survivors. However, the metabolic trajectories for survivors and non-survivors were similar as the metabolic concentration changes over time occur in the same direction in the two clinical groups. Patients with more severe organ failure had the highest levels of metabolites whilst the treatment analysis revealed no significant metabolite changes between treatment arms in the clinical trial population. In summary, this study further contributes to the sepsis field by providing an understanding of the dynamic nature of metabolism in patients with septic shock.

Presentations

Poster Presentations

Hussain H, Gordon AC, Jiménez B, Antcliffe DB. Metabolic phenotyping for exploring metabolic phenotypes related to patient outcomes in Sepsis. Metabolomics Society 16th Annual Conference. October 2020.

Publications related to PhD

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Other Publications

Albreht A, **Hussain H**, Jiménez B, Yuen AHY, Whiley L, Witt M, et al. Structure Elucidation and Mitigation of Endogenous Interferences in LC-MS-Based Metabolic Profiling of Urine. *Analytical Chemistry*. 2022;94(3):1760-8.

Whiley L, Chekmeneva E, Berry DJ, Jiménez B, Yuen AHY, Salam A, **Hussain H**, et al. Systematic Isolation and Structure Elucidation of Urinary Metabolites Optimized for the Analytical-Scale Molecular Profiling Laboratory. *Analytical Chemistry*. 2019;91(14):8873-82.

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A special thanks to my parents and my whole family for understanding and motivating me throughout the course of my study.

Covid-19 Impact Statement

The original research plan of my PhD was impacted by COVID-19 pandemic. The metabolic profiling of serum samples from LeoPARDS trial, which is the validation cohort for my study was supposed to be performed by the NPC in April 2020. However, due to COVID-19 pandemic LeoPARDS sample analysis was delayed and postponed from April 2020 to April 2021, as the lab was shut for a while and another COVID project was also on the priority list to be acquired before LeoPARDS. All the profiling data for LeoPARDS became fully available to me by the end of January 2022 and I had only couple of months to finish all the statistical analysis before 31st March 2022, as that's when my PhD funding was ending. Therefore, I had to apply for a 6-month extension for my PhD so that I can carry out my research work and finish all the analysis which needed to be done to achieve my research goals.

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List of Abbreviations

14,15- DiHETE - 14,15-Dihydroxyeicosatrienoic Acid

1D - One Dimensional

¹H NMR - Proton Nuclear Magnetic Resonance Spectroscopy

1-MH - 1-Methylhistidine

2D - Two-Dimensional

2-Py - N1-Methyl-2-Pyridone-5-Carboxamide

4-Py - N1-Methyl-4-Pyridone-3-Carboxamide

AB - Apo-B100

ACAT - Acyl-Coenzyme A : Cholesterol Acyltransferase

Acetyl-CoA - Acetyl-coenzyme A

ACTH - Adrenocorticotrophic Hormone

APACHE II - Acute Physiology and Chronic Health Evaluation II

APC - Activated form of Protein C

Apo-A1 - Apolipoprotein-A1

Apo-A2 - Apolipoprotein-A2

Apo-B100 - Apolipoprotein-B100

ATGL - Adipose Triglyceride Lipase

ATP - Adenosine Triphosphate

AUC - Area Under Curve

BCAAs - Branched Chain Amino Acids

B-H - Benjamini-Hochberg

BI LISA - Bruker In Vitro Diagnostics Research Lipoprotein Subclass Analysis

BMI - Body Mass Index

C-20 - Carbon-20

CAR - Carnitine

Cers - Ceramides

CEs - Cholesteryl Esters

CETP - Cholesteryl Ester Transfer Protein

CH - Cholesterol

CKD - Chronic Kidney Disease

COPD - Chronic Obstructive Pulmonary Disease

CPMG - Carr-Purcell-Meiboom-Gill

CRH - Corticotropin Releasing Hormone
CRP - C-Reactive Protein
CVD - Cardiovascular Disease
D₂O - Deuterium Dioxide
DAMPs - Damage Associated Molecular Patterns
DG - Diacylglycerol
DHA - Docosahexanoic Acid
DIC - Disseminated Intravascular Coagulation
DNA - Deoxyribonucleic Acid
DPA - Docosapentaenoic Acid
EMMs - Estimated Marginal Means
EPA - Eicosapentaenoic Acid
EPCR - Endothelial Pattern Protein C Receptor
EPIC II - Extensive Prevalence of Infection in Intensive Care II
ETA - Eicosatrienoic Acid
FAs - Fatty Acids
FC - Free Cholesterol
FDR - False Discovery Rate
FIDs - Free Induction Decays
FiO₂ - Percentage of Inspired Oxygen
FPR - False Positive Rate
GC - Gas Chromatography
GCS - Glasgow Comma Scale
GLMs - Generalised Linear Mixed Effect Models
HDL - High Density Lipoprotein
HILIC - Hydrophilic Interaction Liquid Chromatography
HILPDA - Hypoxia-Inducible Lipid Droplet-Associated Protein
HMDB - Human Metabolome Database
HPA - Hypothalamic-Pituitary Adrenal
HPOS - HILIC Positive
ICU - Intensive Care Unit
IDL - Intermediate Density Lipoprotein
IL-1 - Interleukin-1

IL-1 β - Interleukin 1 β

IL-6 - Interleukin-6

IV - Intravenous

IVDr - In Vitro Diagnostics Research

J-res - *J*-resolved

LacCer - Lactosylceramide

LCAT - Lecithin Cholesterol Acyltransferase

LDL - Low Density Lipoprotein

LeoPARDS - Levosimendan for the Prevention of Acute Organ Dysfunction in Sepsis

LMMs - Linear Mixed-Effects Models

LNEG - Lipid Negative

LOS - Length of Stay

LPCs - Lysophosphatidylcholines

LPEs - Lysophosphatidylethanolamines

LPG - Lysophosphatidylglycerol

LPI - Lysophosphatidylinositol

LPIs - Lysophosphatidylinositols

LPL - Lipoprotein Lipase

LPOS - Lipid Positive

lps - Lipopolysaccharide

LPS - Lysophosphatidylserine

LTR - Long Term Reference

m/z - Mass to Charge ratio

MAP - Mean Arterial Pressure

MGs - Monoacylglycerols

MNA - 1-Methylnicotinamide

MS - Mass Spectrometry

MUFAs - Monounsaturated Fatty Acids

Na₂HPO₄ - Disodium Hydrogen Phosphate

NAD⁺ - Oxidised Nicotinamide Adenine Dinucleotide

NaN₃ - Sodium Azide

Neutrophil Extracellular Traps

NF- κ B - Nuclear Factor-Kappa B

NMR - Nuclear Magnetic Resonance

NO - Nitric Oxide

NOD - Nucleotide-binding Oligomerisation Domain

NOESY - Nuclear Overhauser Effect Spectroscopy

NPC - National Phenome Centre

PA - Phosphatidic Acid

PAI-1 - Plasminogen Activator Inhibitor Type 1

PAMPs - Pathogen Associated Molecular Patterns

PaO₂ - Partial Pressure of Arterial Oxygen

PAR1 - Protease Activated Receptor 1

PARPs - Poly-ADP-Ribose Polymerase

PCA - Principle Component Analysis

PCs - Phosphatidylcholines

PEs - Phosphatidylethanolamines

PGE2 - Prostaglandin E2

PGs - Phosphatidylglycerols

PIs - Phosphatidylinositols

PL - Phospholipid

PLS - Partial Least Squares Projection To Latent Structures

PLS-DA - Partial Least Squares Projection To Latent Structures - Discriminant Analysis

PN - Particle Number

ppR - peakPanther

PRRs - Pattern Recognition Receptors

PUFAs - Polyunsaturated Fatty Acids

Q-Q plot - Quantile-Quantile plot

qSOFA - Quick Sequential Organ Failure Score

QC - Quality Control

REML - Restricted Maximum Likelihood

RNA - Ribonucleic Acid

RIG-I - Retinoic Acid Inducible Gene I

ROC - Receiver Operating Characteristic

ROS - Reactive Oxygen Species

RPC - Reversed Phase Chromatography

SAAs - Sulphur Containing Amino Acids
 SDMA/ADMA - Symmetric/Asymmetric Dimethyl Arginine
 SFAs - Saturated Fatty Acids
 SIRS - Systemic Inflammatory Response Syndrome
 SMoESY - Small Molecule Enhancement Spectroscopy
 SMs - Sphingomyelins
 SOFA - Sequential Organ Failure Assessment
 SPMs - Specialized Pro-Resolving Mediators
 SR - Study Reference
 STOCSY - Statistical Total Correlation Spectroscopy
 SulfoHexCer - Sulfohexosylceramide
 SVR - Systemic Vascular Resistance
 TCA - Tricarboxylic Acid
 TFP-1 - Tissue Factor Pathway Inhibitor
 TGs - Triglycerides
 TLR4 - Toll-Like Receptor 4
 TLRs - Toll-like Receptors
 TMAO - Trimethylamine N-oxide
 TNF- α - Tumour Necrosis Factor Alpha
 TOF - Time-of-flight
 TP0 - Timepoint 0
 TP1 - Timepoint 1
 TP2 - Timepoint 2
 TP3 - Timepoint 3
 TPR - True Positive Rate
 tRNA - transfer Ribonucleic acid
 tRNA - transfer Ribonucleic Acid
 TSP - 3-(Trimethyl-silyl) Propanoic Acid
 UHPLC - Ultra-High Performance Liquid Chromatography
 UK - United Kingdom
 VANISH - Vasopressor vs Norepinephrine as Initial Therapy in Septic Shock
 VLDL - Very Low Density Lipoprotein
 χ^2 - Chi Squared

Chapter 1 – Introduction

1.1 Sepsis

1.1.1 Definition and limitations

Sepsis is a heterogenous clinical syndrome (1) and the current definition of sepsis is Sepsis-3, which defines sepsis as a dysregulated immune response of a host's body to an infection causing life threatening organ dysfunction (2). This is a development from the previous Sepsis-2 definition (figure 1.1). Sepsis is regarded as a medical emergency which could lead to death if not given an appropriate and effective treatment on time (3). Septic shock, the most severe form of sepsis is characterised by persistent hypotension in response to overwhelming infection (4), which needs vasopressors for attaining a mean arterial pressure (MAP) of 65mmHg along with a high blood lactate level (i.e., >2mmol/L) after receiving an adequate fluid resuscitation (2, 5). Our ability to understand this complex pathophysiological condition has improved along with the way it's defined over the last few decades. However, due to the heterogeneity of this health condition, defining sepsis is not straightforward and the physiological and clinical parameters for diagnosing sepsis using the old definition (i.e., Sepsis-2) and the current definition (i.e., Sepsis-3) are shown in figure 1.1 (2, 6).

Sepsis-3 is an improvement from Sepsis-1 and Sepsis-2, as it accounts for the suspected or documented infections which can occur with or without systemic inflammatory response syndrome (SIRS) positivity (7-10). Sepsis-3 aims to use objective parameters to help with patient assessment. However, the current definition had some major limitations (7-9) which are as follows: 1) It only focusses on adult population not the paediatrics; 2) The data is mostly accumulated from the hospitals in the United States of America, but not from developing or non-developed countries who do not commonly use lactate parameter which is a part of new definition of septic shock; 3) It relies more on the quick sequential organ failure assessment (qSOFA) score to determine organ dysfunction but organ dysfunction may occur due to reasons unrelated to sepsis; 4) It includes only two clinical stages (sepsis and septic shock) and excludes SIRS from the Sepsis-3 definition which could be used to predict infections, thus making it difficult to compare current sepsis research with the prior that used different definitions (7-10). At present, there is still no ideal method for distinguishing patients with sepsis and those without even after the Sepsis-3 definition (10).

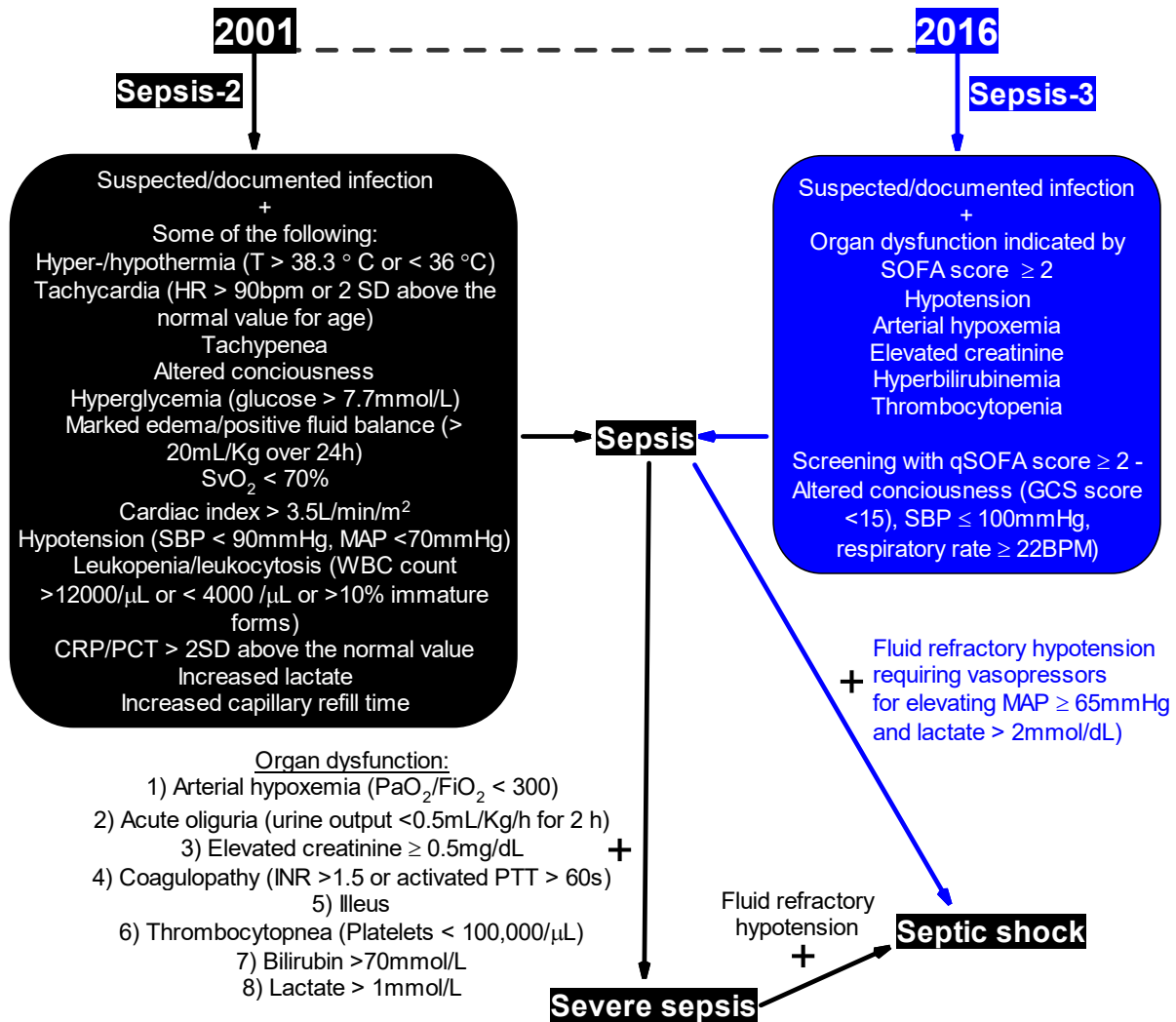


Figure 1.1: Evolution in the definitions of sepsis and its diagnostic criteria from 2001 (black path) to 2016 (blue path) (2, 6). Abbreviations: T = Temperature; SBP = Systolic blood pressure; MAP = Mean arterial pressure; WBC = White blood cell; bpm = beats per minute; HR = Heart rate; BPM = Breaths per minute; INR = International normalised ratio; FiO_2 = Fraction of inspired oxygen; PaO_2 = partial pressure of oxygen; PTT = Partial thromboplastin time; PCT = Procalcitonin; CRP = C-reactive protein; SD = Standard deviation; SIRS = Systemic inflammatory response syndrome SOFA = Sequential organ failure assessment; qSOFA = quick sequential organ failure assessment; SvO_2 = Mixed venous oxygen saturation; GCS = Glasgow Comma Scale.

1.1.2 Epidemiology and Costs

Sepsis is a global health concern with no single cause and the study of its epidemiology has been quite challenging over the years due to the evolution in the consensus definitions of sepsis as discussed previously (11). In 2017, sepsis affected 48.9 million people which is 18.8% lower than the sepsis incidence in 1990 (12). Sepsis is the most prevalent cause of deaths in hospital (13) and intensive care unit (ICU) (14). The mortality rates related to sepsis and septic shock are relatively high and vary between 22% and 76% across various geographical regions, continents, and countries (15). This broad variation in the mortality

rates might be due to a contribution from several factors including age, comorbidities, access to health care and regional health patterns and several other unidentified genomic impacts (15). Globally, 11 million deaths occur due to sepsis each year (12) and in the United Kingdom (UK), 46 000 people die annually because of sepsis (16).

Over years, sepsis related mortality has gradually fallen (17, 18), probably due to earlier detection and intervention (1). However, it is still a huge economic burden on healthcare systems. In 2013, more than \$24 billion was attributed to sepsis related health care expenditures in the United States of America which contributed to 13% of the total hospital costs, but only represented 3.6% of hospital stays (19). In the UK, the estimated cost of £7.76 billion is attributed to sepsis each year which also includes £830 million from direct costs (i.e., hospital costs and costs from complications)(20).

1.1.3 Risk factors

It is crucial to recognize the factors which play an important role in sepsis development and progression to improve people's health (21). Several risk factors contribute towards the development of sepsis and most of these emphasise on patient's susceptibility to infection as seen in figure 1.2. Infants and elderly patients, genetically immunocompromised people or those with acquired immunosuppression such as AIDS, cancer, diabetes or medications (including immunosuppressants, steroids, or chemotherapeutics) are all predisposed to infection increasing the risk of sepsis (22, 23). Moreover, admission to ICU or prolonged hospitalisations, comorbidities and host genetics also increases the risk of sepsis (22, 24, 25).

The effect of host genetic factors such as single nucleotide polymorphisms on the development of sepsis and infections have also been investigated in numerous studies (26-29). Polymorphisms related to toll-like receptors (TLRs) and tumour necrosis factor alpha (TNF- α) are mostly studied and several genetic studies have found polymorphisms that confer increased risk of sepsis or death from sepsis, but the findings have not yet been consistently reproduced (30).

Low socio-economic status is also a risk for infection which could contribute towards sepsis and several factors including lifestyle aspects (such as exercise, diet, smoking, compliance

with vaccination), exposure to chronic stress, not seeking health care, and household crowding contribute towards the socio-economic risk of sepsis.

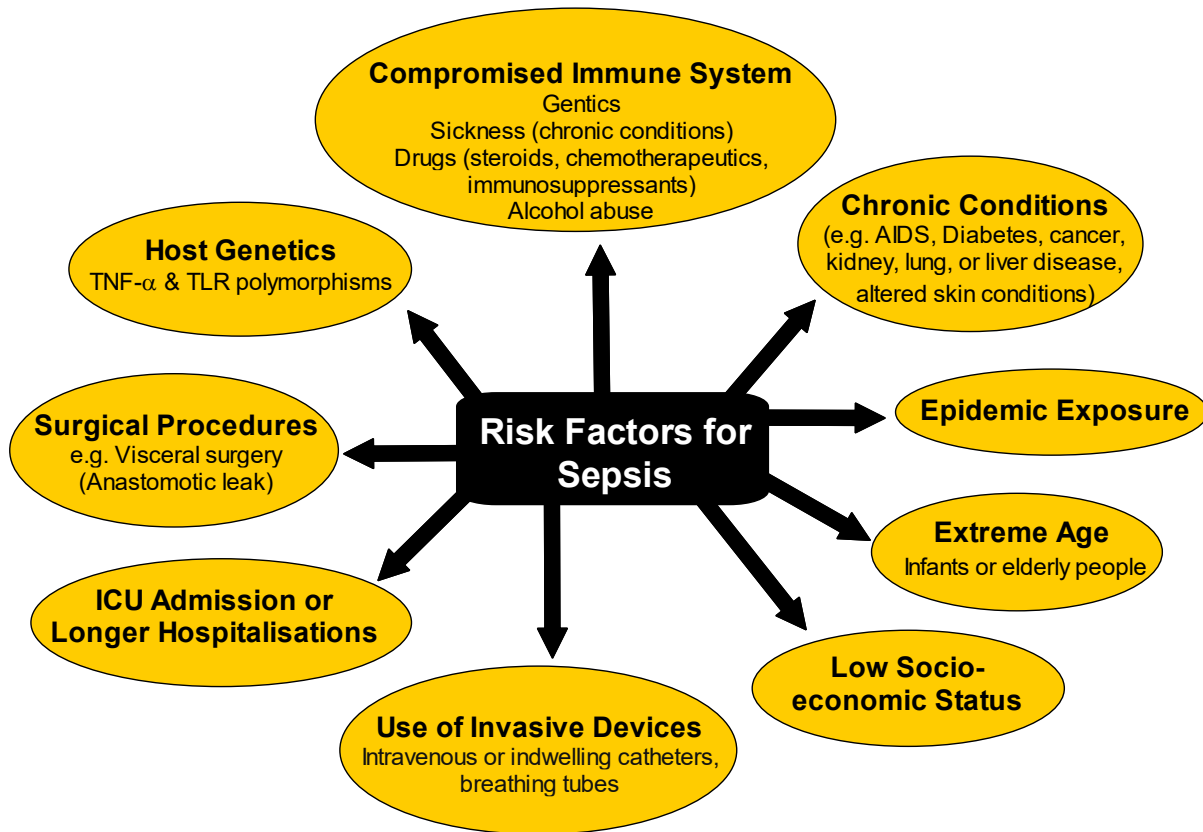


Figure 1.2: Risk factors responsible for the development of sepsis.

1.1.4 Aetiology

The aetiological agents causing sepsis differ based on the site and the source of infection (31). Sepsis results from either community or healthcare acquired infection (22). Almost 53-66% of sepsis cases treated in hospitals are community-acquired but recently, an elevation in the cases of hospital-acquired sepsis has been observed (31). The focal sites of infection vary in sepsis and the Extended Prevalence of Infection in Intensive Care II (EPIC II) investigators have reported respiratory system infections to be the most common site of infection contributing almost 64% of all sepsis cases in ICU patients, followed by intraabdominal (20%), bloodstream (15%) and genitourinary system infections (14%) (32). Site of infection is associated with the risk of death with sepsis from urinary tract infections having lower mortality than that from lung or abdominal infection (33-35).

In hospital-acquired sepsis, almost 80% of cases originate from Gram-negative bacteria whilst 56.2% of community-acquired sepsis caused by Gram-positive bacteria (36). Aetiological agents including *Staphylococcus aureus* (a Gram-positive bacteria), *Escherichia coli* (a Gram-negative bacteria) and *Pseudomonas* species are most frequently found in patients with sepsis (32, 37). A few studies have investigated sepsis mortality related to the causative microorganisms and revealed that patients infected with Gram-positive bacteria had lower mortality than those with Gram-negative bacteria (38, 39). Pathogens including *Staphylococcus aureus* and *Pseudomonas* showed higher association with sepsis mortality than *Escherichia coli* and enterococci (40). The EPIC II study also reported that infections caused by *Enterococcus* spp. , *Pseudomonas* spp. and *Acinetobacter* spp. are independent risk factors for sepsis related mortality in hospitals with an odds ratio of 1.56, 1.38, and 1.53 for death respectively (31, 32).

1.1.5 Pathology

Sepsis is a complicated clinical syndrome with a multifaceted pathology (41). After years of research there have been significant advancements in the understanding of the pathological mechanisms leading to organ dysfunction at a cellular, molecular and organ level (42, 43). Various studies have provided an insight into the heterogeneous manifestations of sepsis which mainly originate from the complex association between the pathogen and host. (44).

The normal response of a host's body to an infection causes activation of the innate immune system which is comprised of macrophages, monocytes, granulocytes, natural killer cells, and dendritic cells (42). In sepsis, the immune system becomes overactive and activates both cellular defence and plasma protein mechanisms. Immune cells recognise and bind to components of bacteria, fungi, or viruses known as pathogen-associated molecular patterns (PAMPs) (which include endotoxins and β -glucan), via pattern recognition receptors (PRRs) (42, 45). The binding of microbial components to PRRs (such as TLRs and C-type lectin receptors situated on the cell surface or nucleotide-binding oligomerization domain (NOD)-like receptors and retinoic acid inducible gene I (RIG-I)-like receptors in the cytosol) of the immune cells cause activation of intracellular signal transduction pathways (42, 44). Signalling pathways are triggered by activating cytosolic nuclear factor-kappa B (NF- κ B) and the activated form of NF- κ B binds to the transcription binding sites in the nucleus

and initiates the transcription of a large group of genes involved in the inflammatory response which stimulate the release of inflammatory mediators (such as cytokines, chemokines and type I interferons as shown in figure 1.3) and these mediators activate the cellular defences of a host (31, 42, 46).

Inflammatory	Anti-inflammatory
TNF- α IL-1 β , IL-2, IL-6, IL-8, IL-15 IFN- γ Prostacyclin Thromboxane Prostaglandin Phospholipase A ₂ Platelet activating factor Vasoactive neuropeptides Plasminogen activator inhibitor -1 Neutrophil elastase	Soluble TNF- α receptors IL-1 ra, IL-4, IL-10, IL13 Type II IL-1 receptor Transforming growth factor- β Leukotriene B4 receptor antagonist Lipopolysaccharide binding protein Epinephrine

Figure 1.3: Inflammatory and anti-inflammatory mediators. Abbreviations: TNF= Tumour necrosis factor; IL = Interleukins, IFN = Interferon.

Endogenous molecules such as damage-associated molecular patterns (DAMPs) are released from damaged host cells during inflammatory response and are also recognised by PRRs causing activation of innate immune system (42). DAMPs may be nuclear, cytoplasmic, or mitochondrial (47) and include adenosine triphosphate (ATP), mitochondrial deoxyribonucleic acid (DNA), and high mobility group box 1 (HMGB1) (42).

A host's response to an infection causes the release of proinflammatory mediators however, at the same time the synthesis of acute phase proteins such as C-reactive protein (CRP) is also enhanced, which causes the activation of humoral defence mechanisms including complement, a part of innate immune system (48, 49). The complement system is also a protection mechanism against infection and has several biological roles including tissue repair and regeneration (48). However, inappropriate or uncontrolled activation of complement system can be harmful (48) and can further add to the inflammatory response. The complement system consists of 50 soluble and surface bound proteins and is activated by three main pathways (classical, lectin and alternative) (48). These, all converge onto complement protein C3, which then leads to a common cascade (C5-9) causing the membrane-attack-complex (C5b, C6, C7, C8 and C9 (50)) to be deposited on specific targets releasing proinflammatory peptides, C3a and C5a (51). The chemoattractant C3a binds to its

receptor C3aR, causing enhanced vascular permeability and smooth muscle contraction), whilst C5a which is an activator of polymorphonuclear leucocytes and macrophages binds to its receptor C5aR1 causing the release of proinflammatory cytokines and chemokines, chemotaxis, synthesis of harmful oxygen free radicals, and release of mediators which are both pro- and anti-inflammatory (52).

Overall, all these mediators play a crucial role in the pathology of sepsis and several complex pathways lead to multiorgan failure (31, 53). Evan et al. grouped these pathways into four main features: endothelial dysfunction; coagulopathy; cellular dysfunction and cardiovascular dysfunction. These features are highlighted in figure 1.4 (53).

1) Endothelial Dysfunction

Damage to vascular endothelial cells has been reported to be one of the major contributors to mortality in septic patients by inducing a cascade of events which lead to multiple organ failure. (54). Firstly, the release of endotoxins (e.g. lipopolysaccharide (LPS)) by bacteria causes activation of the immune effector cells by attaching to TLRs and then causing the release of inflammatory chemokines and cytokines (55) including TNF- α (42) and interleukin-1 β (IL-1 β) (54). These signalling molecules cause cell damage by activating endothelial cells resulting in increased leucocyte adhesion and enhanced transmigration into tissues thereby impairing the function of the endothelial cells (53, 54). The complement protein C5a stimulates the release of the endothelial surface proteoglycan heparan sulphate which disrupts the normal function of the glycocalx, (a gel-like layer on the vascular endothelial surface which contributes to antithrombogenicity and anti-inflammation) thus increasing vascular permeability and impairing the function of antithrombin and tissue factor pathway inhibitor -1 (TFP-1) (50). As endothelial permeability is increased it leads to organ dysfunction such as pulmonary oedema in the lung and bacterial translocation in the gut, further worsening cell injury and the inflammatory process (53).

Other factors also contribute to endothelial dysfunction including increased capillary leakage, alteration in the vascular tone and a shift towards a procoagulant state resulting from the altered haemostatic balance (figure 1.4). All these factors eventually result in irreversible organ failure and cause septic death (54).

2) Cellular Dysfunction

The pathological pathway leading to cellular dysfunction is still not well understood, but there is some evidence that cell death occurs in sepsis. Some studies have shown that during apoptosis, cytokeratin 18 (CK18), type I protein of the intermediate filament widely present in epithelial and parenchymal cells gets cleaved at different sites into CK18 proteolytic fragments (M30 and M65) by caspases which then diffuse into the blood stream (56, 57). CK18 proteolytic fragments include neoantigen M30, which is a caspase cleaved neoepitope of CK18 specific to apoptosis whilst CK18 M65 represents both the total intact and caspase cleaved forms of CK18 resulting from both apoptosis and non-apoptotic cell death (58). Septic patients have been reported to have increased levels of neoantigen M30 as a result of an early caspase cleavage of CK18 at Asp396 which reflects increased apoptosis of epithelium cells (57) in these patients. In sepsis, there is also a general reduction in energy consumption by cells suggests that they may enter a hibernation like state. This leads to altered cellular function with several metabolic changes including increased catabolism, insulin resistance and hyperglycaemia. (53)

3) Cardiovascular Dysfunction

Numerous studies have shown that septic patients develop the so-called 'hyperdynamic' state of sepsis. In this state, septic patients have reduced systemic vascular resistance (SVR) with a normal or increased cardiac output. Normally, in order to main the cardiac output the left ventricles dilate, with reduced ejection fraction and a reduced left ventricular stroke work index which can lead to the hypotension as considered in septic shock (53). Variations in SVR are most likely to be driven by increased formation of nitric oxide (NO) in the vasculature (59). The vasodilation caused by NO can be problematic to correct with the use of vasopressors. Additionally, poor tissue perfusion could be the most likely cause of the increased lactate seen in septic shock however, other mechanisms such as high circulating catecholamines can also be a cause (53).

4) Coagulopathy

In sepsis, altered coagulation is common (53) and the homeostatic balance between procoagulant and anticoagulant pathways is disturbed. Coagulation is initiated by bacterial endotoxin, lipopolysaccharide (Lps), and other microbial components and the complement system (C5a and the membrane attack complex) (50) which stimulate tissue factor

expression on mononuclear and endothelial cells (43). The activation of proteolytic cascades by tissue factor in the procoagulant pathway (extrinsic pathway) results in the formation of thrombin from prothrombin and fibrin from fibrinogen (43) (figure 1.4).

At the same time, the normal regulatory fibrinolytic mechanisms which are involved in the breakdown of fibrin by plasmin also become impaired due to the presence of increased plasminogen-activator inhibitor type-1 (PAI-1) in the plasma, thus inhibiting the formation of plasmin from its precursor, plasminogen (43). Overall, increased production and decreased clearance of fibrin results in a prothrombotic state which leads to the accumulation of fibrin clots in small blood vessels blocking the microvasculature and giving rise to disseminated intravascular coagulation (DIC) (figure 1.4) (43, 53). DIC also causes bleeding and hinders blood flow in limbs and organs (60) leading to reduced tissue perfusion, organ hypoxia and multiple organ dysfunction (43, 53). DIC is a strong predictor of sepsis related mortality (60).

Endothelial damage also plays an important role in coagulopathy by removing the protective role of the anticoagulant protein C pathway and making the endothelium a prothrombotic surface (53). Under normal conditions, the activated form of protein C (APC) results from protein C forming a complex with thrombin and an endothelial transmembrane glycoprotein, thrombomodulin along with the endothelial protein C receptor (EPCR) (43). The dissociation of APC from EPCR inactivates factors Va and VIIIa and also results in inhibition of PAI-1 activity, thereby reducing protein C levels which causes blockade of the coagulation cascade (43). However, due to endothelial damage this activation of protein C does not take place resulting in APC deficiency (43). The use of EPCR as a cofactor for APC to dissociate protease-activated receptor 1 (PAR1) was also reported (61). Gene profiling revealed that PAR1 signalling can also cause activation of protective genes induced by APC, such as immunomodulatory monocyte chemoattractant protein-1, indicating that PAR1 activation might play a protective role in sepsis (61).

Figure 1.4: Pathophysiological changes in sepsis leading to multiorgan failure via (43, 50, 53). Note: Increased = ↑; Reduced/decreased = ↓, DIC = Disseminated intravascular coagulation; TFP1 = Tissue factor pathway inhibitor-1; PAI-1 = Plasminogen activator inhibitor-1; APC = Activated protein C; FDP = Fibrin degradation products; PRRs = Pattern recognition receptors; red circle with a negative sign indicates inactivation and inhibition by APC.

1.1.6 Prognosis

Scoring systems have been developed to identify the severity of sepsis. Different clinical scoring systems such as Sequential Organ Failure Assessment (SOFA), and Acute Physiology and Chronic Health Evaluation II (APACHE II) can be used to determine the disease severity and prognosis of septic patients (62). SOFA and APACHE II scoring systems use a different combination of parameters to stratify patients (62). The Apache II score is normally used within 24 h of admission of a patient to measure disease severity and it employs a point score based on 12 physiological parameters, age and the previous health condition (63). The SOFA score is primarily designed to evaluate morbidity and is comprised of scores from the following six organ systems: 1) cardiovascular; 2) coagulation; 3) renal; 4) respiratory; 5) liver; 6) central nervous system (64). The criteria for each component SOFA score are listed in table 1.1 (2, 44, 64). Each component of the SOFA score ranges from 0 to 4 based on the extent of organ dysfunction/failure, the higher the score the worse the organ dysfunction/failure (64, 65). SOFA score can be easily calculated to provide information about the status of patients as well as the degree of organ dysfunction/failure in the critically ill patients in the ICUs (64, 65) on daily basis whilst other scoring systems (such as APACHE II) do not individualise the extent of organ dysfunction/failure and are normally taken early after admission (64). The performance of both APACHE II and SOFA scoring systems has never proven enough to predict early mortality or clinical evolution (62), therefore these scoring systems have never been used in direct patient care. Thus newer, better and more reliable methods are urgently needed to support early prognosis of sepsis and at present metabolomics is emerging to be a promising and yet powerful tool for the early prognosis of patients with sepsis (66).

Table 1.1: SOFA score with its six independent component scores (2, 44, 64).

SOFA Score component	Score				
	0	1	2	3	4
1-Cardiovascular Hypotension	MAP $\geq$ 70 mmHg (No hypotension)	MAP < 70 mmHg	Dopamine $\leq$ 5 or dobutamine (any dose)*	Dopamine > 5 or epinephrine $\leq$ 0.1 or norepinephrine $\leq$ 0.1*	Dopamine > 15 or epinephrine > 0.1 or norepinephrine > 0.1*
2-Coagulation Platelets X 10 ³ /mm ³	$\geq$ 150	< 150	< 100	<50	<20
3 - Renal Creatinine, μ mol/L (mg/dL)	110 (<1.2)	110 - 170 (1.2 - 1.9)	171 - 299 (2.0 - 3.4)	300 – 440 (3.5 - 4.9)	> 440 (>5.0)
urine output, mL/day				< 500	<200
4-Respiratory PaO ₂ /FiO ₂ , mmHg	$\geq$ 400	<400	<300	<200 (while on respiratory support)	<100 (while on respiratory support)
5-Liver Bilirubin, μ mol/L (mg/dL)	20 (<1.2)	20 – 32 (1.2 - 1.9)	33 – 101 (2.0 - 5.9)	102 – 204 (6.0 - 11.9)	> 204 (>12.0)
6-Central Nervous system GCS score	15	13 - 14	10 - 12	6 - 9	<6

Each of the six organ systems has a score which ranges from 0 to 4, higher the score worse the organ dysfunction. GCS score ranges from 3 to 15; higher the GCS score the better the neurological function. Abbreviations: GCS = Glasgow Comma Scale; FiO₂ = Fraction of inspired oxygen; PaO₂ = Partial pressure of oxygen; MAP = Mean arterial pressure

Asterisk (*) refers to the dose of adrenergic agents (catecholamines) administered as μ g kg⁻¹ min⁻¹ for a minimum of 1 h.

1.1.7 Disease management and treatment

Though there are various standard strategies which are currently used for the treatment of sepsis and septic shock including infection control via use of effective antibiotics (3), fluid resuscitation (53), vasopressors (5), the use of corticosteroids (67), glycaemic control (53) and support for failing organs (66) such as cardiovascular support (5), no new or specific therapies for sepsis have been developed (68).

1) Antibiotic therapy

It should be our priority to recognize the origin of infection as it encourages an early administration of antibiotics or the focus on the surgical control (69). The delay in the administration of antibiotics for every hour was found to be related with decreased survival of 7.6% (70). The current guidelines recommend treating patients with sepsis with antibiotics within the first 3 h of admission to the emergency unit or within an hour of ICU admission (71, 72). The use of inappropriate antibiotics is linked with increased mortality therefore the selection of appropriate antibiotics is crucial to improve mortality (71, 72). Broad spectrum antibiotics are used as the first line in sepsis patients and then the treatment is reconsidered after the results of microbiological cultures and adjusted to target against the isolated microorganism. However, using broad spectrum antibiotics could result in collateral damage such as increased growth of potentially pathogenic bowel flora (73) increasing antibiotic resistance and adverse drug reactions (5, 74).

2) Fluid resuscitation

The role of fluid resuscitation is well recognised in the initial treatment of sepsis, and some evidence exists that the fluid type and its cumulative dosage given over the course of the ICU admission might have an impact on the patient related outcomes (5). Generally, intravenous saline is provided to all sepsis patients as a part of the Surviving Sepsis Guidelines (75).

3) Vasopressors and support for failing organs

Patients who have persistent hypotension despite fluid resuscitation need to be admitted to a critical care facility and are treated with vasopressors such as noradrenaline (76) or vasopressin. Currently, noradrenaline is the first line of vasopressor for treating patients

with septic shock (67, 77, 78). Noradrenaline, a vasoconstrictor formed from tyrosine, is involved in the stimulation of adrenergic alpha-1 (α_1) receptors causing peripheral vasoconstriction thereby increasing blood pressure (79). Vasopressin, an endogenous nonapeptide also known as antidiuretic hormone is a vasoconstrictor, which is formed in the hypothalamus, stored in the neurosecretory vesicles in the posterior pituitary gland and then released (80). In septic shock, the mechanism of action for vasopressin leading to vasoconstriction is multifactorial, but it mainly exhibits vasoconstrictive activity by binding to G-protein-coupled V1 receptors in the vascular smooth muscle leading to increased intracellular levels of calcium and vasoconstriction which results in increased arterial blood pressure (67, 80).

Sepsis is often linked to reversible myocardial dysfunction (81, 82). It has been recognized that patients in the initial stages of septic shock could also have cardiac dysfunction, both systolic and diastolic whereas previous cardiac function was thought to be intact at the early stages of this disease (83-85). The risks associated with increased stimulation of catecholamines including tachycardia, arrhythmias, myocardial injury and direct toxic effects have been well known (86). Levosimendan, a substitute for catecholamines with several roles including sensitisation of the myocardium to calcium via troponin C binding, enhancing the contractility of the myocardium without rising oxygen requirements, causes vasodilation by opening the potassium channels on the vascular smooth muscle, protection of myocardium from ischaemic insult and also exhibits anti-inflammatory properties (87). In 2016, a clinical trial conducted by Gordon et al., revealed that administration of levosimendan regularly did not improve organ dysfunction in septic shock patients when compared to placebo and it might also be causing some harm (88).

4) The use of corticosteroids

In sepsis, corticosteroids are commonly employed as an adjunctive therapy for treating patients with septic shock (67) and they have anti-inflammatory, immunosuppressive, anti-proliferative, and vasoconstrictive roles (89). The recommended guidelines from the Surviving Sepsis Campaign suggest the use of intravenous corticosteroids such as hydrocortisone at a dose of 200mg/day (i.e., 50mg for every 6 hours) or as a continuous intravenous infusion (67). This treatment should be initiated in septic shock patients at a

dose of vasopressors (i.e., norepinephrine or epinephrine) ≥ 0.25 mcg/kg/min at least 4h after initiation of vasopressor therapy (67). Hydrocortisone is an exogenous glucocorticoid structurally the same as cortisol (89), an endogenous hormone which is secreted by adrenal cortex through the stimulation of adrenocorticotrophic hormone (ACTH) released from the pituitary gland via the stimulation of corticotropin-releasing hormone (CRH) which was in turn released by hypothalamus during circadian rhythm, stress and excessive inflammation (90). Cortisol exerts its effect on the hypothalamic–pituitary–adrenal (HPA) axis through negative feedback either by indirectly suppressing the expression of inflammatory cytokines (such as IL-1 β , TNF, and interleukin-6 (IL-6)) or via the paraventricular nucleus of the hypothalamus and the anterior pituitary by suppressing the release of ACTH and CRH (90). Hydrocortisone can bind to glucocorticoid receptors and has many different effects ranging from short-term effects (such as causing reduced vasodilation, vascular permeability, and leukocyte migration at inflammation sites) to long-term effects such as inhibition of phospholipase A2 (which leads to reduced synthesis of arachidonic acid derivatives), neutrophils apoptosis, inflammatory transcription factors (NF- κ B, activator protein 1 and interferon regulatory factor-3) as well as stimulating the production of anti-inflammatory cytokines (interleukin-10, NF- κ B inhibitor) (89).

There is some evidence that the use of corticosteroids as an adjunctive therapy in patients with septic shock, constantly lowers the shock duration (91-93). The effect of corticosteroids on mortality is still less clear as two largest clinical trials on the use of corticosteroids revealed conflicting results, with one study reporting no difference in the mortality (93) whilst other study showed improved survival in patients who received corticosteroids than those who received placebo (91). Corticosteroids have also been linked with an increased risk of hypernatremia and hyperglycemia in sepsis patients (94, 95).

1.1.8 Clinical need to target and track therapy

Sepsis does not only have complex pathology but it's the heterogeneous nature of this syndrome (such as originating from a variety of causative pathogens, various sites of infection, host response variability and beneficial effect of treatment on some patients not all) which makes it difficult to not only find new therapies but also to target and track response to the treatment. At present there are no reliable ways for this which is why

metabolomics could be useful as it has a potential for patient stratification as metabolic profiles obtained from analytical platforms can reflect human individuality and phenotypic variation (96).

1.2 Metabolomics and Sepsis

1.2.1 Analytical Technologies for Metabolomics

The term “Metabonomics”/ “Metabolomics” has been broadly used, accounting for the global measurements of metabolites (such as small molecules, lipoproteins, and lipids) conducted on biological fluids and tissues. The purpose of this is to understand how these metabolites vary at multiple levels as well as evaluating beneficial or adverse effect of any systematic and temporal changes on these metabolites due to applied factors including diet, drugs, environmental stress, lifestyle, pharmaceutical effects, external stimuli and genetic variation (96). The term metabonomics has been progressively substituted by metabolomics or, more recently metabolic profiling or metabolic phenotyping. Metabolic profiling is non-selective and generates real-time measurements of metabolites (ranging from expected metabolites to potentially unknown and uncharacterized metabolites), which makes this an effective method for investigating and understanding biological processes and discovering new biomarkers (97). Generally, biological fluids (such as urine, plasma, serum, seminal, amniotic, cerebrospinal, synovial, digestive, blister and cyst fluids, lung aspirates, dialysis fluids, and cell or tissue extracts) are typically used for metabolomic studies (98, 99). Biofluids such as urine, plasma and serum are quite easy to obtain in routine clinical studies (99). The use of several of these biofluids is advantageous, as they can give an integrated outlook on the biology of the whole living system (99).

Metabolomic studies are conducted using various analytical platforms such as nuclear magnetic resonance (NMR) spectroscopy, infrared spectroscopy and mass spectrometry (MS) by direct infusion or coupled to other separation techniques including ultra-high performance liquid chromatography (UHPLC), gas chromatography (GC) and capillary electrophoresis. The two key analytical platforms, nuclear magnetic resonance (NMR) and Mass Spectrometry (MS) are the basis of all metabolic phenotyping methods and are highly complementary to each other (99). Therefore, utilising both NMR and MS is often important for full molecular/structural characterization of compounds. (100)

1.2.1.1 NMR Spectroscopy

NMR Spectroscopy is a non-destructive analytical technique widely used for metabolite fingerprinting (101) which uses magnetic properties of certain nuclei which possess magnetic dipoles due to a combination of electric charge and mechanical spin. Atomic nuclei which have spin quantum number > 0 are NMR active and behave like a small bar magnet when placed in an external magnetic field generating different spin states (i.e., parallel, or antiparallel to the magnetic field) for nuclei with spin $\frac{1}{2}$ such as ^1H and ^{13}C .

When a sample is placed in an NMR spectrometer, nuclei which have active magnetic moment (as in nuclei with a spin quantum number > 0) behave like tiny magnets due the external magnetic field and will have a net magnetisation. In a simple 90° pulse NMR experiment, nuclei with spin $1/2$ will have two different orientations in the presence of an external magnetic field and these two orientations will have different energy levels. The nuclei which are aligned with the magnetic field will have lower energy (i.e., those in parallel state) whilst those aligned against the magnetic field will have higher energy (i.e., those in antiparallel state)(102). The difference between the energy levels of these two orientations is small and are in the radiofrequency region as shown in eq. 1.1 and 1.2 (103), hence the population difference will also be minute with a slight preference for the ground state (i.e., parallel state)(104). The spin population differences are dictated by the Boltzmann's statistics and depend on the energy difference between the spin states which is shown in eq. 1.3 (104). As the difference in the spin population between the energetic levels of the spin states is small, there are not many spins that will be excited when irradiated, hence NMR is not a sensitive technique (104).

The difference between the energetic levels of the spin states depends on the nucleus and its nuclear properties (i.e., the gyromagnetic ratio (γ) which is a proportionality constant calculated by the ratio of nuclear magnetic moment (i.e., magnetic field generated by a nucleus) to the angular momentum of a nucleus) as well as the external magnetic field (i.e., B_0) (104). This energy difference is directly proportional to both γ and B_0 at the nucleus (eq.1.2) (102). Therefore, the bigger the gyromagnetic ratio the larger the energy difference between the spin populations, hence the sensitivity is higher. Additionally, a stronger magnetic field also increases the energy difference between the spin populations which increases the sensitivity as the population difference in the spin states also increases. There

is another way to increase NMR sensitivity without modifying the nucleus or the magnetic field and that is by acquiring many experiments to increase the signal to noise ratio. (102-104)

$$\Delta E = h\nu \quad \text{eq. 1.1}$$

$$\Delta E = h \left(\frac{\gamma B_0}{2\pi} \right) \quad \text{eq. 1.2}$$

$$\frac{n_{\text{antiparallel}}}{n_{\text{parallel}}} = \exp \left(\frac{-\Delta E}{kT} \right) \quad \text{eq. 1.3}$$

Where, ΔE = The difference in the energy levels of spin states (i.e., parallel and antiparallel);

h = Planck's constant;

ν = The resonance frequency or the Larmor frequency;

B_0 = External magnetic field;

γ = Gyromagnetic ratio;

k = Boltzmann's constant;

T = Temperature in Kelvin;

n_{parallel} = Spin population of the nuclei in the parallel state;

$n_{\text{antiparallel}}$ = Spin population of the nuclei in the antiparallel state.

When a sample placed in the NMR spectrometer has a radio frequency applied to it, it will cause the nuclei in the ground state (i.e., parallel state) to absorb the electromagnetic radiation making them transition from the lower to higher energy state (i.e., spin flip from ground to excited state) which corresponds to the resonance frequency or the Larmor frequency of the nuclei. The applied magnetic field strength (B_0) is proportional to the difference in the energy levels between parallel and antiparallel state (i.e., ΔE) hence, different field strengths will cause the protons to resonate at different frequencies. All the NMR resonance frequencies produced by the absorption of electromagnetic radiation in the sample are measured at the same time. Later, when the radiofrequency signal is removed

the excited nuclei relax back to their equilibrium state by releasing energy in the form a signal called free induction decay (FID) also known as the Larmor precession frequency. All the resonances obtained from the sample are superimposed on each other in the FID signal therefore this needs to be decoded with an arithmetic process called Fourier Transformation (FT) which decomposes space or time functions into functions which depend on spatial or temporal frequency thereby generating an NMR spectrum. The signal to noise ratio will be low if generated from single FID as mentioned previously, therefore several FIDs need to be combined and averaged, to obtain NMR spectra with good signal to noise ratios using FT NMR. (105, 106)

^1H nuclei are typically used for NMR based metabolic profiling. Hydrogen is present in all organic molecules (107) such as lipids, carbohydrates and amino acids (107). ^1H is the most abundant isotope of hydrogen and one of the stable nuclei with high gyromagnetic ratio of 42.6 MHz/T, highlighted in table 1.2 (108, 109). Sensitivity in NMR depends on the energy difference in the spin populations as well as the number of nuclei that can present this difference, that is, that are active to NMR. Hence, ^1H is the perfect nucleus to be studied by NMR and the reason why everything is referred to it.

Table 1.2: NMR properties of some of the chosen nuclei (104, 108, 110, 111).

Nucleus	Nuclear spin	Natural Abundance (%)	Gyromagnetic ratio (MHzT ⁻¹)
^3H	1/2	$\sim 10^{-18}$	45.4
^1H	1/2	99.9	42.6
^{13}C	1/2	1.1	10.7
^{17}O	5/2	0.1	5.8
^{19}F	1/2	100	40.0
^{23}Na	3/2	100	11.3
^{31}P	1/2	100	17.2

NMR spectroscopy not only provides detailed information about a chemical entity in either its pure form or in complex mixtures/matrices, but also provides detailed structural information on the absolute/relative concentrations of the metabolites (101). This makes NMR an extremely powerful technique which can be employed for distinguishing isomers, characterization and identification of known and unknown compounds (105). NMR is very robust and reliable analytical platform, easy to automate, very reproducible and inherently quantitative which makes it an ideal technique for metabolic profiling as it even allows direct comparison of results obtained in different laboratories (112). NMR has its own limitations, it has limited sensitivity and resolution (101).

The use of one-dimensional (1D) and two-dimensional (2D) NMR experiments is a standard approach for metabolic profiling of biofluids (101). In metabonomic studies, biofluids are mainly profiled via high resolution 1D (^1H NMR general profiling, and using a spin-echo experiment) and 2D NMR experiments (^1H - ^1H , 2D J-res), thereby providing detailed information about the metabolites in the biological samples (113). Metabolic profiling can be targeted and untargeted. As NMR signal intensity is directly correlated to the concentration of the metabolite the signal belongs to, targeted metabolic profiling allows quantification of specific sets of metabolites whilst an untargeted analysis provides a more global view of metabolic changes in the sample of study (114). Various in house and commercially available approaches exist for targeted metabolic profiling and have been used in this thesis.

1.2.1.2 Mass Spectrometry

Mass spectrometry (MS) is a technique that is used for identification of metabolites within a sample based on their mass to charge ratio (m/z) of charged molecules or fragments generated during the ionisation process. In MS, analyte molecules are first vaporised and then ionised by bombarding with either electrons or ions, breaking the molecule into charged fragments which are sorted based on their m/z ratio and detected via a suitable detector. Several different techniques exist for both ionisation and ion analysis. Generally, MS is coupled with a chromatographic or other separation technique such as UHPLC or GC, especially for the analysis of complex mixtures. (115)

In Time-of flight (TOF)-MS the sample initially undergoes the ionisation step where it is first vaporised then ionised into charged particles via electron impact (i.e., bombardment with a beam of electrons) or electrospray ionisation. This breaks the molecules in the sample into charged particles which can then enter the mass analyser. All these charged particles then enter the ion pulser in the mass analyser where they are accelerated by an electric field. The resulting speed for each charged particle will depend on its mass and charge (i.e., m/z) as each particle has the same kinetic energy which is the same as the electric potential energy. After the acceleration step, all the charged particles (i.e., positive ions) will then enter a flight tube with a known length through a hole in a negatively charged plate. In the flight tube of the mass analyser, all the charged particles will set off at the same time, but lighter ions (i.e., those with smaller m/z values) will travel faster than the heavier ions (i.e., those with larger m/z values) in the field-free drift region (the region without an electric field). Here they will start to separate out with lighter particles reaching the ion detector first before heavier ions. When the positively charged ions hit the negatively charged plate in the detector, they gain electrons and are discharged from the plate which results in the movement of electrons, creating an electric current that is measured by the detector. The size of the current gives an estimate of the number of ions which hit the plate in the detector. (116-118)

There are several advantages of TOF mass analyser which include high mass accuracy, high scan speed, unlimited m/z range, greater sensitivity, and higher resolution in comparison to other mass analysers (such as quadrupole and ion trap) (116, 117, 119). TOF-MS combined with a quadrupole and a collision cell is known as Q-TOF-MS, an expensive instrument which utilises the high fragmentation efficiency of quadrupole mass analyser in conjunction with rapid analysis speed and high mass resolution of TOF mass analyser (116, 120). Q-TOF-MS is not only capable of isolating and fragmenting specific ions, but it also measures their accurate mass, thereby facilitating tandem MS (i.e., MS/MS) and high-resolution MS (116, 117).

Although, TOF-MS has high mass accuracy, which is a measure of how close the experimental mass is to the theoretical mass (i.e., the exact mass) (121, 122). However, in practice, mass accuracy errors can occur in TOF-MS due to short time delays. Firstly, there is a delay from the time a start pulse is sent by the control electronics to the time when a high

voltage pulse is present on the back plate of an ion pulser in the mass analyser. Secondly, there is also a delay from the time when ions reach the detector until the signal is digitalised via the acquisition electronics (118). Due to these delays the accurate measurement of the flight times of an ion is not possible therefore measured flight times need to be corrected by calculating the difference between the measured flight time and the sum of start and stop delay times (118). These errors are usually corrected by using mass calibration such as a lock mass which corrects for mass drift. This lock mass is usually achieved by calibrating the system in advance using a solution of reference compounds with known masses which will provide actual mass and flight time for each of the reference ions over a known mass range. This can then be used to correct for the measured flight time in the samples which will then result in an accurate mass for the detected ions (118).

UHPLC-MS is broadly used not only for the identification and fingerprinting of metabolites, but also this analytical platform has a core role in the pharmaceutical industry aiding molecular identification and quantification of drug metabolites. UHPLC-MS has higher sensitivity than NMR. However, it has some limitations and ion suppression is one of them which arises when more than one analyte is present simultaneously in the ion source causing competition in the ionisation process and subsequently reduction in the MS signals. Another limitation of UHPLC-MS is the inability to discriminate between isomers as they have the same molecular mass, fragmentation pattern and similar chemical properties which would make them elute at the same or very similar retention times (123).

1.2.2 Chemometric Methods

It has always been very difficult to carry out the analysis of metabolomic data due to the complexity caused by having more variables than number of samples. Therefore, there needs to be robust methods that can show underlying trends which exist in the highly complicated and variable metabolomic data sets. The classical approach is to employ multivariate analytical methods to detect these inherent patterns of spectral peaks/features from an NMR or MS spectrum which have biological significance and could be taken forward for further identification/classification via more targeted approaches. Generally, NMR and UHPLC-MS spectra of biological fluids or cell extracts are assumed to be objects with multi-dimensional components of metabolic coordinates, which correspond to spectral intensities

at each data point of a spectrum. These multidimensional spectroscopic spectra are dimensionally reduced by multivariate statistical analysis and subsequently plotted for interpretation. Metabolomic datasets have an excess number of variables corresponding to spectral intensities when compared with the number of observations. The entire collection of observations and variables forms the data matrix. In statistics, having large number of variables and a small number of observations, results in general class of problems, whereby linear regression models become less feasible. For this reason, untargeted metabolomics data needs to be analysed using multivariate methods, such as principle component analysis (PCA) and partial least squares projection to latent structures (PLS), which can deal with collinearity in the data matrix. (124)

Collinearity occurs when two or more independent variables are highly correlated with each other in a regression model (125). Collinearity is a problem as it undermines the statistical significance of independent variables, variable selection approaches, and it affects the standard errors of model parameter estimates (125). For example, when two independent variables are highly correlated the standard error estimates of both their model associated coefficients (e.g., regression coefficients) will be inflated and hinder statistical inference and model selection methods. Since the mean estimate of these model parameters is not stable, it can fluctuate greatly from one dataset to another (125). In summary, collinearity affects the interpretation of the influence of each variable on the model (126) which can lead to incorrect biological conclusions (124, 126).

Metabolomic datasets especially those with spectral data have a large number of variables corresponding to the same metabolite which are generally involved in complex metabolic networks (127) and are often highly correlated. To overcome the collinearity, methods which account for highly correlated variables such as latent-variable multivariate analytical methods are used (127). There are two types of multivariate analytical methods, supervised and unsupervised. In supervised analysis all the input data is labelled, and the algorithm learns to predict the output from the input data matrix with correct classification whilst in unsupervised analysis, input data is unlabelled, and the algorithm learns to identify hidden patterns in the input data matrix (128). In chemometrics, PCA is the most extensively used method for the multivariate analysis of metabolomic data. It is an unsupervised dimensionality reduction method (129) in which the data matrix X is decomposed to reach

at a linear transformation while retaining as much of the variance in the original data as possible in the output data (124) as shown in figure 1.5 and table 1.3. PCA deals with multicollinearity via dimension reduction which reduces these large number of highly correlated variables into a smaller set of orthogonal new “latent” variables, which are called principal components (shown in figure 1.5) (124, 130). The first principal component (i.e., PC1) explains the maximum amount of variation in the dataset as possible whilst other principal components followed by the first, explain the remaining amount of variation in the data (124, 130). PCA generally provides an overview of all the observations and reveals information about groupings, trends and any outliers. Unsupervised PCA results in an unbiased dimensionality reduction and its application only reveals group structure when intra group variation is sufficiently less than inter-group variation. For this reason, supervised discriminant approaches such as PLS and PLS-Discriminant Analysis (PLS-DA) are used, which mainly depend on the class membership of each observation as shown in table 1.3. (124)

PLS-DA is a supervised multivariate method employed for finding differences between predefined classes (129). This is generally achieved by connecting the two key data matrices X (data matrix) and Y (class membership such as sex, disease, mortality etc.) and preserving the co-variance between them (figure 1.5, table 1.3). Scores plots are commonly used to provide visual interpretations of highly complex data showing the separation between groups/classes. Scores are the linear transformation of original variables with weights defined by the loadings. The PLS loadings provide information on how much each original X-variable contributes to a specific model component (i.e., how important each of the original X variables are in the latent variables) whilst weights give information about the variables which are responsible for class separation and relate the original X-variables with the Y outcome (124, 130, 131). Weights and loadings are generally used together to interpret a PLS-DA model. In a typical loading plot for PLS component 1, positive loadings indicate higher values of variables present in samples with positive scores than the samples with negative scores, and vice versa. A loadings plot including weights will provide information on the variables which are important in class discrimination. Variables which have higher weights will be more strongly associated with the outcome than those with smaller weights. (124, 130, 131) In general, scores provide a summary of observations whilst loadings

provide a summary of variables and explain the position of observations in the scores plot. Hence, scores and loadings are complementary to each other, and are often examined together. (132) In addition, loadings may also be explored on a per-component basis as a line plot, with the loading value plotted as dependent upon the spectral variables (such as ppm, m/z). In line plots, loadings of each multivariate model can be viewed as a full 1D spectrum or pseudo-spectrum, with large positive or negative excursions of a variable's loading corresponding to a large positive or negative correlation with class assignment, respectively (124).

Table 1.3: Main Differences between PCA and PLS-DA multivariate models (124, 129, 130, 132).

	PCA	PLS-DA
Multivariate method	Un-supervised	Supervised
Input data	X matrix	X and Y matrices
Data labelling	Input data is not labelled	Input data is labelled
Dependent variable	None	Y matrix
Transformation	Linear and preserves as much variance between original data (X matrix) and the 1 st principal component	Linear and preserves co-variance between original data (X matrix) and its class labelling (Y matrix)
Matrix decomposition	Dimensionality reduction method which decomposes X matrix	Dimensionality reduction, but with complete awareness of class membership and decomposes both X and Y matrices
Projections	Only X matrix (T scores)	X matrix (T scores) as well as Y matrix (U scores)
Application	Used for dimensionality reduction, visualisation, and clustering	Used for prediction/classification, dimensionality reduction and visualisation.

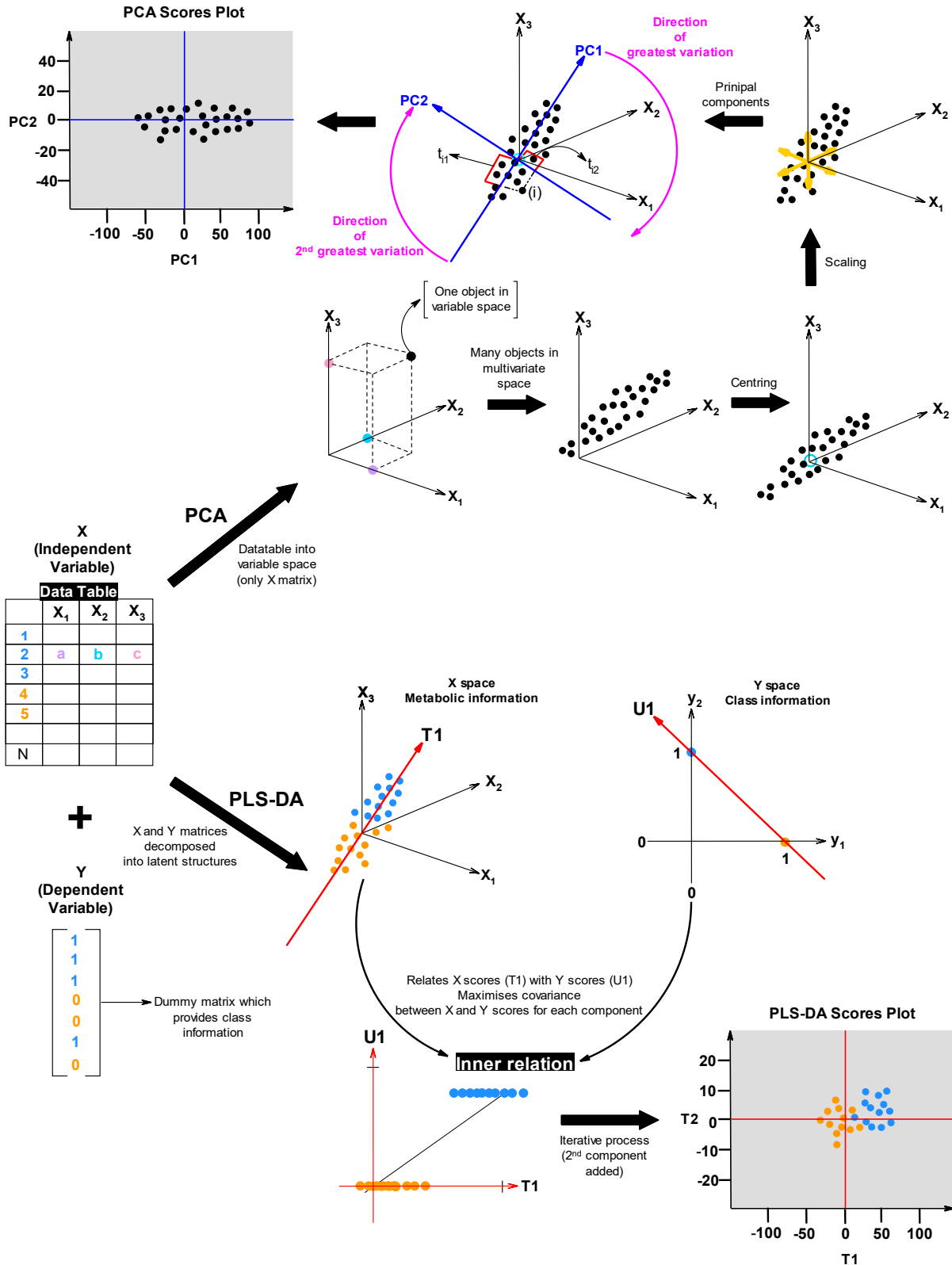


Figure 1.5: Graphical representation of PCA and PLS-DA. Abbreviations: PC1 = Principal component 1; PC2 = Principal component 2; X_1 = Variable 1; X_2 = Variable 2; X_3 = Variable 3; N = last sample in the data matrix; t_{i1} = Distance from the projection of i^{th} point on 1st component to origin; t_{i2} = Distance from the projection of i^{th} point on the 2nd component to origin. Both t_{i1} and t_{i2} are the T scores for the i^{th} point on PC1 and PC1, respectively.

Multivariate models can be validated internally using cross-validation, externally with a test set (which is generally composed of samples not used for building the training model) or with a mix of both whereby cross-validation is performed while training a model for obtaining a better estimate for the classifier performance, which can then be validated on a completely independent external test set (e.g., double cross-validation) (130). Cross validation is carried out to estimate the expected predictive ability of a multivariate model while avoiding the overfitting bias that is likely to occur if the model is benchmarked in the same dataset used to fit it (133). Overfitting is a phenomenon whereby the model learns the data so well in the training set (including noise in addition to true signals) that it fits too closely to the data (i.e., the estimated values match the observed values in the training set), that it negatively impacts the performance on a test set containing samples not previously seen by the model leading to worse performance than in training (134). Overfitting is a problem in supervised multivariate models but can be dealt with to some extent either by limiting the number of components (as increasing the number of components increases the risk of overfitting) or via further resampling (130).

There are several cross-validation approaches which can be performed, and these include leave-one-out cross validation (LOOCV), repeated cross-validation, k-fold, and Monte Carlo cross-validation (130, 133). Normally, K-fold cross-validation is performed whereby the data is generally split into k test sets of equal size. The K-fold method with k typically between 5 and 10 is normally chosen and the size of each fold should be suited to the study design. Repeated cross validation is normally used in datasets with small sample sizes, whereby the cross-validation procedure is repeated several times after re-shuffling the rows in the dataset (130, 133). Overfitting will decrease the predictive ability of models, leading to inaccurate predictions, hence models will not be reliable or robust.

In order to evaluate the goodness of fit for the multivariate models, the following two parameters are considered, and these include R^2 , which is the proportion of the variance explained by the model and Q^2 which is a cross-validated measure of the predictive ability of the model suited for K-fold and LOOCV style cross-validation schemes (130, 133). These two statistical parameters are derived from the sum of squares of the residuals by comparing observed and estimated values of the predictors (X, which is normally spectral data) or outcome (Y, e.g., the predefined class) (130). In an un-supervised analysis both R^2 and Q^2 are

calculated on the metabolic data labelled as X matrix (figure 1.5) hence referred as R^2X (the degree of variation in X explained by the model) and Q^2X . In a supervised analysis, both R^2X and Q^2X can also be calculated, but usually the model predictions which are based on the Y matrix, (a binary variable, figure 1.5) are of particular interest and these are referred to as R^2Y (which is the fraction of the variation explained by the model for the predefined classes) and Q^2Y (the prediction rate of model) (130, 133).

R^2X and R^2Y are calculated on the training data from the whole dataset using eq. 1.8 and 1.9 for X and Y matrices, respectively, which take into account two main parameters: 1) Residual Error Sum of Squares (RESS) (eq. 1.4 and 1.5) which is the sum of squared residual differences between the observed and estimated values of the predictors or the outcome variable summed over all the samples in the training set; 2) Total sum of squares (TSS) (eq.1.6 and 1.7) which is the total sum of all squared differences between the observed value and the arithmetic mean of all the samples in the whole dataset (135). The measured R^2 (i.e., both R^2X and R^2Y) ranges from 0 to 1, with higher values indicative of a good fit (130). The diagnostic statistic Q^2 measures the goodness of fit on the samples left out during the cross validation (i.e., internal test set) or in an external test set. Q^2 therefore checks the goodness of prediction for the multivariate model and is calculated via eq. 1.12 and 1.13 on the left-out samples for X and Y matrices during the cross validation (130, 135). In each cross validation, the sum of squared residuals are calculated by comparing the estimated values with the observed values in the same way as for the training set, but on the left out samples (i.e., test samples). This prediction error is summed over all the samples and is known as Predicted Residual Error Sum of Squares (PRESS) (eq.1.10 and 1.11 for X and Y matrices, respectively) (135) which is then divided by the TSS of all the samples from the whole dataset (135) (eq. 1.6 and 1.7). In multivariate models, R^2 is generally higher than or equal to Q^2 , and values approaching 1 indicate better models. (130, 133, 136)

$$RESS_X = \sum_{i=1}^n (X_{iTrain} - \hat{X}_{iTrain})^2 \quad (\text{eq. 1.4})$$

$$RESS_Y = \sum_{i=1}^n (Y_{iTrain} - \hat{Y}_{iTrain})^2 \quad (\text{eq. 1.5})$$

$$TSS_X = \sum_{i=1}^n (X_i - \bar{X})^2 \quad (\text{eq. 1.6})$$

$$TSS_Y = \sum_{i=1}^n (Y_i - \bar{Y})^2 \quad (\text{eq. 1.7})$$

$$R^2 X = 1 - \frac{RESS_X}{TSS_X} \quad (\text{eq. 1.8})$$

$$R^2 Y = 1 - \frac{RESS_Y}{TSS_Y} \quad (\text{eq. 1.9})$$

$$PRESS_X = \sum_{i=1}^n (X_{iTest} - \hat{X}_{iTest})^2 \quad (\text{eq. 1.10})$$

$$PRESS_Y = \sum_{i=1}^n (Y_{iTest} - \hat{Y}_{iTest})^2 \quad (\text{eq. 1.11})$$

$$Q^2 X = 1 - \frac{PRESS_X}{TSS_X} \quad (\text{eq. 1.12})$$

$$Q^2 Y = 1 - \frac{PRESS_Y}{TSS_Y} \quad (\text{eq. 1.13})$$

Where,

n = Total number of samples;

X = Metabolic variables;

Y = Outcome(i.e., dependent variable);

X_i = Observed value of X for the ith object in the whole dataset;

X_{iTrain} and X_{iTest} = Observed values of X for the i^{th} object in training & test set, respectively;

Y_i = Observed value of Y for the i^{th} object in the whole dataset;

Y_{iTrain} and Y_{iTest} = Observed values of Y for the i^{th} object in training & test set, respectively;

$\hat{X}_{iTrain}$ and $\hat{X}_{iTest}$ = Estimated values of X for the i^{th} object in training & test set, respectively;

$\hat{Y}_{iTrain}$ and $\hat{Y}_{iTest}$ = Estimated values of Y for the i^{th} object in training & test set, respectively;

$\bar{X}$ = Mean of observed X values from the whole dataset;

$\bar{Y}$ = Mean of observed Y values from the whole dataset.

Assessing the goodness of fit for a multivariate model is crucial, but it is equally important to choose the right number of components. In general, the number of components should be lower than the total number of samples or variables in the data. A common approach is to choose the smallest number of components that can explain as much of the variance in the data (in case with PCA) or obtain a best possible prediction of the outcome variable (in case with PLS-DA). The number of components for a multivariate model are usually selected via cross-validation to maximize Q^2 and a new component is only added if there is an increase in explained variance by at least 5% in Q^2 for both supervised and un-supervised multivariate models (130). There is a point when Q^2 reaches a plateau while adding components and then decreases with the addition of more components indicative of a decline in the predictive ability of the model (130, 133). At this stage, the model might be adding noise (i.e., random fluctuations) or fitting dataset characteristics which are not representative of the population under study and hence is overfitting (130, 133). A large difference between R^2 and Q^2 can reflect that a model is overfitted (133).

Linear statistical methods including generalised linear models (GLMs) and linear mixed-effects models (LMMs) provide more advanced analysis (137). GLMs generalise simple linear regression (137) in a way that it relates a linear model to the response variable through a link function while also allowing the magnitude of variance present in every measurement to become a function of its predicted value (138). In simple terms, GLMs determine the best linear relationship between a covariate and the response variable using a simple linear

regression (127). The linear predictors in GLMs are normally wrapped in a transformation function and the error distribution could be Gaussian, Poisson, binomial or gamma (139). GLMs are generally employed in metabolomics to determine whether there are significant differences in the concentrations of metabolite between the groups, or whether there is significant association between the metabolite and a variable of interest (e.g., a clinical outcome). Mixed effect models such as LMMs are broadly employed in several different fields including medical, biological and social sciences (140). As mixed effect models are quite complex it makes the selection of variables and the estimation of parameters challenging (140). As with GLMs, LMMs are also extensions of linear models (141) which illustrate the connection between a response variable and other explanatory variables (142). LMMs contains two types of additional terms, fixed effects (which comprise of fixed factors) and random effects (which comprise of random factors) (141, 142). Using LMMs is helpful in situations when the assumptions related to constant variance and independence are not met (137, 141). LMMs are complex but powerful tools and their use in the analysis of biological data is becoming more and more common (141).

Discriminant metabolites identified from line loading plots of the multivariate models of untargeted datasets can be confirmed using statistical total correlation spectroscopy (STOCSY) (143). STOCSY is based on calculating a full correlation matrix for a set of ^1H NMR spectra. STOCSY shows the correlation between the spectral intensities of several peaks across the whole sample. The main purpose of performing STOCSY is to identify multiple NMR peaks belonging to the discriminant metabolite by utilising the correlation for peaks that originate from the same compound in a complex mixture. In addition, STOCSY also provides added benefit by providing extra information about lower or even negative correlations as this normally leads to the connection between two or more metabolites involved in the same biological pathway. (143)

1.2.3 Review of metabolomic sepsis literature focusing on clinical outcomes

Metabolomics has been previously used in various studies to identify metabolic differences between patients survivors and non-survivors of sepsis with the purpose of aiding prognostication and potentially identifying novel therapies (144). There is much known

about the differences between survivors and non-survivors, and this has been summarised in table 1.4.

Most of the metabolomic studies investigating outcome were blood-based studies conducted on adults and focused on baseline differences (66, 145-169) (table 1.4). However, some studies also looked at other static timepoints (154-156, 159, 163, 165, 167, 168, 170). These metabolomic studies highlighted that pathways related to energy, lipid, amino acid, nucleotide, purine, pyrimidine and steroid metabolism were affected in sepsis (144). The metabolites which were significantly different between survivors and non-survivors are summarised in table 1.4 (66, 145-166). Overall, these studies showed a consistent accumulation of many metabolites in non-survivors with respect to survivors. Important metabolites which consistently increased (66, 145-169) in non-survivors and those which consistently decreased (66, 147-149, 151, 156, 158, 159, 170-172) are listed in table 1.5.

Table 1.4: A summary of metabolomics studies for the prognostication of sepsis patients in relation to mortality via (144).

Study (Year)	Biofluid	Participants (Survivors: Non -survivors)	Age group	Analytical Platform	Statistical Approaches	Increased in non-survivors	Decreased in non- survivors
Jones et al. (169) (2022) ^c	Serum	113:39	Adults	UHPLC-MS (Targeted)	PCA, PLSDA, FET MW- <i>U</i> test, MELR, Cox PHR, KMSA	14,15- dihydroxyeicosatrienoic acid (DHET)	-
Mierzchala-Pasierb et al. (153) (2021)	Serum & Urine	11:4	Adults	UPLC-MS (Targeted)	MW- <i>U</i> test, Cox PHR analysis	Serum 4-hydroxyproline and Glutamine	-
Li et al. (149) (2021) ^c	Serum	74:10	Paediatric	HPLC-MS (Targeted)	ANOVA, t-test, PCA, OPLS-DA, HCA, LR	Adenine, indolelactic acid, LPS(18:1/0:0), Ile-Tyr, kynurenine, glutamate, acetylcarnitine, tyrosine, tryptophan, palmitoylcarnitine, methionine, proline, acetylneuraminate and N2,N2- dimethylguanosine	PC (14:0/0:0,17:0/0:0, O-18:1/0:0), PI (18:0/22:5,18:0/22:6)
Reisinger et al. (173) (2021) ^c	Serum	34 :19 30 :16 on days 3 & 7	Adults	¹ H-NMR (Untargeted) & Bruker IVDr Lipoprotein analysis	LR, LMM, FET, X ² or MW- <i>U</i> test, PCA, PLS & OPLS-DA correlations	-	BCAAs (valine, leucine, isoleucine)
Jaurila et al. (157) (2020)	Serum	33:11	Adults	¹ H – NMR (Targeted)	MW- <i>U</i> or t-test, FET, correlations	Lactate and citrate	-
Khaliq et al. (151) (2020) ^c	Plasma	12:8	Adults	LC-MS/MS (Targeted) and industrial clinical chemistry system	PCA, mixed effects type-III ANOVA, Tukey HSD test, Random forests, linear SVMs	Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and troponin T, putrescine, acylcarnitines (mostly short- chain acylcarnitines), amino acids (aspartate, tyrosine, phenylalanine, histidine) on days 0-3 or at any specific timepoint	HDCH, LDCH, 4 LPCs, 28 PCs, 11 SM-OH (C14:1, C16:1, C22:2, C23:0), SM(C16:0, C18:0,C18:1, C20:2, C22:3, C24:0, C26:0) on days 0-3 or at any specific timepoint

Table 1.4 Cont.

Wang et al. (158) (2020) ^c	Plasma	134 :54	Adults	LC-MS (Targeted)	MW-U test, PLS-DA, ROC analysis	Isoleucine, alanine, acetylcarnitine, lactic acid pyruvic acid	LPG (22:0), and LPC (24:0)
Evans et al. (174) (2019) ^f	Serum	7:4	Adults	LC-MS (Untargeted)	MW-U test, Student's t-test, generalised estimation equations	N-Methyl-phenylalanine, glucosamine, isoleucyl-proline/leucyl proline, histamine, adipoyl-L-carnitine, methoxytryptophol, fibrinopeptide A, N.N-dimethylguanosine, N-(3-acetamidopropyl)pyrrolidin-2-one, allysine	N-Acetyl-L-phenylalanine, phenylalanyl-tyrosine
Chung et al. (175) (2019)^c	Plasma	Derivation - 69:21 Validation - 96:24	Adults	UHPLC-MS (Targeted)	Cox PHR, t-test, LR, ROC curves, KMSA	Acetylcarnitine (in both cohorts)	-
Huang et al. (166) (2019) ^e	Plasma	63:30	Adults	UPLC - UV (Targeted)	X ² , MW-U, KWT & t-test, KMSA, Cox PHR, ROC	Leucine and phenylalanine	-
Liu et al. (66) (2019)^b	Serum	40 :29 at 0h 32:19 at 24h	Adults	¹ H -NMR (Untargeted and targeted)	PCA, OPLS-DA, t-test, ROC analysis	Lactate, pyruvate, alanine, glutamate, glutamine, lysine, 1-methylhistidine, tyrosine, phenylalanine, citrate at 0h and 24h. Methionine, fumarate, acetate, urea and 3-hydroxybutyrate at 0h. Creatinine, 3-hydroxyisovalerate and lipids at 24h	N-acetyl glycoproteins - 0h and 24h
Cambiaghi et al. (154) (2018) ^c	Plasma	9:8	Adults	LC-MS/MS (Targeted)	Elastic net LR, LDA, PLS-DA	Day 7 to Day 1 ratios of PCaa (C34:3, C36:3, C36:6, C42:1, C42:5), PCaeC30:1, SDMA, total dimethylarginine, proline, tyrosine	Day 7 to Day 1 ratios of LPC aC24:0, methionine, PCaa (C40:6, C42:6, C42:2), PCae (C30:2 & C42:5)
Winkler et al. (155) (2018) ^c	Plasma	89:31	Adults	LC-MS/MS (Targeted)	X ² , MW-U test, KWT, KMSA	SDMA on days 1, 3 & 7, ADMA on days 1 & 3	-
Cirstea et al. (171) (2017) ^{c, e}		186:14 – Day 28 172:28 – Day 90	Adults	Photometric analysis	KMSA, ROC analysis	-	HDCH

Table 1.4 Cont.

Dalli et al. (168) (2017) ^b	Plasma	13:9	Adults	LC-MS/MS (Targeted)	Wilcoxon paired signed rank test, FET, PLS-DA	PGF _{2α} (on days 0, 3 & 7), RvD5 (on days 3 & 7), RvE1, 17-HDHA, 18-HEPE, 15-HETE (on days 1 & 3), LTB ₄ , RvE2, 4S, 14S-diHDHA & 5S, 15S-diHETE (on day 7), 17R-PD1, 7-HDHA & 15-HEPE (on day 1), 17-epi-RvD1, 17-epi-PD1, 5-HETE & 5S, 12S-diHETE (on day 3).	-
<u>Wang et al. (176)</u> (2017) ^c	Plasma	CAPSOD - 90:31 HAI – VAP - 20:16	Adults	LC-MS/MS (Targeted)	MW- <i>U</i> test, HCA, SVMs, ROC curves	Methylthioadenosine (MTA) in both cohorts	-
Sharma et al. (177) (2017) ^b	Plasma	20:13 – Day 1 14:9 – Day7	Adults	LC-MS/MS (Targeted) & enzymatic colorimetry	ANOVA, Tukey HSD test	No significant difference in lipoproteins.	
Liu et al. (147) (2016) ^b	Serum	21:29	Adults	UPLC-MS (Untargeted)	ANOVA, Tukey HSD test	Citrate, succinate, malate, α-ketoglutarate, amino acids (proline, BCAAs, glutamine, glutamate, phenylalanine, betaine, creatine, creatinine, tyrosine), lactate, bile acids (GUDCA, GUDCS, GCDCA, GCA, UDCA), acyl carnitines (C6, C10, C12), indoxylactate, indoxysulfate, LPC 14:0	Ornithine, citrulline, argininosuccinate, acetylcarnitine, FFA (16:0, 18:0), LPE (18:0, 18:2, 20:3, 20:4), acylcarnitines (C16, C18)
Ferrario et al. (156) (2016) ^{c, e}	Plasma	9:11 (90-day mortality) 11:9 (28-day mortality)	Adults	LC-MS/MS (Targeted)	Unpaired Wilcoxon & paired Wilcoxon signed rank test, Multivariate Elastic Net regression analysis	Acetylcarnitine (on day 1) and kynurenine (on day 7) - based on 28-Day mortality.	PCs and LPCs species (on days 1 & 7) - based on 28-Day and 90-Day mortality. 6 saturated long-chain LPC (aC16:0, aC18:0) & polyunsaturated very long-chain PC (aaC32:3, aaC34:4, aaC36:4, aeC40:1) at day 7 - on both 28 & 90-day mortality.

Table 1.4 Cont.

Garcia-Simon et al. (159) (2015) ^d	Urine	48:12	Adults	¹ H-NMR (Targeted and untargeted)	ANOVA, PCA, PLS-DA, LR, ROC analysis	Ethanol, glucose, hippurate and an unknown metabolite (located at 1.40–1.45 ppm) – at 0h & 24h	Phenylalanine & arginine at 0h & 24h. Glutamine & methionine at 0h
Su et al (165). (2015) ^c	Serum	20 :15	Adults	LC-MS/MS (Targeted)	ANOVA, X ² test, t-test, Pearson correlations, ROC analysis	α-amino adipic acid, ethanolamine, cystathionine, and phenylalanine - at certain time points	Taurine (on days 10 & 14), BCAA/AAA ratio (on day 14), SAA (on days 7, 10, & 14) whilst arginine, glutamic acid, serine, and tryptophan at certain timepoints
Lee et al (170). (2015) ^c	Serum	65:52	Adults	Commercial kits with automated analysers	LMM, MW- <i>U</i> test or Student's t-test, Cox PHR, ROC analysis	-	Cholesterol, TG, HDL, LDL, and Apo A-I - On days 0, 1, 3, and 7.
Mickiewicz et al. (160) (2015)	Serum & Plasma	8:8	Adults	¹ H-NMR (Targeted) & cytokine and chemokine kits	PCA, OPLS-DA, ROC analysis	2-hydroxyisovalerate, fructose, IL-8, IL-9 and growth regulated oncogene alpha (GRO-α.)	Tumour necrosis factor (TNF)-β, beta-nerve growth factor (β-NGF) and dimethylamine
Kamisoglu et al. (163) (2015) ^c	Plasma	90:31	Adults	LC-Q-orbitrap-MS and DSQ GC-MS (Untargeted)	Welch's t-test, Kolmogorov-Smirnov test	2-methylbutyrylcarnitine, tiglylcarnitine, acetylcarnitine, hexanoylcarnitine, octanoylcarnitine, propionylcarnitine, butyrylcarnitine, decanoylcarnitine, cis-4-decenoyl carnitine at 0h & 24h whilst deoxycarnitine only at 24h.	1-eicosatrienoyl-GPC (20:3), 1-palmitoleoyl-GPC(16:1), 2-palmitoyl GPC (16:0) at 0h & 24h. 1-palmitoyl-GPC (16:0) 1-stearoyl-GPC (18:0), 1-oleoyl-GPC (18:1), 1-linoleoyl-GPC (18:2), 1-arachidonyl-GPC(20:4)at 24h.
Mickiewicz et al. (178) (2014)	Serum	4:4	Adults	¹ H-NMR (Targeted)	PCA, OPLS-DA, ROC analysis	20 metabolites were significant in differentiating survivors from non-survivors (Results not reported)	-

Table 1.4 Cont.

<u>Rogers et al. (146)</u> (2014) ^c	Plasma	RoCI - 60:30 CAPSOD - 115:34	Adults	GC-MS & LC- MS (Targeted)	LR, Bayesian networks	Kynurenine lactate, p-HPHLa, ornithine, 3-hydroxyisovalerate, 2-hydroxyisovalerate, N-acetylalanine, sucrose, N-acetylserine, xanthine, allantoin, N2,N2-dimethylguanosine, 1-methylimidazoleacetate, glycocholate, GCDCA, TCDCA, taurocholate, cortisol, carnitines (C3, C4, C5, C5-OH, C5:1 & C6), γ -glutamylphenyl-alanine, γ -glutamyl-tyrosine - In both cohorts.	1-arachidonoyl-GPC(20:4), 1-arachidonoyl-GPE(20:4),, 1-palmitoyl-GPC (16:0), 2-palmitoyl-GPC (16:0), 1-linoleoyl-GPC (18:2), 1-stearoyl-GPC (18:0))- In both cohorts
Su et al. (150) (2014) ^a	Serum	26:9	Adults	LC-MS/MS (Targeted)	MW-U, ROC analysis, PCA, PLS & OPL-DA	S-succinyl glutathione, GPC, PG (22:2(13Z,16Z)/0:0), S-(3-methylbutanoyl) dihydrolipoamide-E.	-
Mickiewicz et al. (179) (2013)	Serum	10:10-Model 1 13:10-Model 2	Infants, Toddlers, School Age	¹ H-NMR (Untargeted & Targeted)	PCA, PLS-DA, OPLS-DA, ROC analysis	11 metabolites from model 1 & 18 metabolites from model 2 were significant in separating survivors from non-survivors. (Metabolites not reported)	-
<u>Langley et al. (148)</u> (2013) ^c	Plasma	Derivation set - 90:31 CAPSOD – 34:18 RoCI - 36:25	Adults	UPLC-MS/MS (Targeted & untargeted) and GC-MS (Untargeted)	ANOVA, LR, SVMs	17 amino acid catabolites (Lactate, p-HPHLa, 4-hydroxyproline, 3-methoxytyrosine), 16 carnitine esters (hexanoylcarnitine ,Cis-4-decenoylcarnitine, 2-methylbutyrylcarnitine, butyrylcarnitine), citrate, malate, pyruvate, dihydroxyacetone, phosphate, 11 nucleic acid catabolites, and 4 FFAs	Seven GPC and GPE esters, anabolic steroids, cortisone
<u>Seymour et al. (145)</u> (2013) ^e	Plasma	15:15	Adults	UHPLC-MS/MS and GC-MS (Untargeted)	Wilcoxon signed rank & t-test, random forests with supervised classification	Urea, cortisol, cortisone, fumarate, Kynurenate, 2-Py, pyridoxate, cofactors/vitamins, taurocholate, sulfated bile acids, sulfated hormones, N2, N2-dimethylguanosine, N1-methyladenosine, pseudouridine, allantoin, 10-hepatodecenoic acid, N6-carbamoylthreonyladenosine	GPEs and xenobiotics (paraxanthine and caffeine)
Gaddnas et al. (180) (2009) ^d	Serum	33:11	Adults	Radioimmuno- logical assays	X ² test, FET, MW- U, ROC	Procollagen type III aminoterminal propeptides and crosslinked telopeptides of type I collagen	-

Table 1.4 Cont.

Chien et al. (172) (2005) ^d	Serum	44:19	Adults	Enzymatic & turbidimetric methods using kits	MW- <i>U</i> , FET, multivariate LR, KMSA	-	HDCH and Apo-A1 (on days 1 to 4)
Vermont et al. (181) (2005)	Serum	46:10	Paediatrics	Enzymatic colorimetric assay	Non-parametric test, FET	-	Total cholesterol
Drobnik et al. (167) (2003) ^d	Plasma	63:39	Adults	LC-MS/MS (Targeted)	MW- <i>U</i> test, ROC curves	Cer-SM ratios – at day 4 and day 11. Cer-SM to LPC-PC ratios – at day 1, 4 and 11	LPC-PC ratios – at day 4 and day 11
van Leeuwen et al (182) (2003)	Plasma	10:7	Adults	DGU & enzymatic methods	ANOVA, t- test, MLRA	No significant differences in lipoproteins	-
Sprung et al. (164) (1991)	Plasma	10:5	Adults	Postcolumn IEC with ninhydrin detection	Spearman's correlations, t- test	AAAs (tyrosine, phenylalanine), SAAs (Taurine, methionine, and cysteine), ammonia and GABA	-
Roth et al. (152) (1982)	Plasma	7:7	Adults	Automatic amino acid analyser (Liquimat III)	Student's t - test	Muscle valine and leucine and plasma levels of glucose, glucagon, phosphoserine, cysteine, valine, phenylalanine, and 3-methylhistidine	Muscle glutamine, proline, and lysine

Studies highlighted in bold and underlined had a validation cohort. Abbreviations UPLC-MS: Ultra-performance liquid chromatography-mass spectrometry; UPLC-UV: Ultra-performance liquid chromatography coupled to ultraviolet detector; UHPLC-MS: Ultra-high performance liquid chromatography-mass spectrometry; GC-MS: Gas chromatography coupled to time-of-flight mass spectrometry; DSQ GC-MS: Dual-Stage Quadrupole Gas chromatography-mass spectrometer; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; LC-Q-orbitrap-MS: Liquid chromatography with quadrupole orbitrap mass spectrometry; IEC: Ion-exchange chromatography, DGU: Density gradient ultracentrifugation; PHR: Proportional Hazards Regression; MW-*U*: Mann-Whitney *U*; LR: Logistic regression; LMM: Linear mixed model; MELR: Mixed effect LR; MLRA: Multiple-level regression analysis; HCA: Hierarchical clustering analysis; LDA: Linear discriminant analysis; PLS-DA: Partial Least Squares Discriminant Analysis; PCA: Principal Component Analysis; OPLS-DA: Orthogonal Projections to Latent Structures Discriminant analysis; ANOVA: Analysis of variance; HSD: Honestly significant difference; MW-*U*: Mann-Whitney – *U*; H-MCR: Hierarchical multivariate curve resolution; KMSA: Kaplan-Meier survival analysis; ROC: Receiver operating characteristic; χ^2 : Chi-square; FET: Fisher's exact test; KWT: Kruskal–Wallis test; SVMs: Support Vector machines; AAAs: Aromatic amino acids; BCAAs: Branched chain amino acids; aa: diacyl; ae: acyl-alkyl; PC: Phosphatidylcholine; PG: Phosphatidylglycerol LPC: Lysophosphatidylcholine; PI: Phosphatidylinositol; LPE: Lysophosphatidylethanolamine; GPE: Glycerophosphatidylethanolamine; GPC: Glycerophospholcholine; Cer: Ceramide, SM: Sphingomyelin; Rv: Resolvins; PGF_{2 α} : Prostaglandin F_{2 α} ; LT: Leukotrienes; PD1: Protectin D1; p-HPHLa: p-(hydroxyphenyl)lactic acid; 2-Py=N1-methyl-2-pyridone-5-carboxamide; TCDCA: Taurochenodeoxycholate; GCDCA: Glycochenodeoxycholate; FAs: Fatty acids; FFAs: Free fatty acids; TG: Triglyceride; HDL: High density lipoprotein; LDL: Low density lipoprotein; HDCH: HDL cholesterol; LDCH: LDL cholesterol; Apo-A1 = Apolipoprotein-A1; TCA: Tricarboxylic acid; SDMA: Symmetric dimethylarginine; ADMA: Asymmetric dimethylarginine; GABA: gamma-aminobutyric acid; HDHA: Hydroxydocosahexaenoate; HEPE: Hydroxyeicosapentaenoate; HETE: Hydroxyeicosatetraenoate; CAPSOD: Community Acquired Pneumonia and Sepsis Outcome Diagnostics; RoCI: Brigham and Women's Hospital Registry of Critical Illness. Symbols ^{a-e} represent censoring time point of mortality assessment; ^a24-h mortality; ^b7-Day mortality; ^c 28-Day mortality; ^d30-Day mortality; ^e90-Day mortality; ^f 1- Year mortality.

Table 1.5: Important metabolites related to mortality outcome. Abbreviations: PCs = Phosphatidylcholines; LPCs = Lysophosphatidylcholines; Pls = Phosphatidylinositols; PGs = Phosphatidylglycerols; NAD⁺ = Nicotinamide adenine dinucleotide; LPGs = Lysophosphatidylglycerols; SMs = Sphingomyelins; Cer = Ceramide; SDMA = Symmetric dimethyl arginine; DMA= Dimethylarginine; HDL = High-density lipoprotein; LDL = Low density lipoprotein; DiHETE = 14,15-dihydroxyeicosatrienoic acid.

<i>Type of metabolite</i>	<i>Increased in non-survivors</i>	<i>Decreased in non-survivors</i>
<i>Energy and urea cycle metabolites</i>	Lactate, pyruvate, citric acid, succinate, malate, fumarate, glucose, sucrose, NAD ⁺ , N1-methyl-2-pyridone-5-carboxamide	Arginine, ornithine, urea argininosuccinate, citrulline, ammonia
<i>Amino acids and derivatives</i>	Tyrosine, phenylalanine, aspartate, betaine, histidine, glutamine, glutamate, proline, lysine, creatine, creatinine, SDMA, total DMA	
<i>Lipids and Lipoproteins</i>	Free fatty acids, PGs, LPS (18:1/0:0), bile acids, catbolic steroids, cortisol, cortisone, Cer/SM ratio	HDL, LDL, Apo-A1, cholesterol, HDL cholesterol, PCs, Pls, LPCs, LPGs, SMs, N-acetylglycoproteins
<i>Purine and pyrimidine metabolites</i>	Pseudouridine, N1-methyladenosine, N2, N2-dimethylguanosine	
<i>Lipid mediators</i>	Resolvins, protectins, oxylipin (14,15- DiHETE)	

Besides the consistent findings reported in these metabolomic studies, there were some metabolites such as branched chain amino acids (BCAAs) (such as leucine, isoleucine and valine) (147, 152, 158, 166) and sulphur-containing amino acids (SAAs) (such as cysteine, taurine and methionine) whose findings were not universally confirmed in relation to mortality and these amino acids were found to be both lower (154, 165, 173) and higher (66, 147, 149, 152, 158, 164, 166) in non-surviving patients.

Despite the large number of studies exploring the metabolomics of sepsis, there is very little known about the metabolic trajectories of survivors, and non-survivors over the course their ICU stays. Only two metabolomic studies have investigated differences in metabolites in blood over time between survivors and non-survivors. However, these studies have used

comparisons at static timepoints and did not investigate true trajectories. One study revealed that survivors had decreased levels of amino acids (including glutamate, glutamine, phenylalanine, alanine), glycolysis and TCA cycle metabolites (lactate, pyruvate, citrate) after 24 hours of ICU admission with respect to baseline whilst non-survivors had increased levels of amino acids (glutamate, glutamine, phenylalanine, alanine), citrate and creatinine after 24 hours of ICU admission. Only N-acetyl glycoproteins were found to be reduced after 24 hours of ICU admission (66) in the non-surviving patients. However, the other study investigated 28-and 90-day survivors and non-survivors and looked at the metabolite differences between days 1 and 7 (156). This study reported accumulated levels of phospholipids (including 17 diacyl-Phosphatidylcholines (PCs), 30 acyl-alkyl PCs, 10 lysophosphatidylcholines (LPCs)), acyl carnitines (carnitine and butyrylcarnitine), and 4 long-chain sphingomyelins (SMs) on day 7 in comparison to day 1 survivors whilst kynurenine and two polyunsaturated diacyl-PCs (PC aa C38:6, PC aa C40:6) were lower in survivors from day 1 to day 7 (156). Non-surviving patients also followed the same pattern in this study and were reported to have an overall increase of LPCs, PCs and SMs from day 1 to day 7 (156).

All these metabolomic studies except for three (158, 167, 169) included small samples and only six studies validated their findings (66, 145, 146, 148, 175, 176). Of those that attempted to validate their findings, two studies utilised mouse models for validation (145, 176). Some of the inconsistencies between studies might be due to the limitations of the current studies, such as experimental design (including sampling time and criteria for defining sepsis), choice of biofluids and the use of different analytical platforms. Most of these studies employed either, NMR or MS coupled to other chromatography techniques such as GC, UHPLC, however only one study benefited from the complimentary power of both analytical platforms (183).

1.3 Hypothesis and Aims

1.3.1 Hypothesis

This thesis explores the hypothesis that there are differences in the metabolic profiles and trajectories of septic shock survivors and non-survivors and that the metabolic changes over time will aid in earlier prognostication of sepsis patients in relation to outcome.

1.3.2 Aims and Objectives

The aim of this study is to explore the metabolic trajectories of patients with septic shock from two clinical trial cohorts. To do this I will address the following objectives:

- 1) Compare baseline differences in the metabolic profiles of patients with septic shock who survive and do not survive to understand the baseline differences that could influence trajectory analysis.
- 2) Compare metabolic trajectories of patients with septic shock between survivors and non-survivors to determine if these trajectories could map patient's clinical courses in the ICU.
- 3) Determine the relationship between metabolic profiles in sepsis and organ failure.
- 4) Determine if dynamic metabolic profiles could be used to map and monitor response to the therapeutic intervention.

Chapter 2 – Methods

2.1 Clinical Trials

2.1.1 VANISH Trial

The VANISH (The Vasopressor vs Norepinephrine as Initial Therapy in Septic Shock) trial (92) was a double-blind randomised clinical trial, with a 2X2 factorial design. Ethics for this trial were approved by Oxford A Research Ethics Committee (12/SC/0014 Oxford A REC) (184). This trial was designed to test the hypothesis that the early use of vasopressin with or without corticosteroids would improve kidney outcomes when compared with noradrenaline with or without corticosteroids (92)

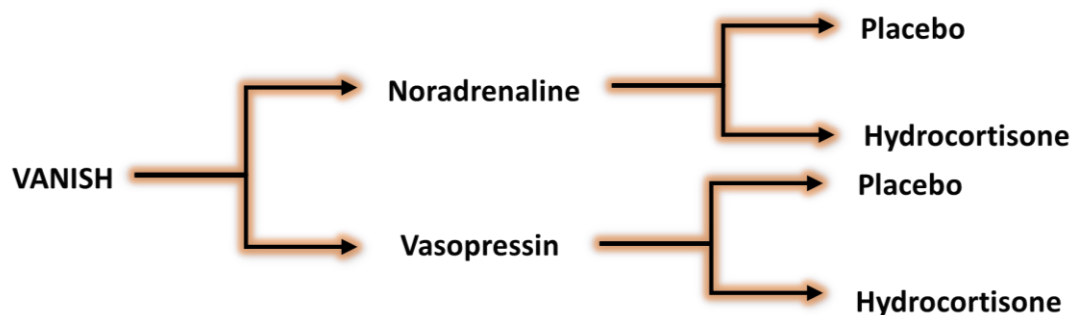


Figure 2.1: VANISH trial design for the allocation of two different treatment drugs. Patients were randomised to receive noradrenaline or vasopressin followed by either hydrocortisone or placebo.

409 patients were recruited to the VANISH trial in the UK between February 2013 and May 2015 (92). Septic shock patients were randomized to receive vasopressin or noradrenaline (study drug 1) as an infusion, titrated to keep the recommended mean arterial pressure (MAP) between 65-75mmHg followed by either hydrocortisone or placebo (figure 2.1). Patients were given study drug 2 (hydrocortisone or placebo) once the study drug 1 had been titrated up to reach its maximum infusion rate. Study drug 2 was administered every 6 hours (h) for 5 days, then every 12h for a period of 3 days and later once daily for up to 3 days. The results from this study showed that there were no significant differences between the early vasopressin use when compared with noradrenaline as it did not improve the kidney failure-freedays. (92)

2.1.1.1 Inclusion criteria

In the VANISH trial, inclusion criteria based on the Sepsis-2 consensus definitions of sepsis were used for adult patients (age ≥ 16 years) with sepsis who needed vasopressors to support the circulation despite adequate fluid resuscitation (92, 184) in order to be eligible for the clinical trial the following two inclusion criteria needed to be fulfilled by all the patients (92, 184):

1. Meet 2 of the 4 SIRS criteria (fever or hypothermia, tachycardia, tachypnoea, and abnormal leucocyte count which are previously listed in detail in Sepsis-2 consensus definition in chapter 1, figure 1.1) due to documented/suspected infection.
2. Shock (i.e., hypotension despite adequate fluid resuscitation requiring a minimum of 1L fluid in the previous 4 h).

2.1.1.2 Exclusion criteria

In this trial, patients were not eligible for the trial and were excluded if they met one or more than one of the following exclusion criteria (92, 184):

- 1) Not fulfilled the 2 of the 4 SIRS criteria
- 2) Received open-label vasopressor for more than 6 h
- 3) Received vasopressor infusion during their current ICU admission except for those who needed vasopressors as an emergency treatment for < 6 h
- 4) Received a regular corticosteroid therapy (excluding inhaled steroid therapy) within the last three months.
- 5) Had adrenal insufficiency/dysfunction.
- 6) Had mesenteric ischemia.
- 7) Had End-stage kidney failure.
- 8) Known pregnancy.
- 9) Had vasospastic diseases (such as Raynaud phenomenon, systemic sclerosis, or others).

- 10) Enrolled in another clinical trial concerning a medicinal drug in the previous 30 days or enrolled on another interventional study which might have potential interactions with the study drugs.
- 11) Denied consent or were not able to consent.
- 12) Medical team and physicians are not committed to full active treatment.
- 13) A history of anaphylaxis or hypersensitivity to any of the study drugs in the clinical trial.

2.1.1.3 Sample Collection

Sample collection from patients enrolled on the VANISH trial was conducted in 18 general ICUs (92). Serum samples were collected from each patient in tubes with no additives. These tubes were left in ice for 30 minutes to allow the blood clot to form and then centrifuged at 4500 rpm for 10mins. Afterwards, serum was immediately pipetted into cryo tubes in the following aliquots: 5x 1.mL serum. These samples were collected at four different timepoints; 1) TP0 - at the time of randomisation/ICU admission prior to starting study drug 1; 2) TP1- after 24-36 hrs of ICU admission; 3) TP2 after 48-72 hrs of ICU admission; 4) TP3 - after 96-120 hrs of ICU admission. A total of 175 patients got sampled from VANISH trial.

2.1.2 LeoPARDS Trial

The LeoPARDS (The Levosimendan for the Prevention of Acute Organ Dysfunction in Sepsis) trial was a prospective, double-blind randomised placebo-controlled clinical trial conducted in 34 general adult ICUs. (88) The Ethics for this trial were approved by the independent National Research Ethics Committee London - Harrow (13/LO/0365) and it is registered as ISRCTN12776039 in the International Standardised Randomised Controlled Trial Registry (185). This trial was designed to test the hypothesis that the addition of levosimendan (an inodilator with pharmacological effects including calcium-sensitization of troponin C, vasodilation and cardioprotection) (186)) to the standard treatment would cause a decline in the severity of organ dysfunction as well as assessing its safety profile in patients with septic shock.(88)

516 patients were recruited into the LeoPARDS trial in the UK between January 2014 and December 2015. In addition to receiving standard treatment, patients were randomised to

receive levosimendan (N = 259) or placebo (N = 257) as an infusion for a period of 24 h. The infusion of levosimendan or placebo was initiated at a rate of $0.1 \mu\text{gKg}^{-1}\text{min}^{-1}$ and when no rate-limiting side effects were observed for 2 to 4 h then the infusion rate was raised to $0.2 \mu\text{gKg}^{-1}\text{min}^{-1}$ for 20 to 22 hours. In the event of rate-limiting side effects (such as hypotension or severe tachycardia) the infusion rate for the drug was reduced to $0.1 \mu\text{gKg}^{-1}\text{min}^{-1}$ from $0.2 \mu\text{gKg}^{-1}\text{min}^{-1}$ and was even reduced to $0.05 \mu\text{gKg}^{-1}\text{min}^{-1}$ or discontinued to prevent these severe side effects if necessary. The results from LeoPARDS trial indicated that adding levosimendan in standard care treatment does not contribute towards less severe organ dysfunction or decreased mortality and those patients who received levosimendan had a less likelihood to be successfully weaned from mechanical ventilation and had an increased risk of supraventricular tachyarrhythmia when compared to those who received placebo. (88)

2.1.2.1 Inclusion criteria

Inclusion criteria based on Sepsis-2 consensus definitions of sepsis were used for adult patients (age ≥ 18 years) with documented or suspected infection requiring vasopressors for the management of sepsis despite adequate fluid resuscitation (88, 185). The patients need to fulfil both of the following inclusion criteria to be eligible for their inclusion onto the trial (88, 185):

1. Meet 2 of the 4 criteria of SIRS (fever or hypothermia, tachycardia, tachypnea, abnormal leukocyte count which are previously listed in detail in Sepsis-2 consensus definition in chapter 1, figure 1.1).
2. Shock i.e., hypotension despite adequate fluid resuscitation which needs a continuous intravenous infusion of vasopressors (such as noradrenaline, adrenaline, or vasopressin analogue) for at least 4 h as well as an ongoing need for vasopressor at the time of randomisation.

2.1.2.2 Exclusion criteria

In this trial, patients were excluded if they meet one or more than one of the following exclusion criteria (88, 185):

- 1) Did not have septic shock.

- 2) Age < 18 year.
- 3) Infection not present.
- 4) Did not fulfil SIRS criteria (2 of the 4).
- 5) Fallen outside 24-h window since meeting the inclusion criteria.
- 6) Had end-stage kidney failure.
- 7) A history of torsades de pointes.
- 8) Had severe chronic liver impairment.
- 9) Known cardiac mechanical obstructions affecting ventricular flow (such as filling, outflow, or both).
- 10) Weight of more than 135 kg.
- 11) Known pregnancy.
- 12) Received levosimendan within the last 30 days.
- 13) Hypersensitivity to levosimendan or any of the excipients.
- 14) Made decision based on the limitation of the treatment.
- 15) Enrolled onto another clinical trial with a medicinal drug within last 30 days or currently a part of interventional study which might potentially interact with the study drugs.
- 16) Non-enrolment based on physician's decision.
- 17) Consent declined or the consent was not obtained.
- 18) There were problems concerning the availability of staff.
- 19) Other reasons for non-enrolment (such as the language barrier, unavailability of the study drugs (levosimendan or placebo), mental health problems, and some unspecified reasons).

2.1.2.3 Sample Collection

Sample collection from patients enrolled into the LeoPARDS trial (88) was conducted in 34 general adult ICUs (92). Serum samples were collected from each patient at four different timepoints: 1) TP0 - before randomisation on the first day of inclusion into the trial; 2) TP1 - After 24 h of ICU admission; 3) TP2 - After 96 h of ICU admission; 4) TP3 - After 120 h of ICU admission. All the samples were centrifuged at 1000 g for 10 min, frozen, stored and then sent to NPC for the later analysis (187). A total of 472 patients were sampled from LeoPARDS trial.

2.2 Metabolic Profiling Platforms

The metabolic profiling of serum samples was undertaken by the National Phenome Centre (NPC) on two different analytical platforms, NMR and UHPLC-MS using the experimental methods which are discussed below. Sorting and formatting of serum samples was also carried at the NPC.

2.2.1 NMR profiling

2.2.1.1 Experimental

The NMR profiling of serum samples was carried using methods previously described by Dona et al (113). In brief, serum samples were initially centrifuged (12000g at 4°C for 5 min) and then prepared into 96 well-plate format by adding 350 µL of sample. The NMR sample preparation was automated using a Gilson 215 liquid handling robot. 300µL of each serum sample was first aspirated from each well via the Gilson needle and then transferred into 4'' 5 mm NMR tubes. Afterwards, 300 µL of water (H₂O): deuterium dioxide (D₂O) buffer (containing 75mM disodium hydrogen phosphate (Na₂HPO₄), 6.2mM sodium azide (NaN₃), and 4.6mM TSP at pH 7.4) was added to the NMR tube via Gilson needle. Following this, the NMR tubes were sealed and shaken to homogenise the samples prior to experimental setup and were immediately loaded onto a refrigerated SampleJet robot (Bruker Corporation, Germany) and kept at 6 C until measurement. (113) A Bruker Avance III 600 spectrometer, working at 14.1 T, and equipped with a BBI probe was calibrated (temperature calibration, water suppression test, and external reference setup) prior to sample setup to check the performance of the spectrometer as well as to enable easy metabolite quantification by calibrating the signal intensity via an external reference sample. For each serum sample,

three experiments were performed: 1D ^1H -NMR general profiling using the 1D-Nuclear Overhauser Effect Spectroscopy (NOESY) pre-saturation pulse sequence (with a relaxation delay of 4s and water pre-saturation pulse applied during this period to cancel the water signal); a spin echo experiment using a 1D Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence (with a spin echo delay of 76.8ms) to attenuate the resonances belonging to macromolecules from ^1H NMR spectrum and a 2D *J*-res (with a relaxation delay of 2s and a continuous irradiation wave of 25 Hz radiofrequency strength was applied during this period for suppressing the water signal) to get the signal multiplicities were acquired in automation at 310K. Both 1D ^1H -NMR general profiling and CPMG experiments were acquired with a total of 32 free induction decays (FIDs) with 96k data points in a spectral window of 30 ppm and 20 ppm centred at 4.78 ppm for ^1H -NMR general profiling and CPMG experiments, respectively. 2D *J*-res experiments were acquired with only 2 scans over 40 increments in the indirect dimension (F2 i.e., proton channel). All the spectral processing such as line broadening of 0.3 Hz, Fourier transform, phasing and baseline correction was carried via the automated processing in TopSpin 3.6 (Bruker Corporation, Germany). QC monitoring was carried by acquiring a study reference (SR) sample for every 40 study samples for both cohorts.(113)

2.2.1.2 NMR Targeted data

Targeted analysis of the serum ^1H NMR experiments was also performed by the NPC using the commercially available Bruker *In Vitro* Diagnostics research platform (IVDr) quantification methods from Bruker Biospin (112). This platform provides quantification reports for 41 small molecules (Plasma/Serum B.I.Quant-PSTM) and 112 lipoprotein parameters (Bruker IVDr Lipoprotein Subclass Analysis (B.I.LISATM)). The small molecule quantification was done by peak fitting a series of known metabolite signals and integrating them. While the lipoprotein quantitation was done based on a PLS-2 regression model constructed using ^1H NMR spectra for the lipoprotein subfractions of human blood plasma obtained by ultracentrifugation (the gold standard for lipoprotein characterisation and quantitation. (112)

As the quantification of small molecules via Plasma/Serum B.I.Quant-PSTM provides many values which are zero if below the limit of detection while still being present in the serum

samples therefore, I also used Small Molecule Enhancement Spectroscopy (SMoESY) – select V1.1, a computational lightweight algorithm (188) to obtain quantitative measurements of 22 small molecules by integrating ^1H NMR spectra of serum samples from both cohorts. SMoESY - select provides reliable quantification of 1D ^1H NMR spectra by carrying out quick signal deconvolution and integration supported by enhancing spectral resolution as well as suppression of macromolecule signals (188).

2.2.1.3 Quality control assessment and pre-processing

A Quality control (QC) check was performed for both targeted (B.I.Quant-PSTM and B.I.LISATM) and untargeted (1D NMR general profile and the CPMG data) data by the NPC using nPYc toolbox v 1.2.1 (189) in Python which is developed to perform reproducible processing of the NMR profiling datasets (NMR and UHPLC-MS) which has been optimized to automatically assess the quality of the profiling data. Following the data pre-processing and QC assessment, 12 study samples from the general profile and 27 from the spin-echo profile were excluded from a total of 509 samples from the VANISH trial whilst in LeoPARDS 13 study samples from both general profile and spin-echo profile were excluded from a total of 1452 samples. These exclusions were based on low volume, wrong sample matrix as well as the following four set of previously defined QC criteria: i) quality of solvent suppression, ii) line width, iii) baseline consistency and iv) chemical shift calibration (113). For both experiment, solvent suppression was checked by observing the stability of the base line between the regions [(4.4, 4.5), (4.85, 5)] ppm, line width was measured using quartet resonance for lactate at [4.08, 4.14] ppm with a threshold value of 1 Hz. All spectra were calibrated to the doublet belonging to the anomeric proton of alpha-glucose and that should appear at 5.233 ppm with a 20K spectral resolution points and the stability of the baseline on the edges of the spectral window was checked between the regions [(-2, -0.5), (9.5, 12.5)] ppm. During the data processing, the spectral regions between [(-0.2, 0.2), (4.5, 4.85)] ppm were removed as these may influence the normalisation of the spectra. Those spectra which failed on any one the four criteria were considered for exclusion by the nPYc pipeline, and these spectra were excluded after checking the raw data to ensure they were excluded correctly. The values for small molecules and lipoproteins from the NMR targeted data (B.I.Quant-PSTM, B.I.LISATM and SMoESY-select) were extracted for the samples which passed the QC check for 1D NMR general profile for both study cohorts.

2.2.2 UHPLC-MS profiling

2.2.2.1 Experimental

Serum samples from both cohorts were prepared and run by UHPLC-MS via previously described methods (190-192). Serum samples were analysed by hydrophilic interaction liquid chromatography (HILIC) which is tailored for small polar metabolites whilst reversed-phase chromatography (RPC) was used for the separation of complex lipids. For VANISH, metabolite analysis was performed by aliquoting 50 μ L from each study sample for HILIC profiling and 100 μ L for lipid RPC profiling. For LeoPARDS metabolite analysis was performed by aliquoting 25 μ L from each study sample (diluted 1:1 v/v with ultrapure water) for HILIC profiling and 50 μ L (diluted 1:1 v/v with ultrapure water) for lipid RPC profiling. Prior to the sample analysis, protein removal was carried out by the addition of organic solvent (sample/isopropanol in 1:4 v/v ratio for lipid RPC profiling and diluted sample/acetonitrile in 1:3 v/v ratio for HILIC profiling) followed by a mixing and centrifugation step, resulting in a homogenous supernatant. Mixtures of method specific authentic reference materials and internal standards were added (at the dilution stage for HILIC and protein precipitation stage for RPC) to monitor data quality during the acquisition run. All the analyses were performed on ACQUITY UPLC instruments, (Waters Corp., Milford, MA, USA) coupled to mass spectrometry (Xevo G2-S TOF mass spectrometers, Waters Corp., Manchester, UK) through a Z-spray electrospray ionisation source operating in positive and negative ion modes to generate lipid positive (LPOS) and lipid negative (LNEG) datasets and HILIC positive (HPOS) dataset. Two types of QC samples were used. Study references (SR) and long-term references (LTR) were acquired during the run at regular intervals for supporting the QC assessment of the profiling data for LeoPARDS. For VANISH only SR samples were acquired during the run at regular intervals for supporting the QC assessment. An SR sample for both cohorts was prepared by pooling equal parts of each study sample for every 10 study samples and an SR dilution series, created by diluting pooled SR samples to different concentrations (i.e., 10x 100%, 5x 80%, 3x 60%, 3x 40%, 5x 20%, 10x 1%) was also acquired at the beginning and end of the acquisitions for QC assessment and data pre-processing. Full details about the QC samples, dilution series, method authentic reference materials and internal standards have been previously reported. (190, 191).

2.2.2.2 Feature extraction and annotation

UHPLC-MS datasets are formed by thousands of features making them very information rich. However, their annotation requires a labour-intensive process involving MS/MS and standard injection experiments. These were out of the scope of this thesis, where the main focus was the biological interpretation of the analytical data in the context of sepsis. Hence, we focused on the analysis of the UHPLC-MS features extracted with peakPanther (193) as the results allow easier interpretation. However, fully untargeted LC-MS data may provide additional information not available in the targeted datasets and may be analysed in future analysis.

Feature extraction in UHPLC-MS datasets was performed in a targeted manner using the R package, peakPanther. The .Raw data for UHPLC-MS profiling datasets (HPOS, LPOS and LNEG) was converted from the Waters .RAW format to .mzML (194), an open source format, with the ProteoWizard msconvert tool (13, 14). During conversion, all signals below an absolute intensity threshold of 100 were removed. Features were then extracted from UHPLC-MS profiling data (HPOS, LPOS and LNEG) using peakPanther v1.12.1, an R package for targeted annotations and integration of chemical signals (193) which generates the UHPLC-MS peakPanther datasets (HPOS ppR, LPOS ppR and LNEG ppR). This package requires two main inputs 1) A comma separated file with defined region of interest (ROI) containing information on expected retention time and theoretical m/z value of a compound to integrate signal; 2) raw MS data files in mzML or other open format and works by fitting a chromatographic line-shape model to the extracted ion chromatograms (EIC) from each ROI (193). The metabolite annotations were achieved using biologically relevant metabolites which are detectable in human biofluids, manually curated and compared to the reference material to achieve the highest level of confidence (192).

2.2.2.3 Data quality control and batch correction

Feature filtering and mitigation of any potential run order and batch effects was performed on all the UHPLC-MS peakPanther datasets (HPOS ppR, LPOS ppR and LNEG ppR) by the NPC via nPYc Toolbox v 1.2.1 (189). During the QC assessment, 26 samples from a total of 1479 samples from LNEG ppR, 28 and 30 samples from a total of 1477 samples from HPOS and LPOS ppR, respectively, were excluded from the LeoPARDS dataset. These exclusions

were based on samples not available mainly due to the low volume, suspected to have different sample matrix (i.e., urine; N = 2 in all three datasets), or raw data file corrupted (N = 1 only in LPOS ppR).

The features were selected from all ppR datasets (HPOS, LPOS, LNEG) from both cohorts based on the following criteria: 1) High precision (i.e., Pearson correlation to dilution in the SR dilution series > 0.7) 2) High accuracy (residual standard deviation in the SR samples < 30%) (189). In LeoPARDS, 65 features from HPOS ppR, 89 features from LNEG ppR, and 259 features from LPOS ppR passed the feature selection criteria whilst in VANISH, 50 features from HPOS ppR, 94 features from LNEG ppR, and 309 features from LPOS ppR passed this feature selection criteria. All these selected features were kept for biological analysis.

2.3 Statistical analysis methods

All analyses (except for the multivariate and trajectory analysis of NMR untargeted data) were carried in the R programming language v3.6.1 (The R foundation) (195). Statistical analysis of targeted data, including univariate and trajectory analysis was carried out on the common/matching features (i.e., small molecules, lipids, and lipoproteins) between the two study cohorts. In total, there were 22 small molecules from SMolESY-select, 39 small molecules from HPOS ppR data, 110 lipoproteins from BI LISA, 246 lipids from LPOS and 63 lipids from LNEG ppR. The univariate analysis and the validation of univariate results from the targeted datasets was performed separately for each NMR and UHPLC-MS targeted dataset whereas all the trajectory analysis and the analysis focusing on metabolic changes with organ failure scores were all performed on the combined datasets from both NMR and UHPLC-MS. The combined targeted dataset for small molecules resulted by combining SMolESY-select and HPOS ppR data whilst the combined dataset for lipids and lipoproteins resulted by combining BI LISA, LPOS ppR and LNEG ppR. All the figures and boxplots related to the statistical analysis were generated in R-studio 3.6.1 (The R foundation) (195) using ggplot2 (196) unless specified. Data related to additional drugs, nutrition and fluid therapy were not available for either study so could not be included in any of the statistical analysis.

Linear regression analysis for tyrosine association with albumin and noradrenaline dosage for VANISH was performed on the tyrosine and albumin spectral regions integrated from NMR untargeted data. Spectral regions for tyrosine (7.18-7.20 ppm and 6.88-6.91 ppm)

were integrated from CPMG spectra and the albumin regions (0.3-0.5 ppm, 6.0-6.50 ppm, and 9.0-10.0 ppm) were integrated from ^1H -NMR spectra of 287 samples (TP0 = 154 and TP1 = 133 from TP1) in MATLAB R2020a (197). Three samples were excluded from this analysis due to high albumin concentration.

2.3.1 Baseline characteristics

Continuous variables for baseline characteristics of patients were compared using a non-parametric Mann-Whitney's U test (198) while categorical variables were compared via Chi squared (χ^2) test, with Yates' continuity correction applied to those variables where the frequency of one of the cell was less than 5 in a 2x2 contingency table (199, 200). The p values obtained from Mann-Whitney U test, and χ^2 with test were adjusted with Benjamini-Hochberg (B-H) correction which controls the false discovery rate (FDR) ($\text{FDR} = \text{False Positives} / (\text{False Positives} + \text{True Positives})$) (201) i.e. the expected proportion of false discoveries).

2.3.2 Multivariate analysis of untargeted data (CPMG)

SIMCA 15.0 (Umetrics, Sweden) was used for the multivariate statistical models (PCA and PLS-DA) looking for metabolic differences between survivors and non-survivors at all timepoints using CPMG data from VANISH. In multivariate models, analytes which were present in higher concentrations are generally associated with higher variance and may dominate the multivariate model as the models focus on the variation between the samples. For this reason, CPMG data was scaled to unit variance, so that every variable including those which were less abundant had the same weight as those variables with higher concentrations. Initially, CPMG data was explored with PCA to look for natural clustering and to detect outliers before performing supervised multivariate analysis using PLS-DA. Multivariate PLS-DA models were generated to optimally detect the metabolites responsible for differentiating between the prespecified groups; survivors and non-survivors at day-29. Cross-validation for PLS-DA models was performed using seven-fold cross validation method. Receiver operating characteristic (ROC) analysis was also performed on the cross validated data to determine the discriminant potential of the PLS-DA models. Loading line plots for each multivariate model were also generated and examined to identify important spectral signals and the discriminating metabolites were selected based on the

correlation coefficients i.e., $p(\text{corr})$. A combination of several different techniques was employed to identify the important signals belonging to the discriminatory metabolites from CPMG data. Initially, STOCSY was performed in R 3.6.1 (The R foundation) (195) via in-house R-script to look for peak correlation within the acquired spectra (143). Metabolites were then found in the SBASE database using AMIX 3.9.11 software or in published literature (202, 203).

2.3.3 Univariate analysis of Targeted data

Univariate analysis for all the NMR (SMoIESY-select and BI LISA) and UHPLC-MS (HPOS ppR, LPOS ppR and LNEG ppR) targeted datasets from both cohorts was performed using Welch two-sample t-test. The normality of the features (i.e., small molecules, lipids and lipoproteins) from targeted datasets was checked using histograms, Quantile-Quantile plot (i.e., Q-Q plot) and Shapiro-Wilk normality test (204). NMR targeted data (SMoIESY-select and BI LISA) was not log transformed as the data passed the normality check. Conversely, since there were no consistent results for the Shapiro-Wilk normality test for all the features from UHPLC-MS datasets, UHPLC-MS data (HPOS ppR, LNEG ppR and LPOS ppR) was log transformed to avoid heteroscedasticity and make the distribution prior to further statistical testing. The p-values obtained from the Welch two-sample t-test were adjusted with B-H correction. Box plots for the selected variables were generated using the R package ggplot2 (196) and the reshape2 package (205) for data formatting.

2.3.3.1 Prediction of outcome based on metabolic changes in the first 24 h

The predictive models (i.e., logistic regression models, fitted with generalised linear models (GLMs)) were generated for the validation of mortality results for both NMR (SMoIESY-select, BI LISA) and UHPLC-MS targeted datasets (i.e., HPOS ppR, LPOS ppR and LNEG ppR). In this analysis samples from TP0 and TP1 were used, as the metabolic differences in relation to outcome were found at these two timepoints. The predictive models were built separately for each timepoint using the raw intensity values from the NMR targeted data and log transformed intensity values from UHPLC-MS targeted data. The validation of mortality results was performed in two ways, one using cross-validation within the study cohort (i.e., within LeoPARDS or VANISH as shown in figure 2.2) and the other by using the other cohort as an external test set. Mortality validation within the study cohorts (i.e.,

LeoPARDS and VANISH) was performed by splitting samples from the first and second timepoint separately into a training and test set using 7-fold cross validation (figure 2.3). Afterwards, a mortality model was built by first using Welch two sample t-test on the training set from each cohort at individual timepoints to select features based on their statistical significance ($p < 0.05$) and then use only the selected metabolites to build a logistic regression classifier (i.e., GLM fitted) on the training set. This model was then tested on the cross-validation test (from the same cohort) and a confusion matrix obtained. The whole procedure was repeated 7 times and at the end a cross validated ROC curve was obtained for each timepoint of the two study cohorts by using an R package called pROC (206) and the receiver operating characteristics (ROC) curve was plotted. The R caret v6.0-88 package (207) was used to implement model fitting and cross-validation schemes.

The confusion matrix is a comparison of sample assignments against true class membership. Samples can be assigned into four groups: 1) True Positives (TP); 2) True Negatives (TN); 3) False Positives; 4) False Negatives (208). TP are the samples which are classified as positives and belong to the positive class whilst TN are the samples which are classified as negatives and belong to the negative class. FP are the samples which actually belong to the negative class but are incorrectly classified as positive whilst FN are the samples which actually belong to the positive class but are incorrectly classified as negatives. (208, 209)

Sensitivity is used to test how well a model is able to correctly classify the samples so it is calculated as the ratio of true positives with respect to the total number of samples actually in the class (equation 1 i.e., eq.2.1) (135). Specificity is the proportion of samples which do not belong to a class that are tested negative (eq. 2.2) (135). Sensitivity and specificity are crucial parameters for evaluating the performance of a test, however, it is also important to know how likely a sample is to truly belong to the class given a positive test (210). Positive predictive value is the probability that the sample is truly positive given a positive test (eq. 2.3) whilst negative predictive value is the probability that the sample is truly negative given a negative test (eq. 2.4) (210).

$$\text{Sensitivity} = \frac{TP}{TP+FN} \quad (\text{eq. 2.1})$$

$$\text{Specificity} = \frac{TN}{TN+FP} \quad (\text{eq. 2.2})$$

$$\text{Positive Predictive Value} = \frac{TP}{TP+FP} \quad (\text{eq. 2.3})$$

$$\text{Negative Predictive Value} = \frac{TN}{TN+FN} \quad (\text{eq. 2.4})$$

The ROC curve is one of the analytical approach which is used to assess the performance of a diagnostic classification method for a binary classifier (209). It plots sensitivity against 1-specificity for a test. A ROC curve can be constructed from a typical PLS-DA model as such that the estimated values of y (i.e., outcome) can be converted back into the original class membership (such as 1 and 0's for cases and controls, respectively) by relating them to a set discriminative threshold (135). To perform the predictions for the two classes, the discriminative threshold is usually set somewhere at 0.5 when y is a vector of 0 and 1's for samples belonging to class of controls and cases, respectively. If the estimated y value for a sample is greater than or equal to 0.5 then the sample is assigned to class of cases and if the predicted y value of a sample is less than 0.5 then the sample is assigned to class of controls. Once all the samples have been predicted and assigned to a class membership at a set threshold, then the total number of samples which are TP, TN, FP, and FN can be computed into a confusion matrix. Following which sensitivity (i.e., true positive rate (TPR)) and 1-specificity (i.e., false positive rate (FPR)) can be determined and plotted as a point in a graph with "1-specificity" as the x-coordinate and "sensitivity" as the y-coordinate. The threshold used for the class prediction can be adjusted so that both sensitivity and specificity are maximized following which pairs of sensitivities and specificities are obtained which can then be plotted as a ROC curve which summarises all the confusion matrices produced at each discriminative threshold. (135, 209, 211).

The overall performance of a tests is assessed by calculating the area under the ROC curve (AUC). The closer the ROC-curve to the upper top-left corner of the graph and the closer an AUC is to 1.0 the better the performance. In the ROC curve, a straight diagonal line (i.e., $y = x$) plotted from the origin with coordinates (0,0) (for sensitivity and FPR, respectively) to the extreme top-right corner (coordinates (1,1) for sensitivity and FPR, respectively) represents the performance of a random classifier where the TPR = FPR. AUC values close to 0.5 indicate a model with no discrimination. In general, an AUC of 0.8 is acceptable for binary classifier and when comparing the diagnostic performance of two tests, the ROC curve with the larger AUC will have better diagnostic performance for a classifier. (135, 209, 211)

ROC curves have some strengths and weaknesses. Firstly, they provide a concise visualisation of a test's performance (209). Secondly the ROC curve is not affected by the prevalence of a disease as it is a function of sensitivity and specificity (209). However, ROC curves also have weaknesses. ROC curves do not directly display the threshold value for differentiating cases from controls or the number of samples used in analysis (209). The AUC of a ROC curve lacks clinical interpretability as it does not reflect the effect for an individual patient and it evaluates performance across an entire range of classification thresholds both clinically relevant and irrelevant (212). Additionally, the AUC might be the same for different tests despite their performance differing at clinically relevant thresholds. Utilising only AUC can be misleading especially when comparing the ROC curves of two diagnostic tests which cross each other. (212)

External validation for mortality was performed with a similar procedure as used for the within cohort validation shown in figure 2.2 but with the training and test sets obtained from two separate cohorts (i.e., VANISH and LeoPARDS) for each individual timepoints as shown in figure 2.3 without splitting the data at any stage. In external validation, the univariate model for mortality was trained (i.e., univariate feature selection and fitting of the logistic regression classifier i.e., GLM) using the dataset from one cohort for each timepoint separately (e.g., in VANISH) and then testing it on the dataset from the other cohort (e.g., LeoPARDS) and vice versa (figure 2.3). A ROC curve was obtained at the end for each timepoint for both external validation cohorts.

The model optimised in figure 2.2 was not applied in figure 2.3. Cross validation was performed internally to determine if the model was worth further investigation. Once this was confirmed, external validation was performed using the full dataset from one cohort to build a model which was then tested on the independent dataset from the alternative cohort.

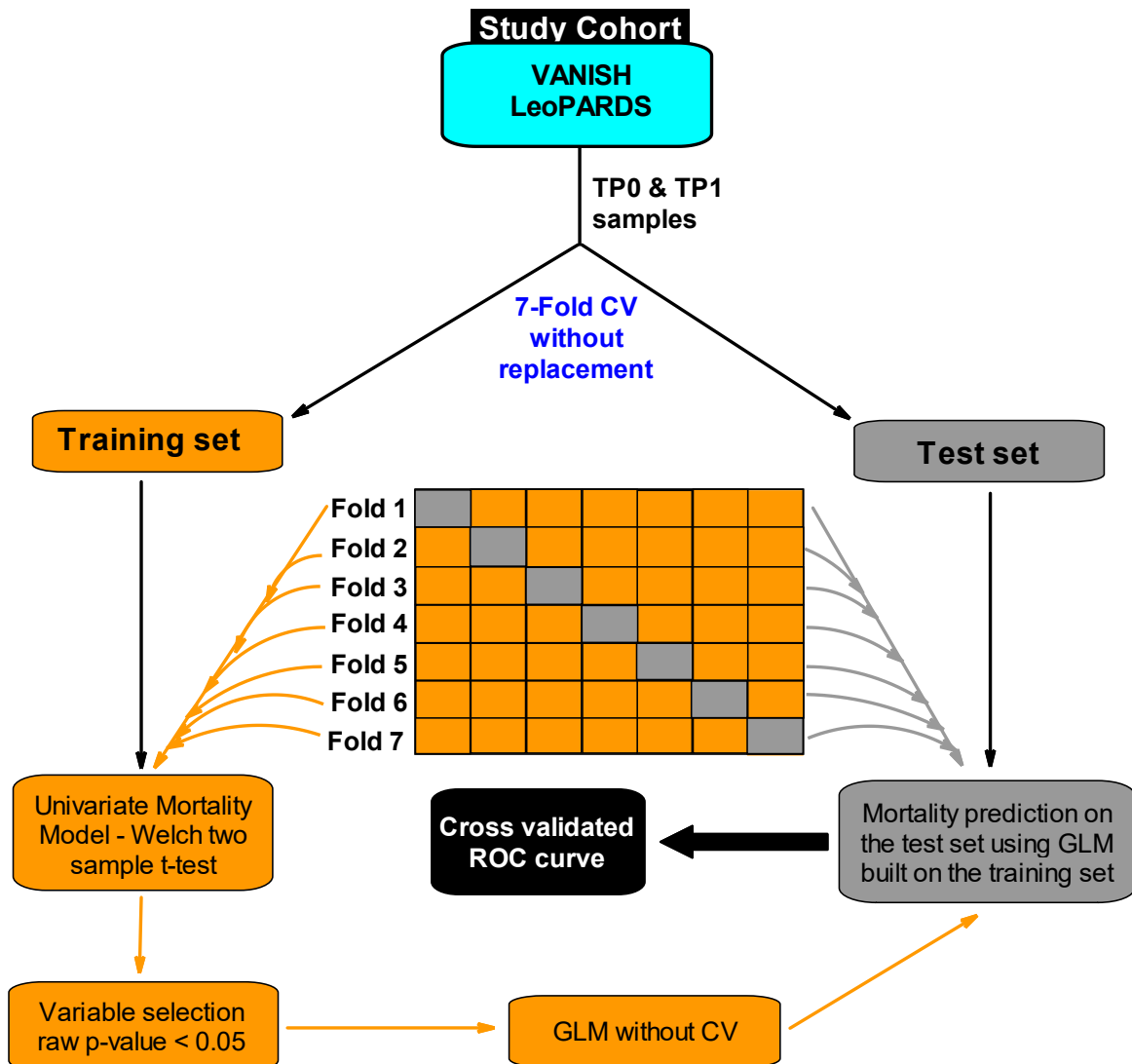


Figure 2.2: A flow chart for the procedure involved in validating mortality results within the study cohorts (VANISH and LeoPARDS) for TP0 and TP1 samples. Abbreviations: GLM = Generalised linear model; CV = Cross validation; ROC = Receiver operating characteristics; TP0 = At ICU admission / at time of randomisation; TP1 = After 24h of ICU admission.

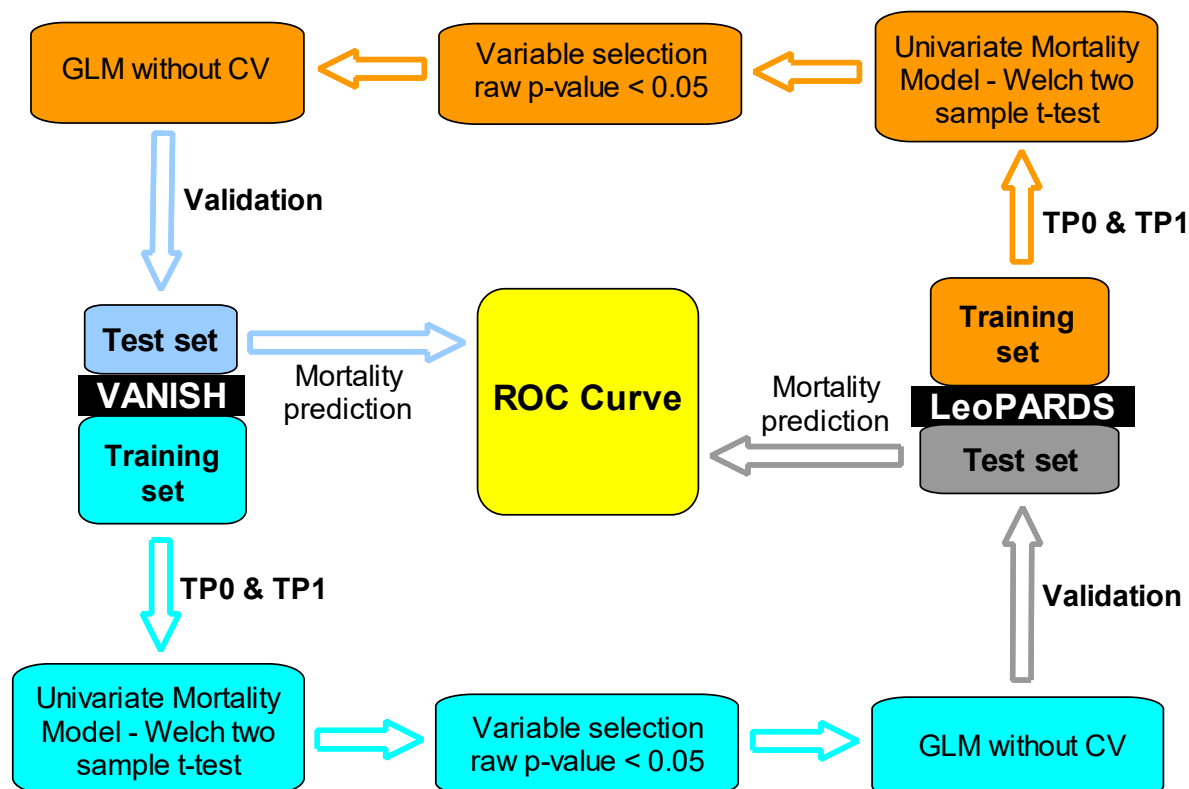


Figure 2.3: A flow chart for the procedure involved in validating mortality results of TP0 and TP1 samples from LeoPARDS in VANISH (highlighted in orange and pale blue) and vice versa (highlighted in turquoise and grey). Abbreviations: GLM = Generalised linear model; CV = Cross validation; ROC = Receiver operating characteristics; TP0 = At ICU admission / at time of randomisation; TP1 = After 24h of ICU admission.

2.3.4 Trajectory analysis of untargeted data (CPMG)

To understand temporal variation of CPMG metabolic profiles, data from VANISH, where difference spectra were obtained by subtracting spectral data (i.e., intensities) of the baseline samples from the last timepoint sample available (24h, 48h, or 96h of ICU admission) for that patient. In this analysis, only matching pairs of samples were used. The spectral data for the difference spectra was scaled to unit variance. Supervised and unsupervised multivariate models (PCA and PLS-DA) were built for this analysis using the pyChemometrics package (213). Means of the difference spectra for survivors and non-survivors were generated separately to compare the overall metabolic profiles over time, thus providing the direction of the change indicated by a positive or negative signal in the mean difference spectra, figure 2.4. Metabolites which were found to have different trajectories from PCA and PLS-DA multivariate models were then viewed by plotting a mean spectra of the difference spectra between these two groups and were then identified via the SBASE database using AMIX 3.9.11 software or in published literature (202, 203).

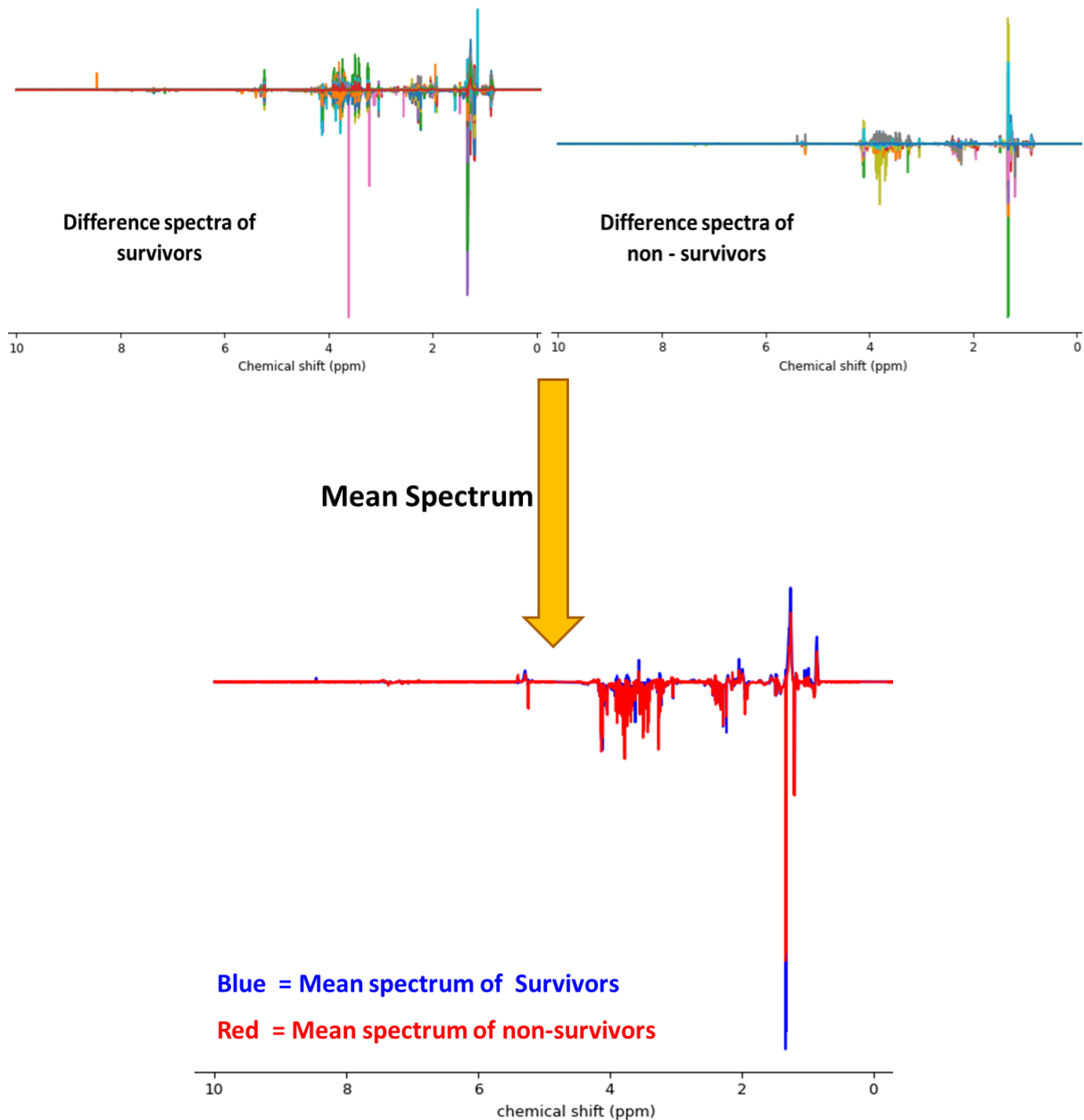


Figure 2.4: Mean spectra of the difference spectra for survivors and non-survivors.

2.3.5 Linear mixed effect model analysis

Linear mixed effects (LME) models are regression models (also known as hierarchical, nested, panel data, or multi-level models) which extend standard linear regression models by adding the capacity to model clustered observations (e.g., repeated samples from a patient) via estimation of variance parameters (the random effects) in addition to the standard regression coefficients (the fixed effects). Fixed effects are effects which estimate

the mean effect (i.e., regression coefficients) for all the possible levels of interest (such as age, sex, outcome, etc) being incorporated in an experiment, in the same manner as a standard regression model. Random effects are effects which are considered to be sampled randomly from an infinitely large population of levels, and the estimate of interest is the variance associated with that population, not the specific mean effects for each patient. The random effect variance estimates can also be used to obtain shrinkage estimates of the mean coefficients (designated as best linear unbiased predictor, or BLUP estimates) for each observed level of a random effect, for example, the mean intercept or slope across time for each patient. This feature is used in mixed effect models to obtain patient specific estimates. (127, 141, 142, 214-216)

A basic LME model can be written as in eq.2.5, whereby y is a n -vector of observed records (i.e., responses), X is a known $n \times p$ design matrix which links observed records in y with the p -vector of fixed effects coefficients β , Z is a known $n \times q$ design matrix which links observed records in y with q -vector of random effects coefficients b , and e is a vector of residual or random errors (127, 215).

$$y = X\beta + Zb + e \quad (\text{eq. 2. 5})$$

These models are particularly useful in the case of longitudinal data as it allows both inter- and intra- individual variability to be accounted for where the assumptions related to constant variance and independence are generally not met (127, 137, 141). Therefore, LME models are more capable of accounting for a wide range of nuisance variables and are better at estimating the fixed effect of interest than standard linear models (217).

All the LME models for the targeted data from both cohorts were generated using the lme4 R package (218). The targeted data used in all the LME models from both cohorts was log transformed to handle heteroscedasticity and to reduce each distribution's skewness.

The variables used in LME models are as follows:

- 1) Metabolite: The intensity of each metabolic profile feature (such as small molecules, lipids, and lipoproteins), modelled as the dependent variable from the NMR and UHPLC-MS datasets for each sample from each patient. Each metabolite was log transformed with an offset of 1 to handle zeroes during log transformation.

- 2) Time post TPO (days): A numeric variable which contains the difference between the timing of each sample from baseline/ICU admission (i.e., TPO) in days.
- 3) Mortality at Day 29: A variable with mortality outcome for each patient at day 29 with two categories, survivors and non-survivors.
- 4) Sitename: A variable containing hospital sites where the samples were collected.
- 5) Subject Number: A variable with unique patient ID for the samples collected across four different timepoints.
- 6) Age: A variable containing information about the age of each patient.
- 7) Sex: A categorical variable with information about the sex of each patient.
- 8) SOFA score: A variable which contains information on the SOFA scores for each patient at each timepoint.
- 9) Drug1_Administered: A binary variable which indicates whether drug 1 (i.e., noradrenaline or vasopressin) had been administered or not at the time of sample collection.
- 10) Drug 1: A variable which indicates which type of allocated drug 1 based on the study protocol (i.e., noradrenaline or vasopressin) had been given at the at the time of sample collection.
- 11) Drug2: A variable with three categories (Hydrocortisone, Placebo and Pre drug2) which indicates whether the study drug2 had been given, and if so, which drug had been used at the time of sample collection.
- 12) Hydrocortisone: A binary variable which indicates whether the hydrocortisone had been given at the time of sample collection.
- 13) Placebo: A binary variable which indicates whether placebo had been given at the time of sample collection.

All LME models used a random effect structure with a random intercept per individual, another random intercept per study site, and a random slope per individual for time. The random intercept and random slope per individual terms model inter-patient variability at baseline and changes with time (more specifically, the “Time post TPO (days)” variable),

while the random intercept per site accounts for deviations from different hospital sites. Confounding by age and sex, was accounted by modelling these as fixed effects in all LME models. All the LME models were fitted using a restricted maximum likelihood (REML) method. The R package *emmeans* (219) was used to estimate the slopes/trends and marginal means (EMMs) for contrasts of interest in all the LME models. The Satterthwaite approximation method was used to estimate the degrees of freedom to use in the statistical tests of contrasts, using the *pbkrtest* package functions via the *emmeans* package interface for contrast coding (220-222). The Welch *t*-test was used to test for differences between means or trends in all the pairwise contrasts comparisons (from mortality analysis, drug analysis) and identity contrasts (from the organ failure score analysis). To obtain multiple test corrected metabolic signatures, the p-values for each contrast were pooled together (across all metabolites for a given assay) and were adjusted with the Benjamini-Hochberg (B-H) FDR correction (201). In all the figures related to LME analysis, metabolites (small molecules, lipids and lipoproteins) were grouped based on the compound classes obtained from the Human Metabolome Database (HMDB) (223) and then arranged in a specific order which was kept the same throughout chapters 4 and 6.

2.3.5.1 Trajectory analysis of outcome

LME models for metabolites from targeted data from both cohorts were generated according to the formula: `Outcome_model <- log(Metabolite + 1) ~ 1 + `Time post TP0 (days)`*`Mortality at Day 29` + `Age` + `Sex` + (`Time post TP0 (days)`|`Sample Number`) + (1|`Sitename`)`. In LME analysis of mortality, the two variables including Time post TP0 (days) and Mortality at Day 29 and the interaction between these two were the main predictor variables (i.e., the fixed effects). The metabolites from the combined targeted data were the main response variables for these LME models. The change in the metabolite levels over time in survivors and non-survivors was obtained as an overall slope/trend for these two clinical groups, based on Time post TP0 (days) as a function of Mortality at Day 29 and a pairwise comparison was performed to obtain the difference between the slopes of these two groups (i.e., an estimate) via *emtrends*. Estimated marginal means (EMMs) based on the fitted LME models were obtained for survivors and non-survivors at days 0 and 4 via the *emmeans* package and day 4 was used in these comparisons as it was the latest sampling timepoint from both cohorts. Additionally, estimates (i.e., the difference between

the estimated marginal mean levels of metabolites) of four pairwise comparisons were calculated: [(1) Difference between the EMMs of survivors at days 4 and 0 (4,Alive-0,Alive0); 2) Difference between the EMMs of non-survivors at days 4 and 0 (4,Dead-0,Dead); 3) Difference between the EMMs of survivors and non-survivors at day 0 (0 Alive-0,Dead); 4) Difference between the EMMs of survivors and non-survivors at day 4 (4,Alive-4,Dead)] were also obtained. Un-adjusted and adjusted p-values were calculated as discussed previously. As several studies have revealed that low body mass index (BMI) is associated with increased mortality whilst high BMI is linked to reduced mortality in patients with sepsis (224-227), for this reason the same trajectory models for mortality were also generated, but with BMI as a confounder to account for this. The metabolic trajectories of survivors and non-survivors were determined again with the introduction of BMI as a fixed effect variable into the main model formula whilst keeping everything else constant in the LME model. The results for LME model with and without BMI were compared.

2.3.5.1.1 Prediction of outcome based on metabolic changes from baseline

In this analysis TP0 samples matching with TP1 and TP2 were used. Fold-changes between TP0 paired samples with TP1 (TP0TP1) and TP2 (TP0TP2) for combined data from both cohorts were obtained. In cases where the minimum value was zero, a missing data imputation rule was applied and for that variable zero was replaced with the half of the non-zero minimum value for that metabolite. The fold-change data from both cohorts was log transformed in R 3.6.1 (The R foundation) (195) to symmetrize the distribution.

The predictive models (i.e., logistic regression models, fitted with GLMs) were generated for the validation of mortality results from the LME models for both NMR (SMoIESY-select, BLISA) and UHPLC-MS targeted datasets (i.e, HPOS ppR, LPOS ppR and LNEG ppR). In this analysis fold-change data from TP0 samples matching with TP1 and TP2 was used. The mortality prediction models were built separately for each fold-change data using the log transformed intensity values from the combined datasets (SMoIESY-select and HPOS ppR; BLISA, LPOS ppR and LNEG ppR) from both cohorts. The validation of mortality results was performed in two ways, one within the same study cohort (i.e., within LeoPARDS or VANISH as shown in figure 2.5) and the other with the other cohort.

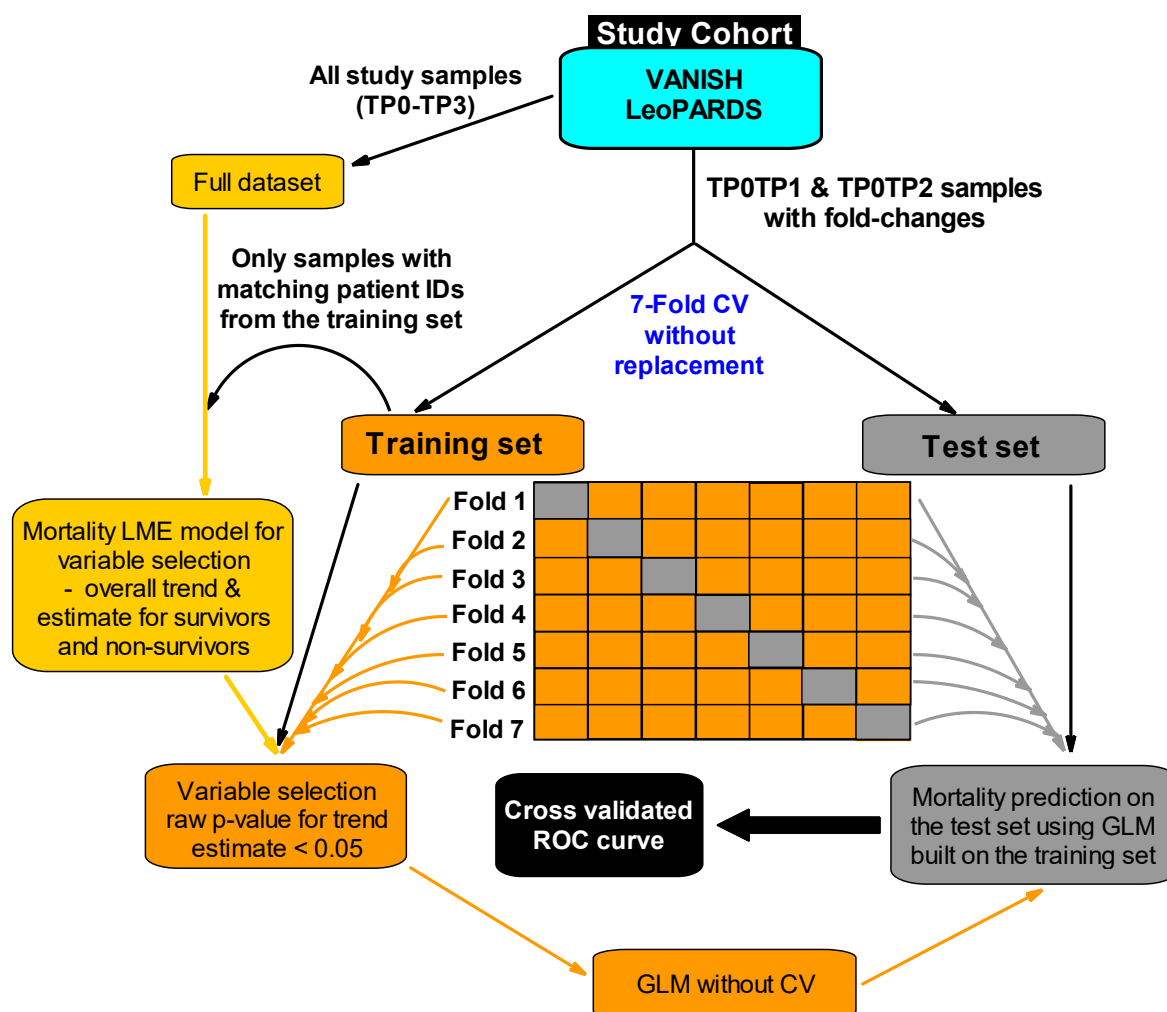


Figure 2.5: A flow chart for the procedure involved in validating mortality results within the same study cohorts (VANISH and LeoPARDS) for TP0TP1 and TP0TP2 samples with fold changes. Abbreviations: GLM = Generalised linear model; CV = Cross-validation; LME = Linear mixed effects; ROC = Receiver operating characteristics; TP0TP1 = TP0 matched TP1 samples with fold changes; TP0TP2 = TP0 matched TP2 samples with fold changes.

Validation within the same study cohorts (i.e., LeoPARDS and VANISH) was performed by splitting foldchange values into training and test set (6 parts in the training and 1 part in the test set) using 7-fold cross validation (figure 2.5) via carets package (207). Afterwards, lme4 (228), an R package was used to build an LME model for variable selection on the full dataset with only the samples which had unique patient IDs matching with TP0TP1 and TP0TP2 samples with fold-changes from each cohort. This LME model was based on mortality and also had the same model structure as previously mentioned in section 2.3.5.1, with the same confounders (age and sex), random and fixed effects as before. Contrast coding was also performed via emtrends which gave trend estimates based on outcome for each metabolite of all matching samples from the whole dataset of each cohort. Then, the

metabolites which had significant difference between the slopes of survivors and non-survivors over time based on the raw p-values were selected ($p < 0.05$) from the full model of each cohort and then same metabolites were also selected from the data with samples with fold-changes of each cohort. These metabolites were used to build a logistic regression model which was fitted as a GLM on the training set from TP0TP1 or TP0TP2 samples with fold-changes, which was then tested on the test set and a confusion matrix summarising the performance of the classification model showing the actual and predicted patients (i.e., survivors and non-survivors) was obtained. The whole procedure was repeated 7 times and at the end a cross validated ROC curve was obtained for each paired timepoints (i.e., TP0TP1 and TP0TP2) of the two study cohorts by using an R package called pROC (206).

External validation was performed with a similar procedure as used for the within cohort validation shown in figure 2.5, but with the training and test sets obtained from alternative cohort as shown in figure 2.6 but without splitting the data at any stage. In external validation, the same LME models focusing on mortality as reported in section 2.3.5.1, with the same confounders (age and sex), random and fixed effects as before were built on the full dataset from one cohort (e.g., in VANISH). Contrast coding was also performed via emtrends which gave trend estimates based on outcome for each metabolite from the whole dataset of one cohort (e.g., in VANISH). Then, the metabolites which had significant difference between the slopes of survivors and non-survivors over time based on the raw p-values were selected ($p < 0.05$) initially from the whole dataset from one cohort (e.g., in VANISH) and then the same metabolites were also selected from the samples (i.e., TP0TP1 and TP0TP2) with fold-changes from that cohort (e.g., VANISH) followed by building a logistic regression model fitted as a GLM on the fold-change data from this cohort using the significant metabolites ($p < 0.05$) selected from the LME models on the whole dataset. These GLMs were then tested on the fold-change data of the external dataset (e.g., LeoPARDS) and vice versa (figure 2.6). A ROC curve was obtained at the end for each timepoint for both external validation cohorts.

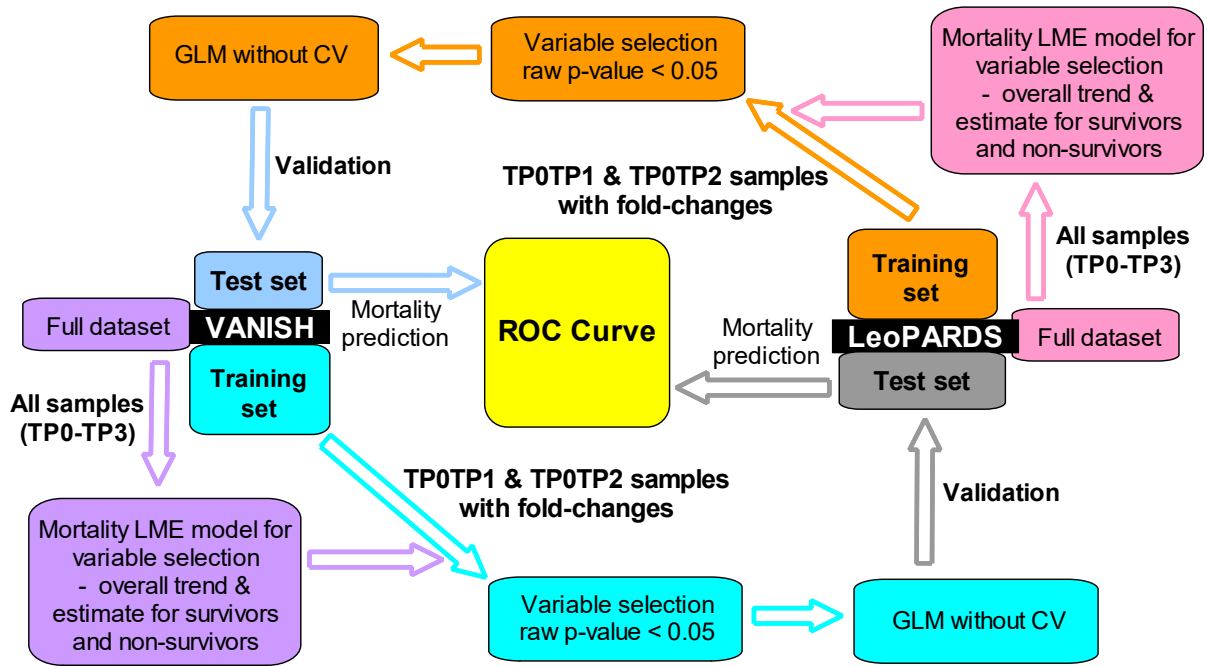


Figure 2.6: A flow chart for the procedure involved in validating mortality results of TP0TP1 and TP0TP2 samples with fold changes from LeoPARDS in VANISH (highlighted in orange, pink and pale blue) and vice versa (highlighted in turquoise, purple, and grey). Abbreviations: GLM = Generalised linear model; CV = Cross-validation; LME = Linear mixed effects; ROC = Receiver operating characteristics; TP0TP1 = TP0 matched TP1 samples with fold changes; TP0TP2 = TP0 matched TP2 samples with fold changes.

2.3.5.2 Metabolic changes with organ failure scores

LME models were also built on the targeted datasets to find out how the metabolites change with organ failure defined by SOFA scores (including total SOFA, cardiovascular, coagulation, renal, respiratory, and liver scores). In this analysis, each sample was matched with the SOFA score recorded on the day of sampling. LME models for the total and each component of the SOFA score were built independently and were based on the same model structure with formula: $\text{SOFA_model} \leftarrow \log(\text{Metabolite} + 1) \sim 1 + \text{Time post TP0 (days)} + \text{SOFA Score} + \text{Age} + \text{Sex} + \text{Time post TP0 (days)} | \text{Sample Number} + (1 | \text{Site name})$. In these LME models the same random effects and confounders (i.e., age and sex) were added to the model as before. In these LME models, SOFA score was the main predictor variable and Time post TP0 (days) was added as a numeric covariate. Later, identity contrast was performed for each element of the SOFA score via emtrends. This provided slope (i.e., an overall slope for each metabolite in relation to the SOFA score for its relationship with each metabolite as well as the estimate (i.e., the difference of slope from baseline i.e., 0) and the p-value was obtained via identity contrast using the Welch two sample t-test with Satterthwaite approximation method for degrees of freedom and indicated whether this

slope is significantly different from 0 or not. The adjusted p-values were calculated via B-H correction. As previously mentioned in section 2.3.5.1, that low BMI is associated with worse outcomes (as in mortality) (224-227) and sepsis patients have various degree of organ dysfunction/failure, so the same LME models for all the SOFA scores were also generated but with the BMI as a confounder.

2.3.5.3 Metabolic changes and trajectories in response to trial drugs

LME models for the trajectory analysis of small molecules focusing on the treatment given to the patients enrolled onto the VANISH trial were generated on the combined NMR and UHPLC-MS targeted datasets. As mentioned previously, that patients enrolled onto the VANISH trial were randomised to receive drug 1 (i.e., noradrenaline or vasopressin) followed by drug 2 (i.e., hydrocortisone or placebo). The random effects and confounders (i.e., age and sex) were kept the same as the models from the trajectory analysis of mortality.

2.3.5.3.1 Noradrenaline vs vasopressin

The trajectory analysis for study drug 1 (i.e., noradrenaline and vasopressin) was performed for small molecule metabolites by generating LME models according to the formula: `Drug1_model <- log('Metabolite' + 1) ~ 1 + 'Time post TP0 (days)' * 'Mortality at Day 29' + 'Drug1_Administered' + 'Time post TP0 (days)' * 'Drug 1' + 'Hydrocortisone' + 'Placebo' + 'Age' + 'Sex' + ('Time post TP0 (days)' | 'Sample Number') + (1 | 'Site name')`. In this LME model the interaction between time post TP0 in days and drug1 allocation (i.e., a variable with two categories i.e., noradrenaline and vasopressin) was the main predictor variable (i.e., the fixed effect) and drug1 administered at each timepoint sample (i.e., a binary variable) was also added as a fixed effect to account for whether the drug1 was given or not at the time of the sample. In addition, the interaction between Mortality at day 29 and Time post TP0 (days) was also introduced in this model as a covariate. Models with and without this covariate did not show any major differences so this covariate was kept in the main drug1 model to account for mortality. Additionally, two binary variables concerning drug2 (i.e., whether the patients had received hydrocortisone or placebo at each timepoint sample or not) were also introduced into the LME model as an additive effect to account for drug 2. An overall trajectory based on Time post TP0 (days) as a function of Drug1 was obtained for each group (i.e., noradrenaline and vasopressin) and the difference between the slopes of

the two drugs was obtained via a pairwise comparison. In addition, the EMMs based on the fitted LME models were obtained via the interaction between the two variables, Drug 1 and Time post TP0 (days) at days 0, 1 and 4 (from Time post TP0 (days) variable) via emmeans. The estimates (i.e., the difference in the EMMs) of 7 desired groups : 1) Difference between the EMMs of noradrenaline at days 4 and 0 (4,Noradrenaline-0,Noradrenaline); 2) Difference between the EMMs of noradrenaline (i.e., Dead) at days 4 and 1 (4,Noradrenaline-1,Noradrenaline); 3) Difference between the EMMs of vasopressin at days 4 and 0 (4,Vasopressin-0,Vasopressin); 4) Difference between the EMMs of vasopressin at days 4 and 1 (4,Vasopressin-1,Vasopressin); 5) Difference between the EMMs of noradrenaline and vasopressin at day 0 (0,Noradrenaline-0,Vasopressin); 6) Difference between the EMMs of noradrenaline and vasopressin at day 1 (1,Noradrenaline-1,Vasopressin); 7) Difference between the EMMs of noradrenaline and vasopressin at day 4 (4,Noradrenaline-4,Vasopressin) were obtained by pairwise comparisons. Unadjusted and adjusted p-values obtained from the pairwise comparisons of slopes and EMMs were calculated as discussed previously.

2.3.5.3.2 Hydrocortisone vs Placebo

Two types of LME models were built for the analysis of drug 2 (i.e., hydrocortisone and placebo) on the combined NMR and UHPLC-MS targeted datasets from VANISH which are as follows:

1) Metabolic changes with drug 2

An LME model for drug 2 was built for every small molecule according to the formula: $\text{Drug2_model1} \leftarrow \log(\text{'Metabolite'} + 1) \sim 1 + \text{'Time post TP0 (days)'} * \text{'Mortality at Day 29'} + \text{'Drug2'} + \text{'Age'} + \text{'Sex'} + (\text{'Time post TP0 (days)'} | \text{'Sample Number'}) + (1 | \text{'Sitenam'})$. These LME models were generated to find out how the metabolome changes with drug 2 allocation (hydrocortisone or placebo). In this model the same random effects and confounders were added as the mortality analysis. The interaction between Mortality at Day 29 and Time post TP0 (days) was also introduced in this model as a covariate. In this model, the variable Drug2 with three categories (Hydrocortisone, Placebo and Pre drug2) was the main predictor variable (i.e., the fixed effect). Later, contrast coding was employed to obtain EMMs for Drug2. The difference in the EMMs (i.e., estimate) of three desired

groups: 1) Difference between the EMMs of patients who had received hydrocortisone and those who had received placebo (Hydrocortisone – Placebo); 2) Difference between the EMMs of patients who had not yet received study drug 2 and those who had received hydrocortisone (Pre drug2 – Hydrocortisone); 3) Difference between the EMMs of patients who had not yet received study drug 2 and those who had received placebo (Pre drug2 – Placebo) were obtained by pairwise comparisons.

2) Metabolic trajectories of drug2

An LME model for the trajectory analysis of drug 2 was built in the same way as the previous LME model using the formula: `Drug2_model2 <- log('Metabolite' + 1) ~ 1 + 'Time post TP0 (days)' * 'Mortality at Day 29' + 'Time post TP0 (days)' * 'Drug2' + 'Age' + 'Sex' + ('Time post TP0 (days)' | 'Sample Number') + (1 | 'Sitename')`, but here the main predictor variable (i.e., the fixed effect) was the interaction between Time post TP0 (days) and Drug2. This predictor variable was introduced to find the trend of metabolites over time for drug2. The random effects, confounders and other covariates were the same as LME model 1. Later, contrast coding was performed using `emmeans` to look for trends based on Time post TP0 (days) as a function of Drug2. This provided slope (i.e., an overall trajectory for each group (Hydrocortisone, Placebo and Pre-samples) as well as the difference between the slopes for each group (i.e., estimate). In addition, EMMs via `emmeans` for Drug2 were also obtained. Estimates (i.e., the difference in the EMMs) of three desired groups (Hydrocortisone – Placebo, Pre drug2 – Hydrocortisone, Pre drug2 – Placebo) were obtained by pairwise comparisons as mentioned previously.

2.3.5.3.3 Drug 2 (Hydrocortisone vs Placebo) and Day29 mortality

The trajectory analysis of study drug2 with mortality at day29 was performed by generating LME models according to the formula: `Drug2_model_withmortality <- log('Lactic acid' + 1) ~ 1 + 'Time post TP0 (days)' * 'Mortality at Day 29' + 'Drug2' + 'Time post TP0 (days)':'Drug2' + ('Time post TP0 (days)':'Mortality at Day 29':'Drug2') + 'Age' + 'Sex' + 'Time post TP0 (days)' | 'Sample Number' + (1 | 'Sitename')`. In these LME models, the interaction between Mortality at Day 29 and Time post TP0 (days) was introduced as a covariate to account for mortality. Drug2 was added as another fixed effect to account for study drug2. In these LME model, the main predictor variable (i.e., fixed effect) was a three-way interaction between

Time post TPO (days), Mortality at Day 29 and Drug2. Trends based on Time post TPO (days) as a function of Drug2 and mortality at day 29 were obtained via `emtrends`. This provided a slope (i.e., an overall trajectory for each group (Alive_Hydrocortisone, Dead_Hydrocortisone, Alive_Placebo and Dead_Placebo) with time as well as the estimate (i.e., the difference between the slopes for each group). In addition, the Satterthwaite method was employed to obtain EMMs via `emmeans` for Drug2 with day 29 mortality at day 2 (from Time post TPO variable). Estimates (i.e., the difference between the EMMs) of 4 desired groups: 1) Hydrocortisone (Alive-Dead); 2) Placebo (Alive -Dead); 3) Alive (Hydrocortisone – Placebo) 4) Dead (Hydrocortisone – Placebo) were obtained by pairwise comparisons at day 2 from Time post TPO (days).

Chapter 3 – Baseline Metabolic Differences in Small Molecules

3.1 Introduction

Metabolic derangements in sepsis have been previously studied using metabolomics and the studies have shown some potential in allowing us to understand the clinical phenotypes of sepsis especially related to mortality (144). Metabolomic studies have revealed that patients who go on to die have accumulation of glycolysis metabolites (lactate, pyruvate), TCA cycle intermediates (citrate, succinate, malate, fumarate and 2-ketoglutarate), amino acids (tyrosine, phenylalanine, leucine, isoleucine, valine, lysine, proline, glutamine, glutamic acid, histidine, 1 & 3-methylhistidine, betaine, methionine, aspartate, symmetric/asymmetric dimethylarginine), carbohydrates (glucose, fructose, sucrose, erythronate and mannitol), acyl-carnitines, catabolic steroids (cortisol and cortisone, pregnenolone sulfate), N-acetylglycoproteins, metabolites from pyrimidine and purine metabolism (allantoin, pseudouridine, xanthine, N2,N2-dimethylguanosine, N6-carbamoylthreonyladenosine and N1-methyladenosine) (66, 145-149, 151, 152, 154, 155, 157-160, 163-166, 229) whilst urea cycle metabolites (ornithine, argininosuccinate, arginine and citrulline) and anabolic steroids were reduced in these patients (147, 148, 159). However, there were some conflicting results from other studies which have shown that BCAAs (leucine, isoleucine, valine), SAAs (cysteine, methionine and taurine), phenylalanine, and proline were lower in non-surviving patients in relation to those who survive (152, 154, 159, 165, 173). These inconsistencies might be due to the limitations of the studies (as in small samples sizes, choice of biofluids and analytical platforms, lack of validation cohort) or it could be due to the complexity of this heterogeneous condition. All studies mentioned previously were small except for Wang et al study (158) (N = 188; 134 survivors & 54 non-survivors) and of those, only four studies (66, 145, 146, 148) validated their findings. (144)

This brings us to the point that we need to perform in depth analysis using larger cohorts to understand the serum metabolome fingerprints of patients with septic shock and see if they differ based on survival. Phenotyping of these patients using a systems biology approach will not only address the heterogeneity of this syndrome but will also allow to determine the metabolic derangements in septic shock and critical illness. The present study uses metabolomics, from two different platforms, NMR and UHPLC-MS to investigate the metabolic differences between survivors and non-survivors of septic shock in serum samples taken from VANISH (92) and LeoPARDS trial (88) with the purpose of understanding the

molecular biology of sepsis. In this study, metabolic signatures related to clinical patient outcome i.e., mortality at day 29 will be explored and understanding these baseline differences between the two clinical groups (i.e., survivors and non-survivors) across the two cohorts was important to understand the metabolic trajectories in the later analysis (Chapter 4). This will be taken a step further where the findings from one cohort will be validated in the other cohort and vice versa. To date this type of validation has not been previously done in other metabolic comparisons of survivors and non-survivors.

3.2 Aims

The main purpose of this study was to use metabolomic techniques to determine the metabolic differences in sepsis patients based on mortality and validate the findings.

The aims of this chapter are as follows:

- 1) To assess whether there are baseline differences in the metabolic profiles of survivors and non-survivors at ICU admission and at later sampling times.
- 2) To validate whether the metabolic differences between the two clinical groups are consistent across the two independent cohorts of sepsis patients studied in this project.
- 3) To evaluate whether the models generated using metabolic signatures of septic shock survivors and non-survivors from one cohort can predict mortality in another cohort.

3.2 Statistical analysis methods

The complete methods for all the analyses are listed in chapter 2 (sections 2.3.1, 2.3.2, 2.3.3).

3.3 Results

3.3.1 Baseline characteristics of patients from VANISH

The baseline demographic and clinical characteristics of all patients are shown in table 3.1. The mean age of 173 patients was 63.8 ± 1.19 years. Age, Sex and BMI were roughly similar between survivors and non-survivors. As expected, SOFA and APACHE II scores were elevated in non-survivors ($p_{Adj} = 2.03 \times 10^{-10}$, $p_{Adj} = 2.25 \times 10^{-3}$, respectively) whilst Glasgow

Comma Scale (GCS) score was lower in non-survivors ($p_{Adj} = 0.017$). The ratio of partial pressure of arterial oxygen (PaO_2) to the percentage of inspired oxygen (FiO_2) was significantly lower in non-survivors than survivors ($p_{Adj} = 3.26 \times 10^{-3}$). Hospital length of stay (LOS) was significantly longer in survivors ($p_{Adj} = 2.32 \times 10^{-12}$) than non-survivors. Presence of renal and respiratory failure were also significantly associated with mortality ($p_{Adj} = 4.61 \times 10^{-3}$, $p_{Adj} = 0.028$, respectively). Lactate and the dose of noradrenaline administered at randomisation were also significantly higher in non-survivors with respect to survivors ($p_{Adj} = 0.047$, $p_{Adj} = 0.012$, respectively). Of 173 patients only 22 patients had received drug 1 (noradrenaline or vasopressin) prior to TP0 sample whilst most patients received it before TP1 also indicated by the average number of 0.02 days for study drug 1 post TP0 sample. A minority of patients (7 out of 114) received study drug2 before TP0 sample was taken and there was no significant association between study drug2 and mortality in these patients ($p_{Adj} = 0.947$).

Table 3.1: Baseline characteristics of patients at ICU admission for the VANISH dataset. Continuous variables are presented as mean $\pm$ standard error of the mean (SEM) and categorical variables presented as numbers (percentages). Note: (*) sign denotes number of patients for which clinical data was available; N= Total number of patients; LOS: length of stay; BMI: body mass index; SOFA: Sequential Organ Failure Assessment; APACHE II: Acute Physiology and Chronic Health Evaluation II; GCS: Glasgow Comma Scale; MAP: mean arterial pressure; PaO_2/FiO_2 : arterial oxygen partial pressure to fractional inspired oxygen; IHD: Ischaemic heart disease; COPD: Chronic obstructive pulmonary disease. The Chi square test for the italicised variables was performed with Yates' continuity correction. Adjusted p-values (p_{Adj}) were calculated with B-H correction for all variables.

Variables	Total (N = 173)	Survivors (N = 127)	Non-survivors (N = 46)	p_{Adj}
Male (%)	109 (63.0%)	82 (64.6%)	27 (58.7%)	0.751
Age, (years)	63.8 $\pm$ 1.19	62.5 $\pm$ 1.44	67.3 $\pm$ 1.98	0.255
BMI (Kg/m ²)	27.8 $\pm$ 0.61 (167)*	27.5 $\pm$ 0.68 (122)*	28.6 $\pm$ 1.34 (45)*	0.926
ICU LOS (days)	10.5 $\pm$ 1.04	11.7 $\pm$ 1.36	7.5 $\pm$ 0.96	0.255
Hospital LOS (days)	29.6 $\pm$ 2.60 (172)*	38.0 $\pm$ 3.24(126)*	8.0 $\pm$ 1.03	2.32×10^{-12}
Mechanical ventilation (%)	91 (52.6%)	64 (50.4%)	27 (59.0%)	0.572
SOFA score	5.6 $\pm$ 0.24 (172)*	4.6 $\pm$ 0.18	8.7 $\pm$ 0.54 (45)*	2.03×10^{-10}
APACHE II score	24.4 $\pm$ 0.61 (172) *	23.1 $\pm$ 0.69	28.1 $\pm$ 1.08(45)*	2.25×10^{-3}
GCS score	10.8 $\pm$ 0.38 (166)*	11.4 $\pm$ 0.42 (122)*	9.0 $\pm$ 0.78 (44)*	0.017
MAP (mmHg)	69.4 $\pm$ 0.85 (172)*	68.8 $\pm$ 0.93(126)*	71.0 $\pm$ 1.92	0.687

Lactate (mmol/L)	3.2 ± 0.21(169)*	2.8 ± 0.18 (123)*	4.3 ± 0.57	0.047
PaO₂/FiO₂ ratio, kPa	29.5 ± 1.13 (166)*	32.0 ± 1.30 (120)*	23.2 ± 2.04	3.26 × 10 ⁻³
Creatinine µmol/L)	163.6 ± 9.77	155.9 ± 11.61	184.8 ± 17.77	0.140
Platelet (G/L)	213.9 ± 10.56 (168)*	211.9 ± 12.29 (122)*	219.28 ± 20.81	0.947
Bilirubin (µmol/L)	34.4 ± 4.59(153)*	31.3 ± 5.03(109)*	42.0 ± 10.1 (44)*	0.413
Noradrenaline dose at randomisation (µg/kg/min)	0.21 ± 0.02 (141)*	0.19 ± 0.02 (103)	0.26 ± 0.03 (38)*	0.012
Study drug 1 post TPO (days)	0.02 ± 0.01	0.01 ± 0.01	0.04 ± 0.03	0.988
Drug 1 Administered	22 (12.7%)	17 (13.4%)	5 (10.9%)	0.926
Noradrenaline	12 (6.9%)	9 (7.1%)	3 (6.5%)	
Vasopressin	10 (5.8%)	8 (6.2%)	2 (4.3%)	
Study drug 2 post TPO (days)	0.21 ± 0.03 (114)*	0.25 ± 0.03 (79)*	0.15 ± 0.05 (35)*	0.147
Drug 2 Administered	7 (6.1%)	4 (5.1%)	3 (8.6%)	0.947
Hydrocortisone	3 (2.6%)	2 (2.5%)	1 (2.9%)	
Placebo	4 (3.5%)	2 (2.5%)	2 (5.7%)	
Source of infection:				
Lung	72 (41.6%)	48 (37.8%)	24 (52.2%)	0.227
Abdomen	35 (20.2%)	29 (22.8%)	6 (13.0%)	0.340
Soft tissue	6 (3.5%)	6 (4.7%)	0 (0 %)	0.549
Other	57 (32.9%)	42 (33.1%)	15 (32.6%)	1.00
Site unknown	3 (1.7%)	2 (1.6%)	1 (2.2%)	
Underlying diseases:				
IHD	28 (16.2%)	18 (14.2%)	10 (21.7%)	0.441
Severe COPD	10 (5.8%)	8 (6.3%)	2 (4.3%)	1.00
Chronic renal failure	8 (4.6%)	6 (4.7%)	2 (4.3%)	1.00
Cirrhosis	11 (6.4%)	7 (5.5%)	4 (8.7%)	0.926
Diabetes Mellitus	40 (23.1%)	33 (26.0%)	7 (15.2%)	0.310
Immunocompromised	10 (5.8%)	6 (4.7%)	4 (8.7%)	0.802
Cancer	21 (12.1%)	15 (14.9%)	6 (13.0%)	0.988
Organ failure:				
Renal	40 (23.1%)	21(16.5%)	19 (41.3%)	4.61 × 10 ⁻³
Haematological	11 (6.5%) (168)*	8 (6.6%) (122)*	3 (6.5%)	1.00
Respiratory	61 (35.6%) (171)*	37 (29.6%) (125)*	24 (52.2%)	0.028
Neurological	55 (33.1%) (166)*	35 (28%) (122)*	20 (45.5%) (44)*	0.140
Liver	12 (7.8%) (153)*	8 (7.3%) (109)*	4 (9.1%) (44)*	1.00

3.3.2 Baseline characteristics of patients from LeoPARDS

The baseline demographic and clinical characteristics of all patients are shown in table 3.2. The mean age of 450 patients was 65.8 ± 0.66 years and non-survivors had significantly higher mean age than non-survivors ($p_{Adj} = 0.028$). Sex was roughly similar between survivors and non-survivors. BMI and MAP were significantly lower in non-survivors ($p_{Adj} =$

0.014, $p_{\text{Adj}} = 3.97 \times 10^{-3}$, respectively). Hospital and ICU LOS were significantly longer in survivors ($p_{\text{Adj}} = 1.28 \times 10^{-32}$, $p_{\text{Adj}} = 8.39 \times 10^{-11}$, respectively). Lactate, creatinine, SOFA and APACHE II scores, noradrenaline dose at randomisation and organ failure SOFA scores including renal, coagulation and liver were significantly higher in non-survivors ($p_{\text{Adj}} = 9.70 \times 10^{-6}$, $p_{\text{Adj}} = 7.38 \times 10^{-5}$, $p_{\text{Adj}} = 4.25 \times 10^{-44}$, $p_{\text{Adj}} = 5.54 \times 10^{-12}$, $p_{\text{Adj}} = 3.98 \times 10^{-4}$, $p_{\text{Adj}} = 5.05 \times 10^{-5}$, $p_{\text{Adj}} = 0.028$, $p_{\text{Adj}} = 0.048$, respectively) in relation to survivors. A past medical history of cardiac failure, immunocompromised patients and the site/source of infection were significantly associated with mortality ($p_{\text{Adj}} = 5.29 \times 10^{-3}$, $p_{\text{Adj}} = 1.93 \times 10^{-3}$, $p_{\text{Adj}} = 0.016$, respectively). Remaining clinical parameters were not significantly different between the two groups.

Table 3.2: Baseline characteristics of patients at ICU admission for LeoPARDS dataset. Continuous variables presented as mean $\pm$ standard error of the mean (SEM) and categorical variables presented as numbers (percentages). Note: (*) sign denotes number of patients for which clinical data was available; N= Total number of patients; LOS: length of stay; BMI: body mass index; SOFA: Sequential Organ Failure Assessment; APACHE II: Acute Physiology and Chronic Health Evaluation II; GCS: Glasgow Comma Scale; MAP: mean arterial pressure; PaO₂/FiO₂: arterial oxygen partial pressure to fractional inspired oxygen; IHD: Ischaemic heart disease; COPD: Chronic obstructive pulmonary disease. The Chi squared test for the italicised variables was performed with Yates' continuity correction. Adjusted p-values (p_{Adj}) were calculated with B-H correction for all variables.

Variables	Total (N = 450)	Survivors (N = 304)	Non-survivors (N = 146)	p_{Adj}
Male (%)	256 (56.9%)	163 (53.6%)	93 (63.7%)	0.068
Age, (years)	65.8 $\pm$ 0.66	64.7 $\pm$ 0.81	68.2 $\pm$ 1.13	0.028
BMI (Kg/m ²)	27.70 $\pm$ 0.31 (441)*	28.3 $\pm$ 0.38 (299)*	26.4 $\pm$ 0.52 (142) *	0.014
ICU LOS (days)	12.00 $\pm$ 0.68	14.4 $\pm$ 0.94	6.85 $\pm$ 0.55	8.39×10^{-11}
Hospital LOS (days)	33.70 $\pm$ 1.84 (446)*	43.8 $\pm$ 2.44 (302)*	12.8 $\pm$ 1.28	1.28×10^{-32}
Mechanical ventilation (%)	356 (79.1%)	229 (75.3%)	127 (87.0%)	0.011
SOFA score	6.43 $\pm$ 0.18	4.48 $\pm$ 0.13	10.48 $\pm$ 0.31	4.25×10^{-44}
APACHE II score	25.83 $\pm$ 0.33	24.14 $\pm$ 0.38	29.34 $\pm$ 0.57	5.54×10^{-12}
GCS score	8.80 $\pm$ 0.26 (381)*	8.97 $\pm$ 0.31 (258)*	8.43 $\pm$ 0.46 (123)*	0.233
MAP (mmHg)	73.65 $\pm$ 0.43	74.46 $\pm$ 0.52	71.96 $\pm$ 0.79	3.97×10^{-3}
Lactate (mmol/L)	3.00 $\pm$ 0.12 (448)*	2.50 $\pm$ 0.11 (302)*	4.03 $\pm$ 0.27	9.70×10^{-6}
PaO ₂ /FiO ₂ ratio, (kPa)	30.93 $\pm$ 0.66 (448)*	31.47 $\pm$ 0.7 (302)*	29.8 $\pm$ 1.20	0.211
Creatinine (μ mol/L)	170.76 $\pm$ 5.74 (449)*	160.72 $\pm$ 7.30	191.81 $\pm$ 8.84 (145) *	7.38×10^{-5}

Platelet (G/L)	243.24 ± 7.34 (447)*	252.27 ± 9.0 (302)*	224.43 ± 12.59 (145)*	0.089
Bilirubin (μmol/L)	23.55 ± 1.63 (441)*	28.31 ± 0.38 (297)*	26.4 ± 0.52 (144)*	0.096
Noradrenaline dose at randomisation (μg/kg/min)	0.38 ± 0.02 (443)*	0.34 ± 0.02 (303)*	0.47 ± 0.04 (140)*	3.98 × 10 ⁻⁴
Source of infection:				0.016
Lung	178 (39.6%)	111 (36.5%)	67 (45.9%)	
Abdomen	163 (36.2%)	120 (39.5%)	43 (29.5%)	
Soft tissue or line	24 (5.3 %)	15 (4.9%)	9 (6.2%)	
Primary bacteraemia	9 (2.0%)	2 (0.66%)	7 (4.8%)	
Urine	26 (5.8 %)	22 (7.2%)	4 (0.9%)	
Other	44 (9.8%)	29 (9.5%)	15 (10.2%)	
Site unknown	1 (0.2%)	1 (0.3%)	-	
Underlying diseases:				
IHD	66 (14.6%)	39 (12.8%)	27 (18.5%)	0.152
Severe COPD	19 (4.2%)	12 (3.9%)	7 (4.8%)	0.724
Chronic renal failure	34 (7.6%)	20 (6.6%)	14 (9.6%)	0.298
Cirrhosis	8 (1.8%)	6 (2.0%)	2 (1.4%)	0.942
Diabetes Mellitus	95 (21.1%)	67 (22.0%)	28 (19.2%)	0.540
Immunocompromised	43 (9.6%)	19 (6.3%)	24 (16.4%)	1.93 × 10 ⁻³
Cardiac failure	43 (9.6%)	20 (6.6%)	23 (15.8%)	5.29 × 10 ⁻³
SOFA Scores:				
Cardio	3.87 ± 0.02	3.88 ± 0.02	3.86 ± 0.04	0.828
Renal	1.16 ± 0.05 (449)*	1.02 ± 0.06	1.46 ± 0.09 (145)*	5.05 × 10 ⁻⁵
Coagulation	0.51 ± 0.05 (447)*	0.41 ± 0.05 (302)*	0.71 ± 0.10 (145)*	0.028
Respiratory	1.81 ± 0.06 (448)*	01.71 ± 0.07 (302)*	2.00 ± 0.10	0.061
CNS	2.21 ± 0.09 (381)*	2.14 ± 0.10 (258)*	2.35 ± 0.15 (123)*	0.178
Liver	0.55 ± 0.04 (443)*	0.47 ± 0.04 (297)*	0.71 ± 0.08 (144)*	0.048

3.3.3 Mortality analysis of untargeted data for VANISH

The multivariate analysis of CPMG data was performed for mortality outcome for each timepoint (TP0-TP3). The aim of this was to analyse the discriminatory metabolites between survivors and non-survivors at each timepoint separately and to describe the evolution of metabolic phenotypes for septic shock survivors and non-survivors during this duration of time. Multivariate analysis was performed on a total of 479 samples after exclusions as shown in figure 3.1. Sample exclusions were performed in the same way as mentioned in chapter 2, section 2.2.1.3. However, three additional samples which had very different metabolic profiles compared to the general profile of a serum sample were excluded from a total of 509 samples during this analysis. Two of those three samples belonged to same patient (TP0 and TP1) and presented very intense signals suspected to belong to drug metabolites whilst the remaining sample belonged to a patient whose metabolic profile

showed an abnormally high level of glucose. Multivariate models were then generated for survivors and non-survivors from all timepoints. Summarized parameters for each multivariate model are shown in table 3.3. Three principal components were used throughout for all the models as the Q^2 values, especially for TP0 and TP1 multivariate models, reached a plateau after 3 PCs were added. We used a threshold of an increase of Q^2 of 5% to determine if additional components improved the model. This was not the case beyond three components.

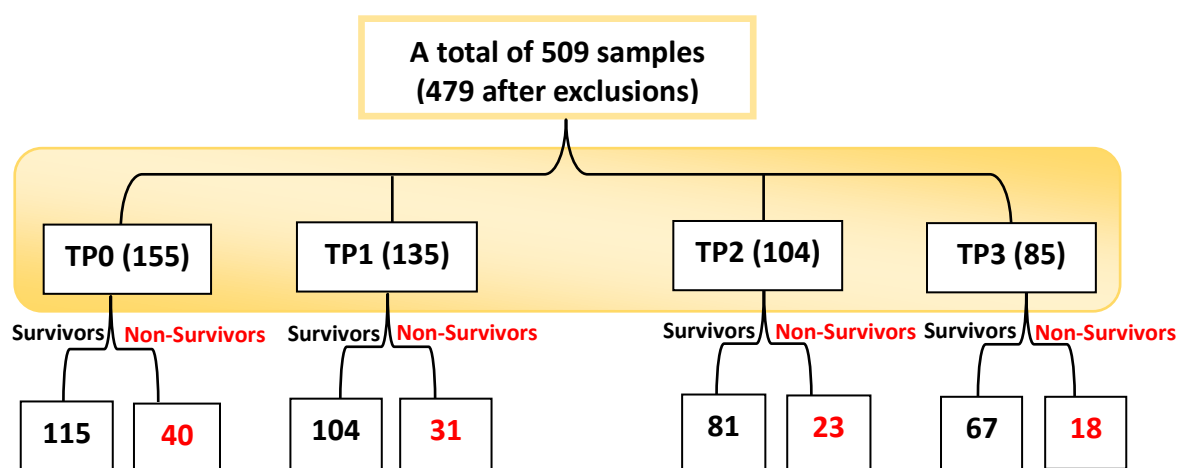


Figure 3.1: Flow chart for all timepoint samples for survivors and non-survivors for the VANSIH dataset.

Table 3.3: Multivariate models for mortality analysis of CPMG data for all timepoints in the VANISH data set. N stands for total number of samples included and analysed.

Timepoint set Survivors vs non- survivors	Multivariate Model	Number of principle components	N	R^2X (cum)	R^2Y (cum)	Q^2 (cum)
TP0	PCA-X	3	155	0.285		0.216
	PLS-DA	3	155	0.223	0.745	0.235
TP1	PCA-X	3	135	0.289		0.233
	PLS-DA	3	135	0.242	0.733	0.269
TP2	PCA-X	3	104	0.286		0.201
	PLS-DA	3	104	0.119	0.927	-0.0862
TP3	PCA-X	3	85	0.282		0.198
	PLS-DA	3	85	0.141	0.943	0.520

Scores plots for PLS-DA models of survivors and non-survivors for TP0 and TP1 are shown in figure 3.2A and figure 3.2C, respectively whilst the score plots for TP2 and TP3 are shown in

figure 3.5A and figure 3.5B. A clear separation between survivors and non-survivors is shown in the two score scatter plots for both TP0 (figure 3.2A) and TP1 (figure 3.2C) multivariate models. The score plots for TP2 and TP3 multivariate models do show separation between survivors and non-survivors however, the PLS-DA model metrics of TP2 and TP3 samples show that these two models are less robust than TP0 and TP1 multivariate models and are overfitted. This might be due to several reasons firstly there is survivorship bias in the model as there are some patients who get discharged or those who die hence a biased selection of samples at later timepoints. The other reason could be the small sample size at later timepoints, hence less statistical power for the multivariate models. The ROC curves for both TP0 and TP1 multivariate models are shown in figure 3.2B and 3.2D respectively. These ROC curves show the TPR against the FPR of the two classes with area under curve (AUC) of 0.874 and 0.795 for TP0 and TP1, respectively indicating a good predictive performance of both PLS-DA models (Figure 3.2A and 3.2C). The loading plots for both PLS-DA models of TP0 and TP1 survivors and non survivors are shown in figure 3.3 and figure 3.4, respectively. In the loading plots, the positive signals correspond to non-survivors whilst negative signals correspond to survivors. The signals on the NMR spectrum that appear to be discriminant between survivors and non-survivors in the PLS-DA models for both TP0 and TP1 correspond to lactate, alanine, phenylalanine, tyrosine, histidine, formate, acetate, creatinine, glycine, glutamate, glutamine, pyruvate, leucine, isoleucine, lysine, valine, methionine, 3-hydroxyisobutyrate, myo-inositol and 1-methylhistidine (1-MH). These metabolites were increased in non-survivors in comparison to survivors. In addition, two unknown metabolites, table 3.4, were also increased in non-survivors compared to survivors with a higher $p(\text{corr})$ value at TP1 (figure 3.4, table 3.4). Furthermore, benzoic acid was also found to be elevated in non-survivors for TP1 PLS-DA multivariate model (figure 3.4, and table 3.4). Similarly, succinic acid was also increased in non-survivors, but only at TP0 (figure 3.3 and table 3.4). However, CH_2 and CH_3 mixed lipoproteins were down regulated in non-survivors compared to survivors for both TP0 and TP1 (table 3.4). In summary, almost all of the same metabolites were more abundant in non-survivors compared to survivors at both timepoints (TP0 and TP1) with tyrosine, lactate, alanine, phenylalanine, creatinine, creatine, lysine, methionine, and unknown metabolites (Unknown 1 and Unknown 2) being the most discriminant metabolites, with a highest $p(\text{corr})$ (table 3.4). The discriminant NMR

information obtained from the loading plots of the PLS-DA models from TP0 and TP1 samples was then confirmed with the STOCYSY results. One STOCYSY was performed per each discriminatory metabolite to confirm the metabolite identity. The highest correlations were obtained with the signals belonging to the same discriminatory metabolite whilst slightly weaker correlations were obtained with other metabolites that change in the same direction as the discriminatory metabolite. STOCYSYs were performed on each discriminatory metabolite, but only STOCYSY results for tyrosine showed the correlation with all the metabolites (listed in table 3.4 and figure 3.6) which were differentiating survivors from non-survivors.

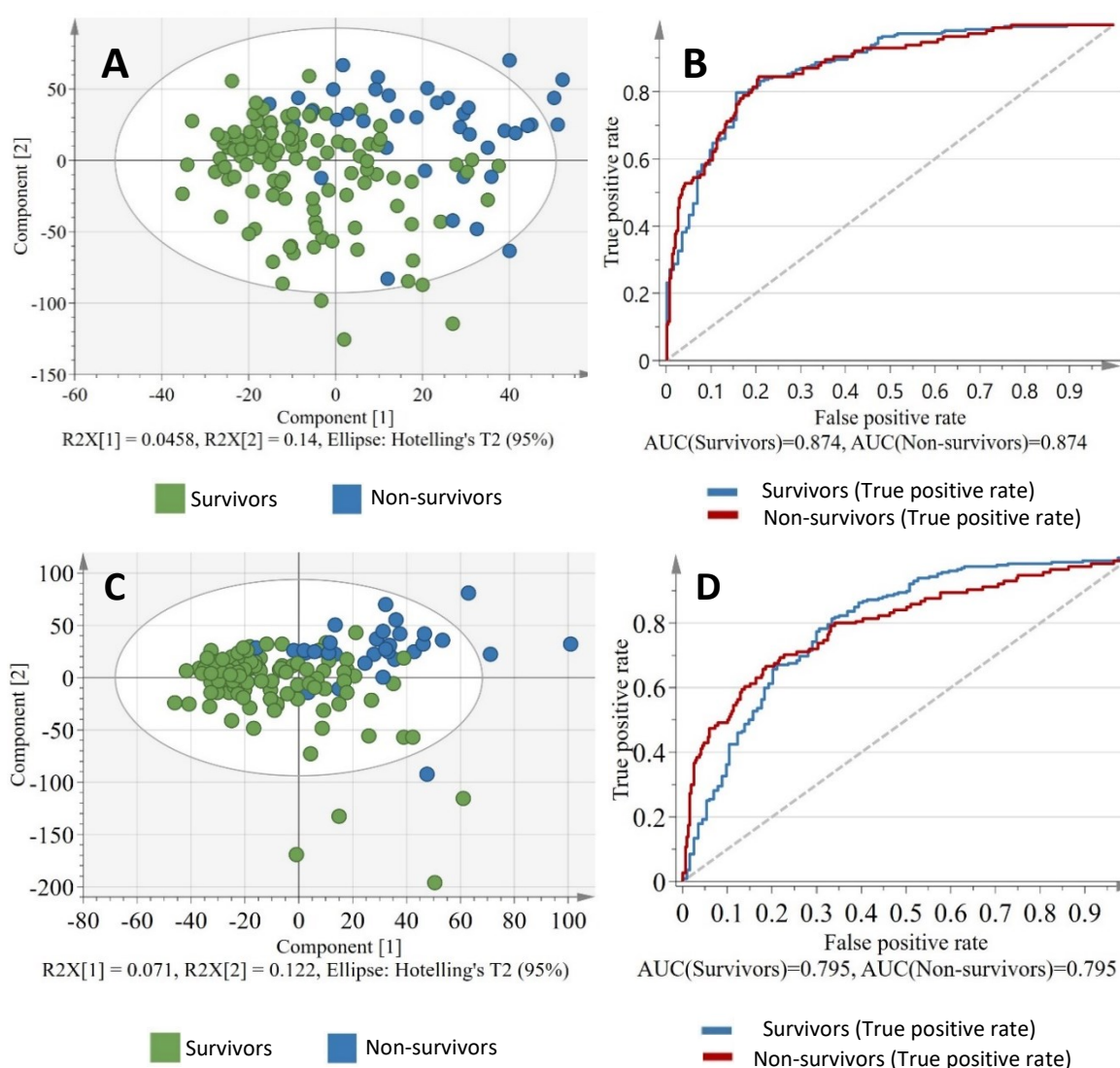


Figure 3.2: Multivariate analysis of TP0 and TP1 survivors versus non-survivors. A) Score scatter plot of PLS-DA model for TP0 survivors and non-survivors ($R^2X = 0.223$, $R^2Y = 0.745$, $Q^2 = 0.235$, model valid); B) ROC curve for PLS-DA model of TP0 survivors and non-survivors; C) Score scatter plot of PLS-DA model for TP1 survivors and non-survivors ($R^2X = 0.242$, $R^2Y = 0.733$, $Q^2 = 0.269$, model valid); D) ROC curve for PLS-DA model of TP1 survivors and non-survivors.

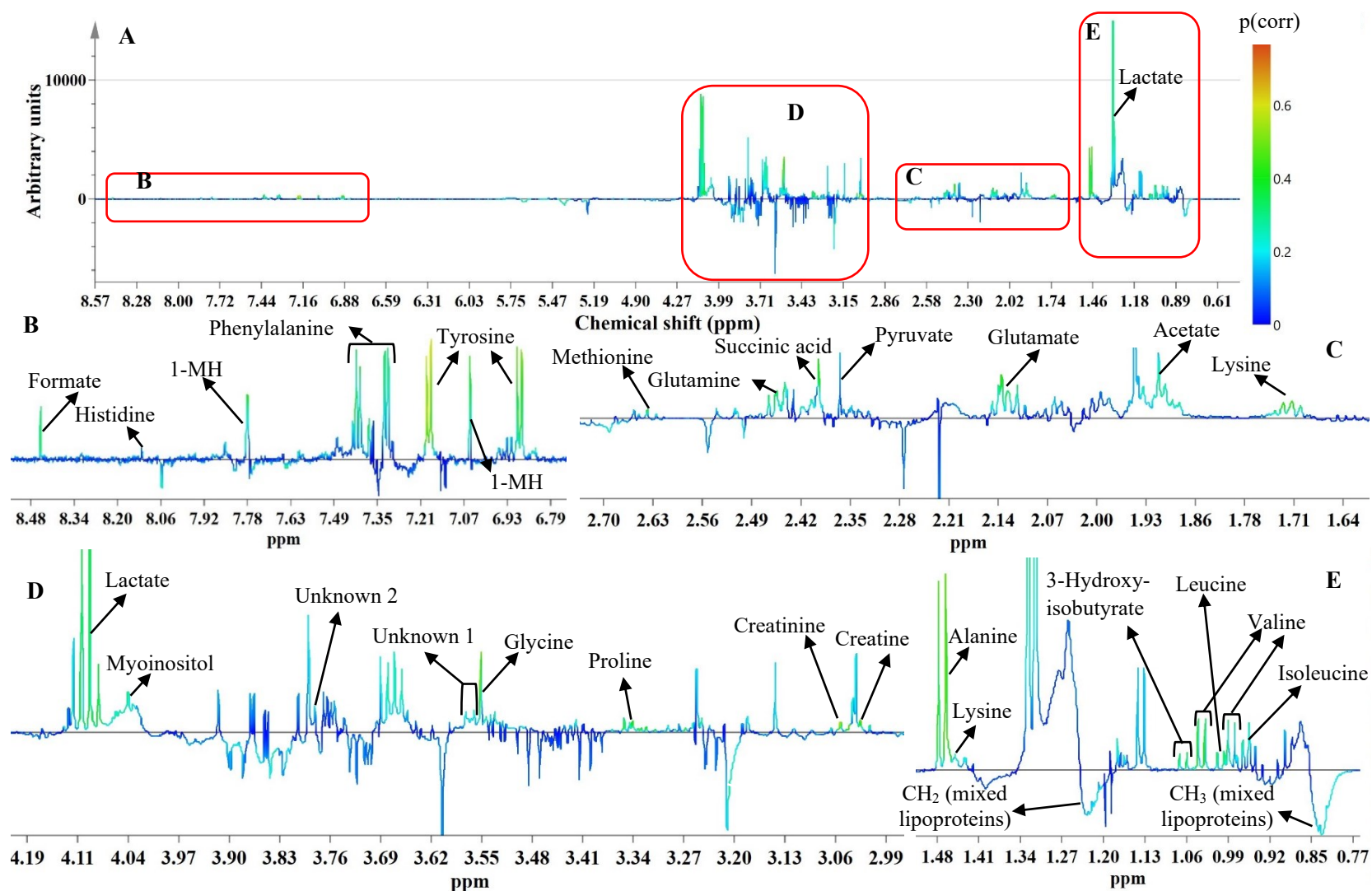


Figure 3.3: Loading plot for PLS-DA model of TP0 survivors and non-survivors (Figure 3.2A). Key discriminatory metabolites are annotated on the plot. The coloured scale indicates the correlation coefficient $p(\text{corr})$. A) Full loading plot; B) Region expansion 6.79-8.48ppm; C) Region expansion 1.64-2.70 ppm; D) Region expansion 2.99-4.19 ppm; E) Region expansion 0.77-1.48ppm.

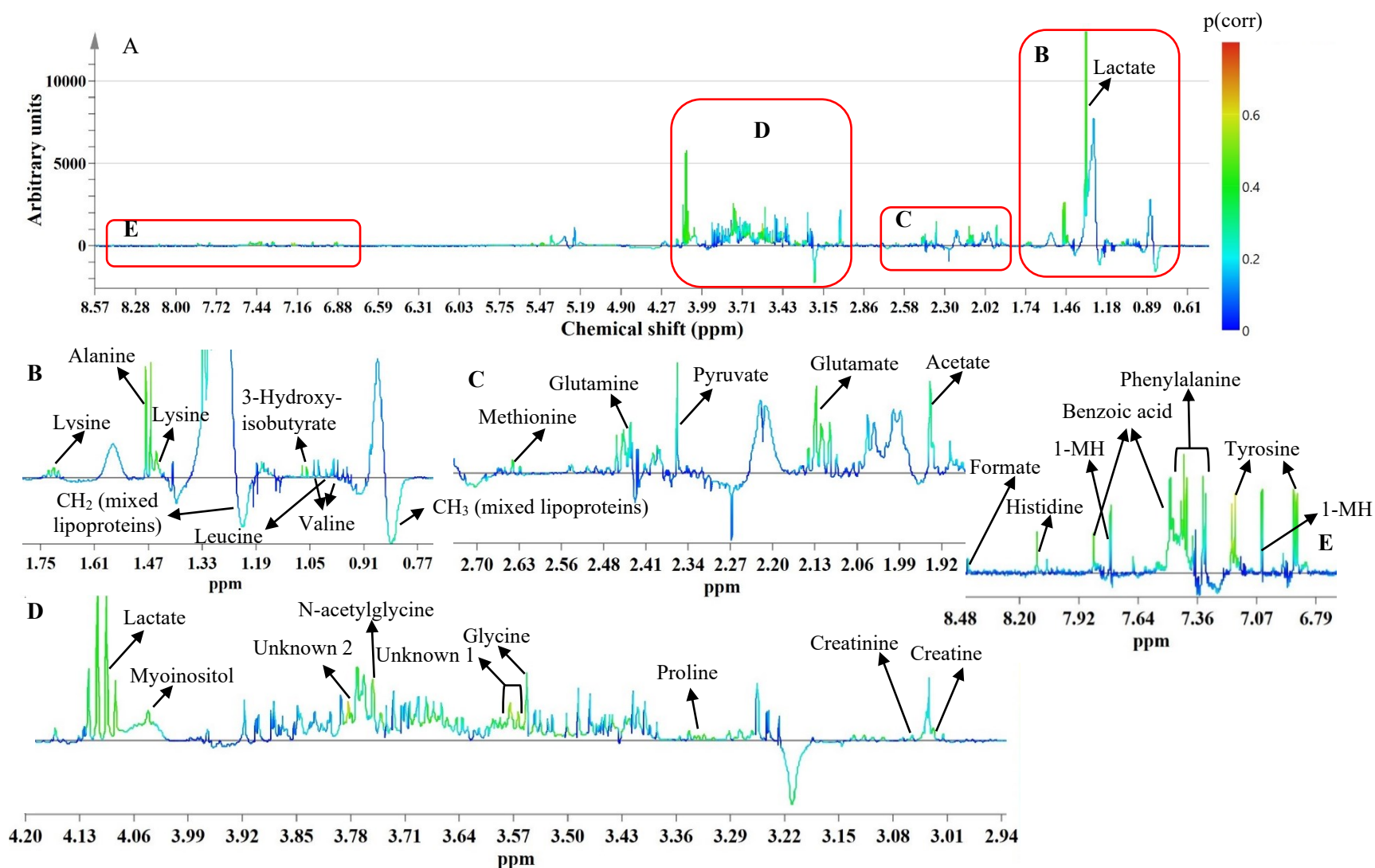


Figure 3.4: Loading plot for PLS-DA model of TP1 survivors and non-survivors (Figure 3.2C). Key discriminatory metabolites are annotated on the plot. The coloured scale indicates the correlation coefficient $p(\text{corr})$. A) Full loading plot; B) Region expansion 0.77-1.75ppm; C) Region expansion 1.92-2.70ppm; D) Region expansion 2.94-4.20ppm; E) Region expansion 6.79-8.48ppm.

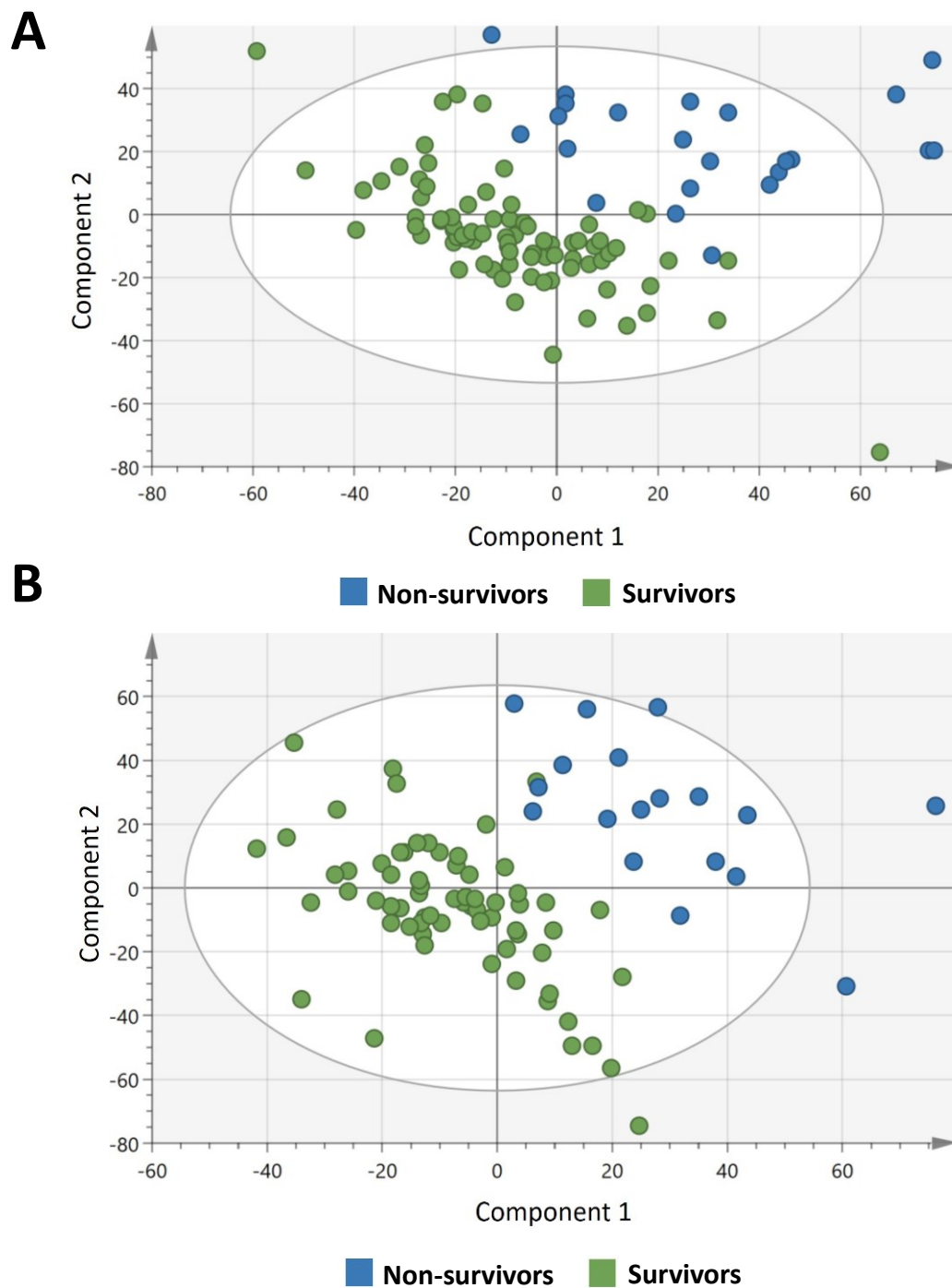


Figure 3.5: Multivariate analysis of TP2 and TP3 survivors and non-survivors. A) Score scatter plot of PLS-DA model for TP2 survivors and non-survivors ($R^2X = 0.119$, $R^2Y = 0.927$, $Q^2 = -0.0862$, model invalid); B) Score scatter plot of PLS-DA model for TP1 survivors and non-survivors ($R^2X = 0.141$, $R^2Y = 0.943$, $Q^2 = 0.520$, model invalid).

Table 3.4: Key discriminatory metabolites for TP0 and TP1 PLS-DA multivariate models of survivors and non-survivors. Note: ↑ denotes increased and ↓ decreased; p(corr): correlation value for each metabolite fragment obtained from the loading plots of TP0 and TP1 PLS-DA and these numbers relate with the values in δ (ppm) column in their respective order for each metabolite peak; s = singlet; d = doublet; t = triplet; m = multiplet (these symbols represent peak multiplicity).

Metabolite	δ (ppm)	Survivors	non-survivors	TP0 p(corr)	TP1 p(corr)
Tyrosine	7.18 (d), 6.89 (d)	↓	↑	0.61, 0.51	0.62, 0.52
1-MH	7.05 (s)	↓	↑	0.43	0.43
Lactate	4.10(q), 1.32 (d)	↓	↑	0.34, 0.33	0.41, 0.44
Glycine	3.56 (s)	↓	↑	0.52	
Unknown 1	3.58(s), 3.57(s)	↓	↑	0.23, 0.24	0.54, 0.55
Unknown 2	3.78(s)	↓	↑	0.18	0.65
N-acetylglycine	3.76	↓	↑	-	0.51
Alanine	1.48(d)	↓	↑	0.48	0.48
Phenylalanine	7.42(m), 7.38(m), 7.33 (m)	↓	↑	0.37, 0.35, 0.34	0.48, 0.43, 0.45
Benzoic acid	7.49(t), 7.85(d)	↓	↑	-	0.35, 0.45
Pyruvate	2.37(s)	↓	↑	0.17	0.35
Succinic acid	2.39(s)	↓	↑	0.40	-
Glutamine	2.46(m)	↓	↑	0.33	0.31
Glutamate	2.14(m)	↓	↑	0.43	0.40
Creatinine	3.05(s)	↓	↑	0.56	0.36
Creatine	3.02(s)	↓	↑	0.37	0.40
Valine	1.04(d), 0.99(d)	↓	↑	0.40, 0.38	0.20
Leucine	1.01(d)	↓	↑	0.34	0.23
Isoleucine	0.97(d)	↓	↑	0.29	0.16
Lysine	1.72(m), 1.45 (m)	↓	↑	0.45, 0.43	0.41, 0.41
Proline	3.33(m)	↓	↑	0.40	0.46
Methionine	2.64(t)	↓	↑	0.37	0.41
3-hydroxyisobutyrate	1.06(d)	↓	↑	0.37	0.43
Formate	8.45(s)	↓	↑	0.31	0.23
Acetate	1.91(s)	↓	↑	0.14	0.31
Histidine	8.12(s)	↓	↑	0.10	0.39

Myoinositol	4.05 (t)	↓	↑	0.29	0.36
CH₂ mixed lipoproteins	1.21	↑	↓	0.17	0.24
CH₃ mixed lipoproteins	0.82	↑	↓	0.23	0.28

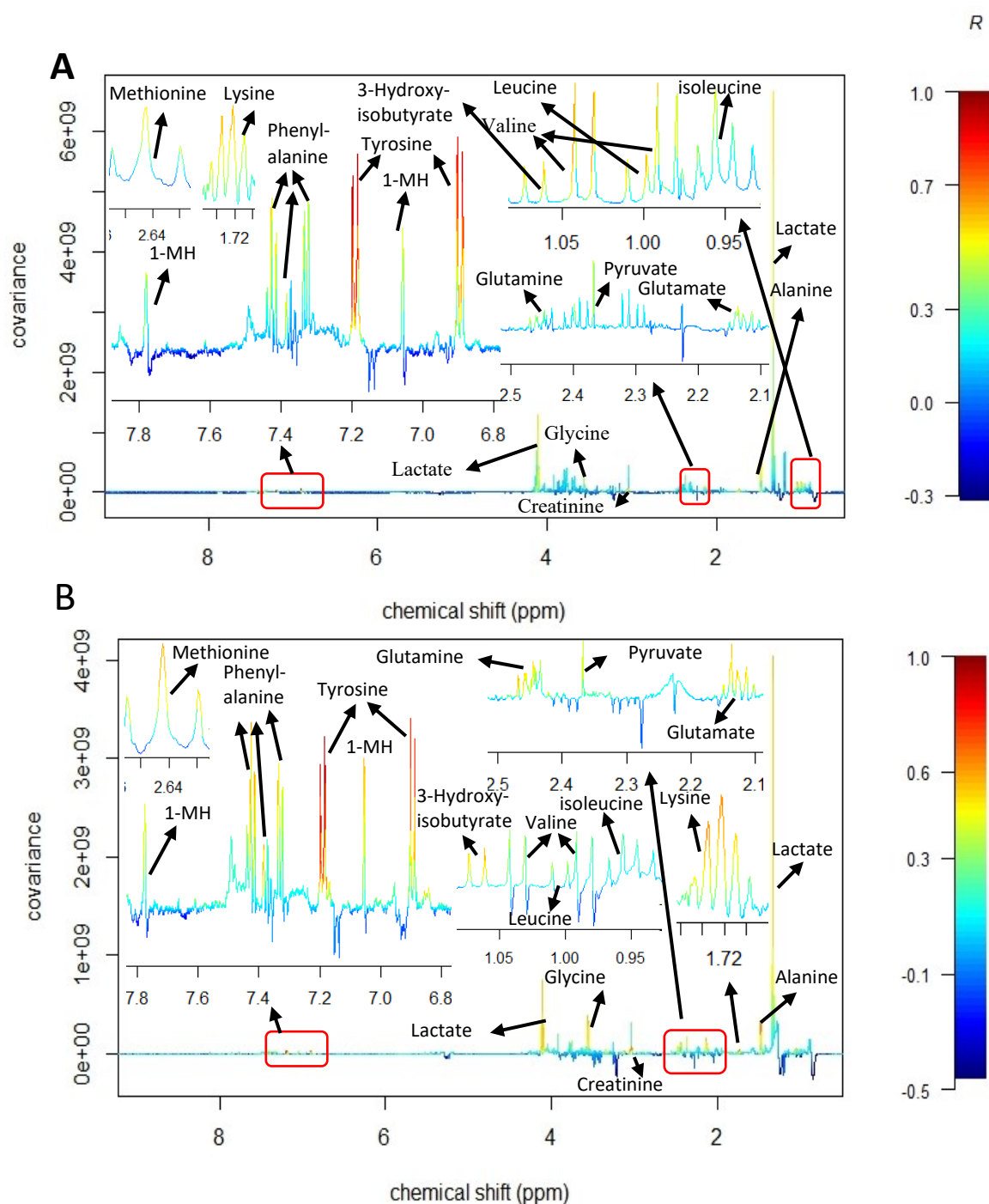


Figure 3.6: STOCSY plot generated for TP0 and TP1 samples using tyrosine as a driver peak (7.185 ppm). A) STOCSY for TP0 samples including some expansion regions; B) STOCSY for TP1 samples including some expansion regions. Colour scale shows the correlation coefficient value from -0.5-1.0; strong correlations (red/orange), slightly weaker correlations (yellow), weak correlations (green/bluish green) and no correlation (dark blue).

3.3.4 Mortality analysis of targeted data

The multivariate analysis of NMR untargeted data revealed some key metabolic differences between survivors and non-survivors. Apart from a couple of signals, the majority of discriminant metabolites from this analysis were already part of the annotated lists of compounds in the targeted data. As targeted data presents the advantage of easy interpretation, hence further analysis was focused on targeted data. Univariate analysis of targeted datasets (including SMoIESY-select and HPOS ppR) was performed on both cohorts, (i.e., VANISH and LeoPARDS) to determine the metabolic differences in small molecules based on mortality.

3.3.4.1 NMR targeted data (SMoIESY-select)

Univariate analysis of 22 small molecules obtained via SMoIESY-select was performed for all timepoints on a total of 472 serum samples from VANISH and 1404 samples from LeoPARDS, figures 3.7 and 3.8. Sample exclusions were performed in the same way as mentioned in chapter 2, section 2.2.1.3 for both VANISH and LeoPARDS. However, additional samples (three samples which were previously excluded from CPMG analysis, 18 samples had no sample timings recorded and four samples belonged to a patient whose samples were collected after several days of receiving the study drug) were excluded from a total of 509 samples from VANISH (figure 3.7). In Leopards 10 additional samples which had no sample timings recorded (figure 3.8) were also excluded prior to univariate analysis.

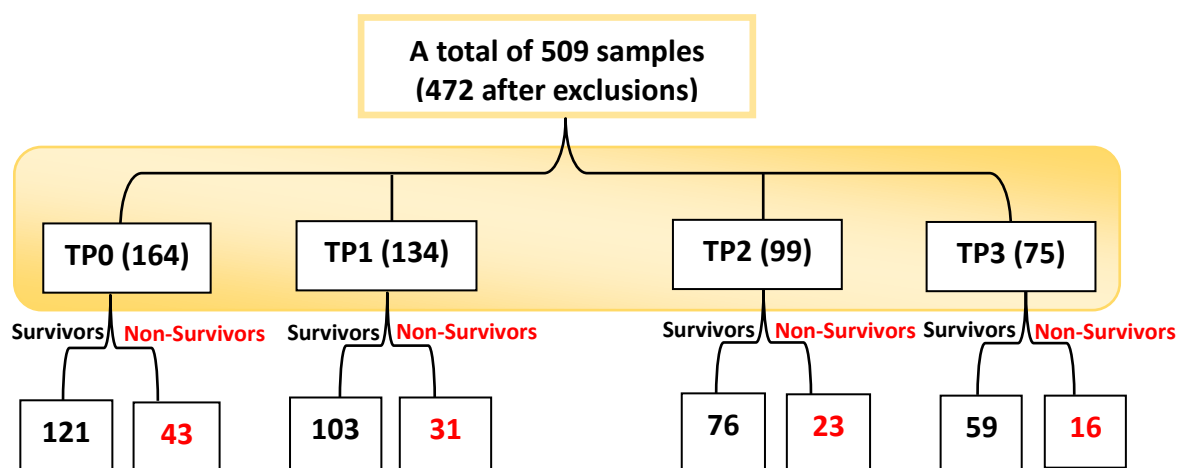


Figure 3.7: Flow chart for the samples included in the univariate analysis of SMoIESY-select data from VANISH.

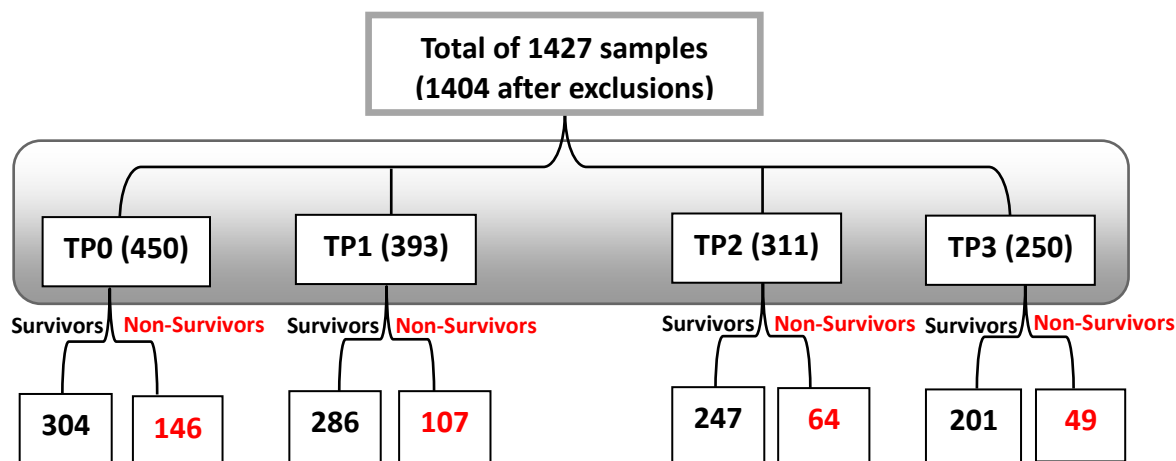


Figure 3.8: Flow chart for the samples included in the univariate analysis of SMoIESY-select data from LeoPARDS.

A summary of univariate results for all timepoints in both cohorts is presented in tables 3.5, 3.6, 3.7 and 3.8, differentiating survivors from non-survivors at ICU admission and after 24, 48 and 96 hours of admission (tables 3.5-3.8).

Nine small molecules including aromatic amino acids (tyrosine and phenylalanine), lactic acid, alanine, glutamine, glycine, histidine, choline, and creatinine at TP0 were found to have significantly higher concentrations in serum of patients who died before day 29 and those who didn't ($p_{\text{Adj}} < 0.05$) in both cohorts (table 3.5, highlighted in green). In addition to the common metabolites between the two cohorts, some metabolites including lysine, formic acid, creatine, glucose and citrate were significantly elevated in non-survivors only in LeoPARDS ($p_{\text{Adj}} < 0.05$) whilst leucine was significantly increased in non-survivors only in VANISH ($p_{\text{Adj}} = 0.047$) at TP0. However, in all cases the trend to change was in the same direction as was seen in the alternate cohort.

At TP1, only five metabolites including tyrosine, phenylalanine, alanine, lactic acid, and creatinine were significantly present in higher concentrations in non-survivors with respect to survivors in both VANISH and LeoPARDS ($p < 0.05$) (table 3.6). Only tyrosine and phenylalanine remained significant after B-H correction in both cohorts ($p_{\text{Adj}} < 0.05$) (table 3.6). In LeoPARDS, nine small molecules including glutamine, glycine, choline, histidine, lysine, branched chain amino acids (valine, leucine), acetic acid, and creatine were significantly raised in non-surviving patients in relation to surviving patients after 24 hours

of admission ($p_{Adj} < 0.05$). All these metabolites except for choline had the same trend in VANISH but the results were not significant.

At later time points, TP2 and TP3, fewer metabolites were significantly different between survivors and non-survivors in both cohorts. At TP2, only tyrosine was significantly higher in patients who went on to die than those who survived in both cohorts ($p_{Adj} < 0.05$). There were some additional metabolites such as lactic acid, choline, alanine, and creatinine which were present in significantly higher levels in non-survivors with respect to survivors at TP2 in LeoPARDS ($p_{Adj} < 0.05$). These metabolites also followed the same trend in non-survivors from VANISH however, results were not significant. At TP3, non-survivors had significantly higher mean levels of three small molecules (choline, tyrosine, and creatine) in LeoPARDS ($p_{Adj} < 0.05$) (table 3.8). These metabolites had the same pattern in non-survivors from VANISH though results were not significant.

In summary, tyrosine was always significantly higher in non-survivors in LeoPARDS at all timepoints ($p_{Adj} = 1.45 \times 10^{-5}$ for TP0, $p_{Adj} = 1.59 \times 10^{-5}$ for TP1, $p_{Adj} = 0.018$ for TP2 & $p_{Adj} = 0.027$ for TP3, respectively) (tables 3.5-3.8) and at three timepoints in VANISH ($p_{Adj} = 0.016$ for TP0, $p_{Adj} = 0.011$ for TP1, $p_{Adj} = 9.73 \times 10^{-3}$ for TP2) (tables 3.5-3.7). Phenylalanine was the second most significant metabolite in both VANISH and LeoPARDS at ICU admission and at TP2 and TP3 (tables 3.5-3.8).

Table 3.5: Welch two sample *t*-test results of NMR SMoIESY-select data for the VANISH and LeoPARDS cohorts at TP0. Adjusted p-values were calculated with B-H correction. Adjusted p-values (p_{Adj}) and un-adjusted p-values (p) <0.05 are highlighted in light green; Mean intensity values are highlighted for survivors and non-survivors (Yellow represents higher mean value; peach represents lower mean value). Metabolites significant in the two cohorts are highlighted in different colours (green = In both cohorts; red = Only in VANISH; blue = Only in LeoPARDS; dark grey = None).

Metabolite	VANISH _{TP0} (N = 164, n = 121 & 43)				LeoPARDS _{TP0} (N = 450, n = 304 & 146)			
	Mean Intensity (a.u)		p	p_{Adj}	Mean Intensity (a.u)		p	p_{Adj}
	Survivors	Non-survivors			Survivors	Non-survivors		
Tyrosine	4.19E+04	6.13E+04	7.46E-04	0.016	4.51E+04	6.04E+04	2.33E-06	1.45E-05
Glutamine	4.72E+05	6.13E+05	1.92E-03	0.021	5.07E+05	6.91E+05	2.83E-05	8.90E-05
Histidine	5.48E+04	6.88E+04	3.85E-03	0.028	6.13E+04	7.48E+04	2.01E-04	5.52E-04
Choline	1.31E+04	1.95E+04	7.99E-03	0.030	1.16E+04	1.85E+04	6.03E-07	6.63E-06
Alanine	3.07E+05	4.31E+05	8.43E-03	0.030	2.82E+05	4.29E+05	2.59E-05	8.90E-05
Glycine	2.30E+05	3.03E+05	0.010	0.030	2.16E+05	2.99E+05	2.64E-06	1.45E-05
Lactic acid	2.83E+06	4.33E+06	0.011	0.030	2.57E+06	4.15E+06	5.38E-07	6.63E-06
Phenylalanine	1.12E+05	1.39E+05	0.011	0.030	1.11E+05	1.38E+05	5.46E-06	2.40E-05
Creatinine	1.24E+05	1.57E+05	0.044	0.088	1.29E+05	1.59E+05	4.20E-04	8.40E-04
Valine	1.97E+05	2.36E+05	0.023	0.051	1.86E+05	2.10E+05	5.06E-03	8.56E-03
Leucine	1.02E+05	1.31E+05	0.019	0.047	1.03E+05	1.14E+05	0.07	0.096
Lysine	1.02E+05	1.29E+05	0.055	0.101	1.05E+05	1.36E+05	2.44E-04	5.98E-04
Formic acid	2.33E+04	2.85E+04	0.079	0.125	9.67E+03	1.45E+04	3.70E-04	8.14E-04
Glucose	7.89E+06	7.22E+06	0.234	0.286	8.41E+06	7.50E+06	2.91E-03	5.34E-03
Creatine	1.19E+05	1.64E+05	0.174	0.225	1.71E+05	2.34E+05	7.91E-03	0.012
Citric acid	8.11E+04	8.47E+04	0.747	0.782	7.32E+04	9.26E+04	0.022	0.032
Isoleucine	5.73E+04	7.07E+04	0.075	0.125	6.51E+04	7.08E+04	0.078	0.100
Acetic acid	4.70E+04	8.71E+04	0.120	0.176	3.56E+04	3.68E+04	0.889	0.889
Acetone	9.54E+04	7.02E+04	0.132	0.182	5.72E+04	7.33E+04	0.322	0.393
Dimethyl sulfone	1.36E+04	3.43E+04	0.383	0.443	2.48E+04	1.49E+04	0.449	0.493
Ethanol	5.92E+04	1.84E+05	0.410	0.451	7.97E+04	1.14E+05	0.375	0.434
3-Hydroxybutyric acid	6.54E+05	6.14E+05	0.833	0.833	4.91E+05	5.35E+05	0.646	0.677

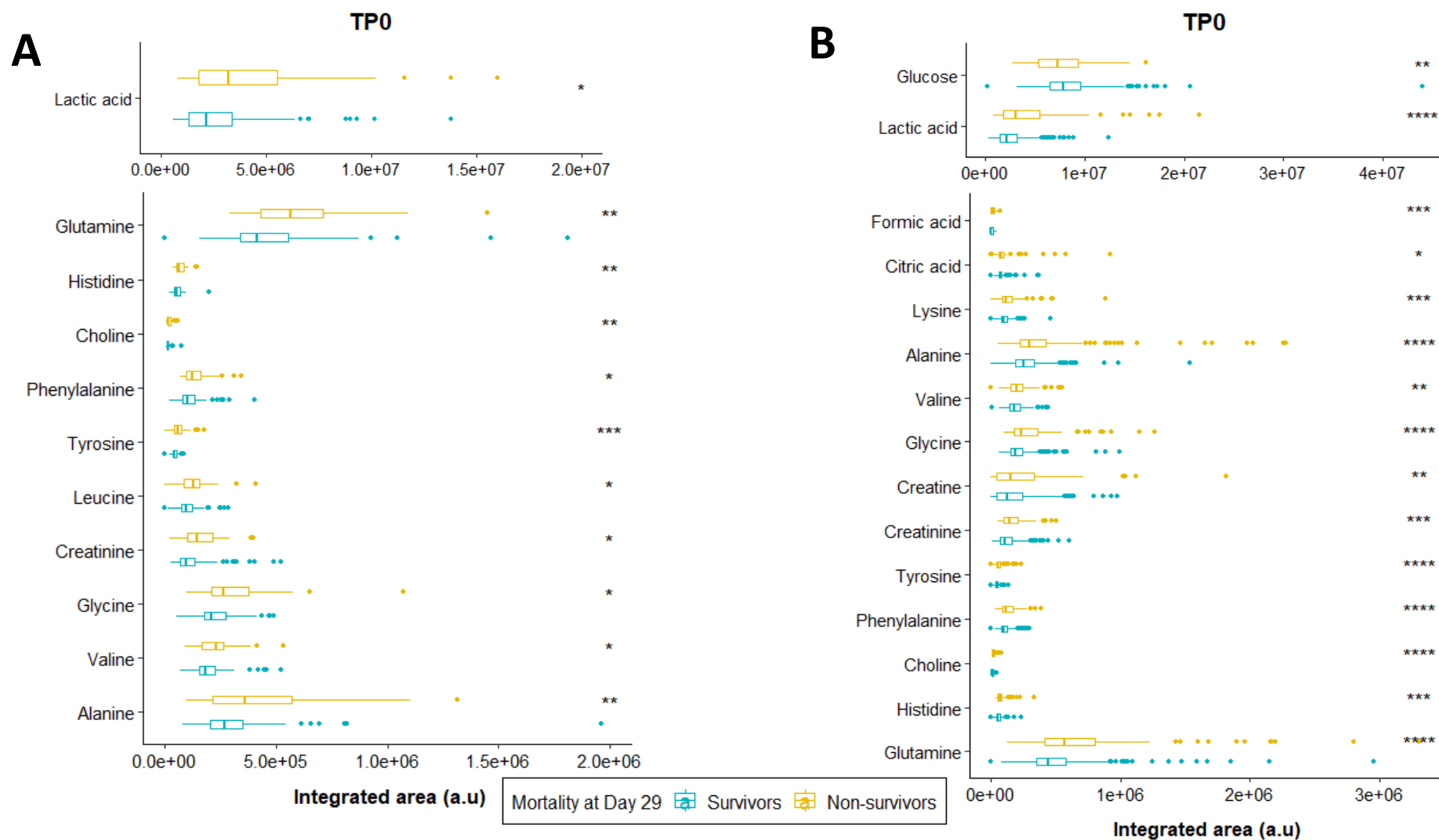


Figure 3.9: Box blots for the metabolites which were significantly different between survivors and non-survivors at TP0 in VANISH (A) and LeoPARDS (B) SMoIESY-select datasets.

Table 3.6: Welch two sample *t*-test results of NMR SMOIESY-select data for VANISH and LeoPARDS cohorts at TP1. Adjusted p-values were calculated with B-H correction. Adjusted p-values (p_{Adj}) and un-adjusted p-values (p) are highlighted in light green; Mean intensity values are highlighted for survivors and non-survivors (Yellow represents higher mean value; peach represents lower mean value). Metabolites significant in the two cohorts are highlighted in different colours (green = In both cohorts; red = Only in VANISH; blue = Only in LeoPARDS; dark grey = None).

Metabolite	VANISH _{TP1} (N = 134, n = 103 & 31)				LeoPARDS _{TP1} (N = 393, n = 286 & 107)			
	Mean Intensity (a.u)		p	p_{Adj}	Mean Intensity (a.u)		p	p_{Adj}
	Survivors	Non-survivors			Survivors	Non-survivors		
Tyrosine	4.54E+04	6.26E+04	5.16E-04	0.011	4.19E+04	5.85E+04	7.24E-07	1.59E-05
Phenylalanine	1.18E+05	1.52E+05	1.81E-03	0.02	1.10E+05	1.39E+05	2.07E-05	1.10E-04
Lactic acid	1.67E+06	3.10E+06	0.021	0.094	1.71E+06	3.07E+06	1.20E-05	8.78E-05
Alanine	2.67E+05	3.79E+05	0.033	0.121	2.33E+05	3.66E+05	2.49E-05	1.10E-04
Creatinine	9.48E+04	1.19E+05	0.048	0.15	1.16E+05	1.39E+05	0.011	0.023
3-Hydroxybutyric acid	4.03E+05	1.75E+05	6.84E-03	0.05	2.25E+05	2.27E+05	0.970	0.970
Dimethyl sulfone	1.08E+04	8.10E+03	0.019	0.094	2.26E+04	1.11E+04	0.365	0.423
Glutamine	4.65E+05	6.46E+05	0.061	0.169	4.38E+05	6.16E+05	1.50E-04	4.73E-04
Glycine	2.41E+05	2.94E+05	0.076	0.169	1.98E+05	3.25E+05	9.50E-04	2.61E-03
Choline	1.27E+04	1.26E+04	0.931	0.931	1.28E+04	1.94E+04	7.31E-05	2.68E-04
Histidine	5.39E+04	6.50E+04	0.140	0.257	5.53E+04	7.45E+04	4.92E-06	5.41E-05
Lysine	1.13E+05	1.43E+05	0.182	0.307	1.07E+05	1.32E+05	3.87E-03	9.47E-03
Valine	2.12E+05	2.14E+05	0.862	0.931	1.81E+05	2.02E+05	0.020	0.032
Leucine	1.08E+05	1.14E+05	0.505	0.686	9.49E+04	1.12E+05	8.99E-03	0.02
Isoleucine	7.05E+04	7.28E+04	0.606	0.741	6.70E+04	7.45E+04	0.043	0.062
Acetic acid	2.85E+04	4.15E+04	0.094	0.188	2.49E+04	3.72E+04	0.013	0.023
Creatine	1.27E+05	1.49E+05	0.389	0.57	1.57E+05	2.21E+05	0.014	0.024
Citric acid	6.65E+04	7.70E+04	0.274	0.431	7.03E+04	8.85E+04	0.045	0.062
Ethanol	4.20E+04	6.45E+04	0.53	0.686	7.43E+04	6.83E+04	0.513	0.564
Formic acid	3.02E+04	3.09E+04	0.787	0.912	1.47E+04	1.80E+04	0.120	0.155
Glucose	7.98E+06	8.05E+06	0.901	0.931	8.49E+06	8.43E+06	0.862	0.903
Acetone	7.18E+04	4.11E+04	0.077	0.169	2.71E+04	4.85E+04	0.276	0.337

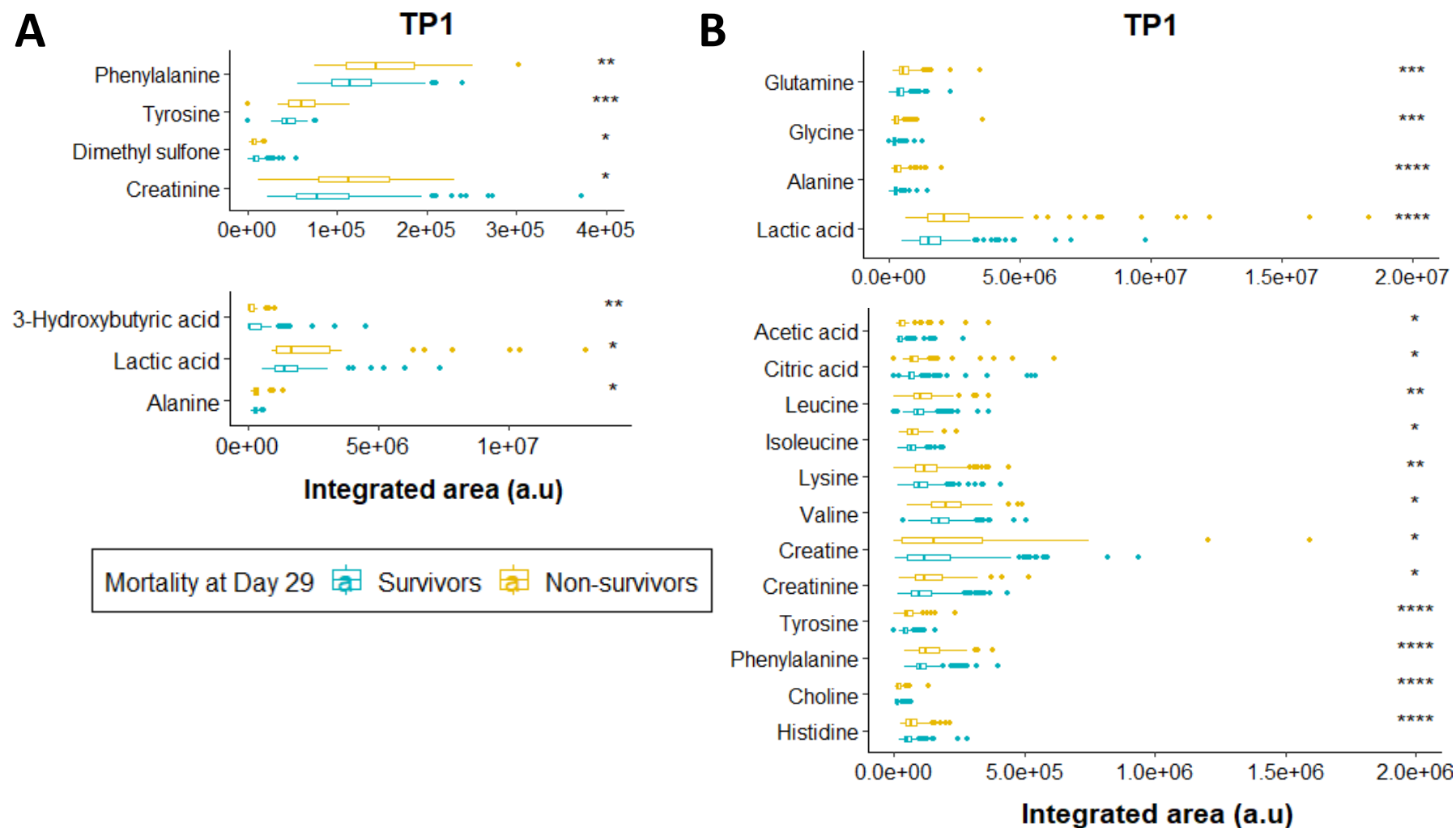


Figure 3.10: Box blots for the metabolites which were significantly different between survivors and non-survivors at TP1 in VANISH (A) and LeoPARDS (B) SMoIESY-select datasets.

Table 3.7: Welch two sample *t*-test results of NMR SMoESY data for VANISH and LeoPARDS cohorts at TP2. Adjusted p-values were calculated with B-H correction. Adjusted p-values (p_{Adj}) and un-adjusted p-values (p) are highlighted in light green; Mean intensity values are highlighted for survivors and non-survivors (Yellow represents higher mean value; peach represents lower mean value). Metabolites significant in the two cohorts are highlighted in different colours (green = In both cohorts; red = Only in VANISH; blue = Only in LeoPARDS; dark grey = None).

Metabolite	VANISH _{TP2} (N = 99, n = 76 & 23)				LeoPARDS _{TP2} (N = 311, n = 247 & 64)			
	Mean Intensity (a.u)		p	p_{Adj}	Mean Intensity (a.u)		p	p_{Adj}
	Survivors	Non-survivors			Survivors	Non-survivors		
Tyrosine	4.74E+04	7.38E+04	4.42E-04	9.73E-03	4.89E+04	6.01E+04	2.40E-03	0.018
Phenylalanine	1.18E+05	1.59E+05	0.045	0.249	1.13E+05	1.35E+05	3.54E-03	0.019
Formic acid	3.21E+04	4.17E+04	0.034	0.249	1.89E+04	2.16E+04	0.292	0.458
Glutamine	5.18E+05	6.27E+05	0.045	0.249	5.27E+05	5.59E+05	0.353	0.471
Lactic acid	1.50E+06	2.91E+06	0.084	0.277	1.36E+06	2.21E+06	2.45E-05	5.38E-04
Choline	1.33E+04	1.60E+04	0.321	0.415	1.29E+04	1.77E+04	3.97E-04	4.37E-03
Alanine	2.96E+05	3.79E+05	0.128	0.352	2.67E+05	3.36E+05	5.23E-03	0.022
Creatinine	8.61E+04	1.09E+05	0.194	0.388	9.46E+04	1.17E+05	5.90E-03	0.022
Histidine	5.21E+04	5.97E+04	0.211	0.388	5.22E+04	6.04E+04	0.023	0.073
Lysine	1.25E+05	1.56E+05	0.074	0.277	1.38E+05	1.56E+05	0.079	0.193
Acetone	3.11E+04	2.03E+04	0.088	0.277	3.07E+04	3.10E+04	0.978	0.978
Glycine	2.57E+05	2.95E+05	0.159	0.388	2.44E+05	2.74E+05	0.124	0.272
Dimethyl sulfone	9.93E+03	8.37E+03	0.204	0.388	1.27E+04	8.30E+03	0.364	0.471
Acetic acid	2.45E+04	3.01E+04	0.276	0.415	2.54E+04	2.90E+04	0.329	0.471
Citric acid	6.49E+04	7.80E+04	0.282	0.415	6.93E+04	8.61E+04	0.239	0.438
Isoleucine	7.53E+04	8.36E+04	0.314	0.415	8.20E+04	8.50E+04	0.615	0.712
Leucine	1.12E+05	1.22E+05	0.315	0.415	1.15E+05	1.28E+05	0.154	0.309
Ethanol	2.15E+04	2.64E+04	0.424	0.519	5.33E+04	8.13E+04	0.054	0.148
3-Hydroxybutyric acid	1.44E+05	1.13E+05	0.457	0.53	1.96E+05	2.34E+05	0.586	0.712
Glucose	8.20E+06	7.79E+06	0.569	0.626	8.06E+06	8.02E+06	0.917	0.96
Creatine	1.22E+05	1.27E+05	0.859	0.899	1.07E+05	1.24E+05	0.276	0.458
Valine	2.29E+05	2.27E+05	0.94	0.94	2.39E+05	2.42E+05	0.817	0.898

Table 3.8: Welch two sample *t*-test results of NMR SMoIESY data for VANISH and LeoPARDS cohorts at TP3. Adjusted p-values were calculated with B-H correction. Adjusted p-values (p_{Adj}) and un-adjusted p-values (p) are highlighted in light green; Mean intensity values are highlighted for survivors and non-survivors (Yellow represents higher mean value; peach represents lower mean value). Metabolites significant in the two cohorts are highlighted in different colours (red = Only in VANISH; blue = Only in LeoPARDS; dark grey = None).

Metabolite	VANISH _{TP3} (N = 75, n = 59 & 16)				LeoPARDS _{TP3} (N = 250, n = 201 & 49)			
	Mean Intensity (a.u)		p	p_{Adj}	Mean Intensity (a.u)		p	p_{Adj}
	Survivors	Non-survivors			Survivors	Non-survivors		
Acetone	2.99E+04	1.84E+04	0.048	0.591	1.98E+04	2.39E+04	0.575	0.635
Choline	1.29E+04	1.78E+04	0.135	0.591	1.39E+04	1.95E+04	1.52E-03	0.017
Tyrosine	5.02E+04	5.99E+04	0.16	0.591	4.78E+04	6.11E+04	3.73E-03	0.027
Creatine	7.64E+04	1.02E+05	0.161	0.591	8.33E+04	1.36E+05	6.78E-04	0.015
Creatinine	7.84E+04	1.13E+05	0.125	0.591	8.95E+04	1.11E+05	0.017	0.094
Leucine	1.09E+05	1.07E+05	0.816	0.898	1.09E+05	1.29E+05	0.046	0.203
Glucose	8.14E+06	6.91E+06	0.082	0.591	8.55E+06	8.47E+06	0.869	0.869
Ethanol	3.25E+04	1.94E+04	0.246	0.691	8.46E+04	5.10E+04	0.102	0.249
Glycine	2.60E+05	2.91E+05	0.289	0.691	3.08E+05	2.71E+05	0.499	0.635
Lysine	1.25E+05	1.39E+05	0.379	0.691	1.40E+05	1.49E+05	0.555	0.635
Lactic acid	1.44E+06	1.58E+06	0.38	0.691	1.50E+06	1.81E+06	0.102	0.249
Formic acid	5.88E+04	3.73E+04	0.39	0.691	1.77E+04	2.26E+04	0.069	0.216
Valine	2.38E+05	2.17E+05	0.391	0.691	2.56E+05	2.64E+05	0.762	0.799
Citric acid	6.64E+04	5.94E+04	0.408	0.691	5.84E+04	7.10E+04	0.135	0.296
Acetic acid	2.75E+04	2.49E+04	0.523	0.796	5.32E+04	3.57E+04	0.577	0.635
3-Hydroxybutyric acid	1.33E+05	1.00E+05	0.561	0.796	1.16E+05	2.03E+05	0.225	0.351
Histidine	5.37E+04	5.07E+04	0.579	0.796	5.12E+04	5.67E+04	0.149	0.298
Glutamine	5.35E+05	5.69E+05	0.624	0.808	4.75E+05	4.99E+05	0.382	0.525
Dimethyl sulfone	7.85E+03	7.38E+03	0.676	0.826	1.39E+04	5.85E+03	0.214	0.351
Isoleucine	7.53E+04	7.35E+04	0.81	0.898	8.04E+04	9.02E+04	0.24	0.351
Alanine	2.88E+05	2.93E+05	0.865	0.906	2.88E+05	3.38E+05	0.21	0.351
Phenylalanine	1.19E+05	1.19E+05	0.988	0.988	1.19E+05	1.40E+05	0.056	0.205

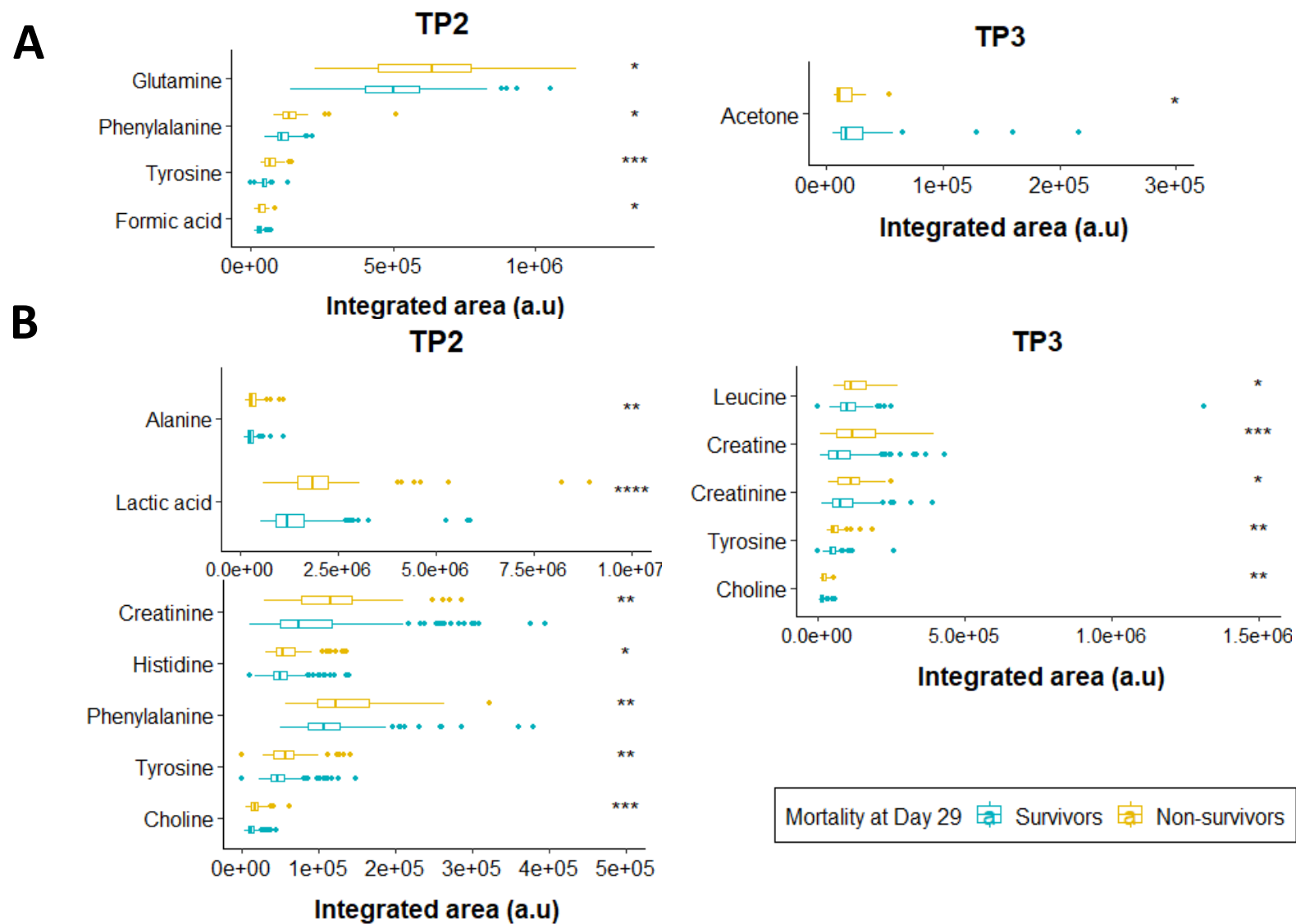


Figure 3.11: Box blots for the metabolites which were significantly different between survivors and non-survivors at TP2 & TP3 in VANISH (A) and LeoPARDS (B) SMoIESY-select datasets.

3.3.4.2 UHPLC-MS targeted data (HPOS ppR)

Univariate analysis of 39 small molecules from HPOS ppR data was performed for all timepoints on a total of 488 serum samples from VANISH and 1439 samples from LeoPARDS as shown in figure 3.12 and figure 3.13, respectively. In total, 23 samples were excluded from VANISH and of those 4 samples belonged to a patient whose samples were collected after several days of receiving the study drug and 19 samples had no sample timing recorded. In LeoPARDS, a total of 10 samples in addition to exclusions during the QC assessment (see section 2.2.2.3) were excluded from this analysis as the sample timings for these were not recorded.

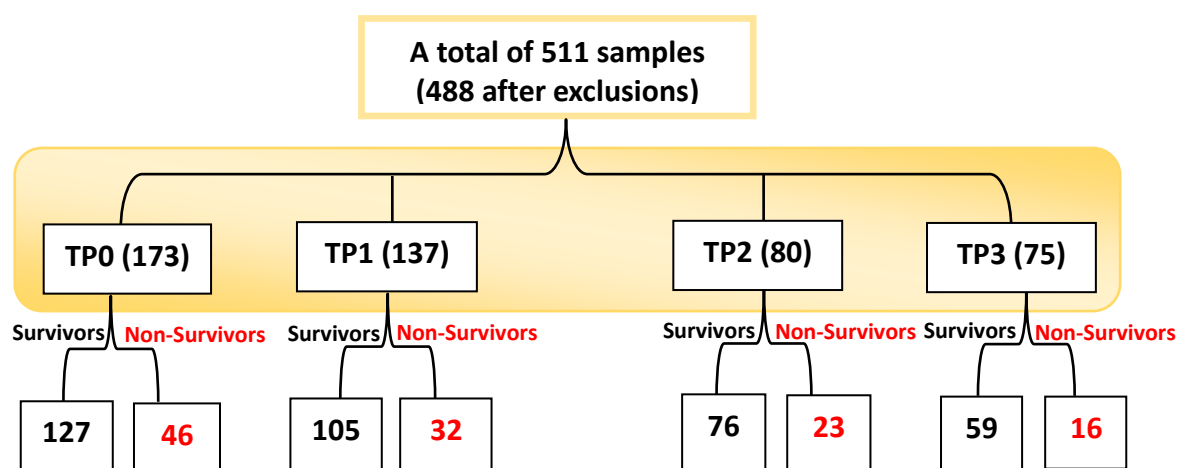


Figure 3.12: Flow chart for the samples included in the univariate analysis of HPOS ppR data from VANISH.

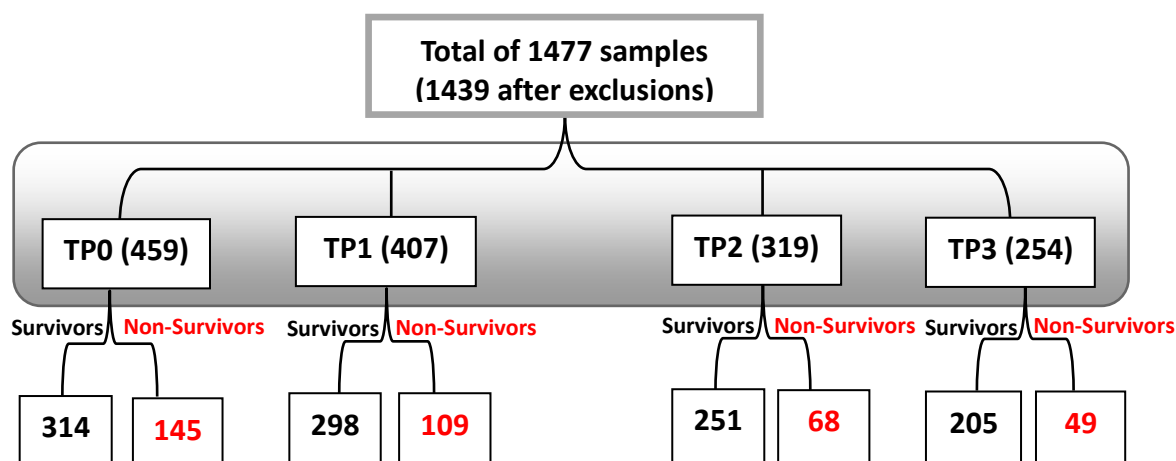


Figure 3.13: Flow chart for the samples included in the univariate analysis of HPOS ppR data from LeoPARDS.

A summary of univariate results for all timepoints (TP0-TP3) in both cohorts is presented in tables 3.9, 3.10, 3.11 and 3.12, differentiating survivors from non-survivors (tables 3.9-3.12).

17 metabolites including 1-methylnicotinamide and its metabolites (N1-methyl-2-pyridone-5-carboxamide and N1-methyl-2-pyridone-5-carboxamide), short-, medium- and long-chain acyl carnitines (hydroxybutyrylcarnitine, decanoylcarnitine, tetradecenoylcarnitine, hexadecenoylcarnitine, stearoylcarnitine, palmitoylcarnitine, oleoylcarnitine), N1-acetylspermidine, betaine, proline, lysine, N6, N6, N6-trimethyllysine, N,N-dimethylglycine and symmetric/asymmetric dimethylarginine were found to have significantly higher levels at ICU admission in serum of patients who died before day 29 compared to those who did not in both cohorts ($p_{Adj} < 0.05$). Several metabolites including creatinine, cortisol and trigonelline were significantly higher in non-survivors whilst hypoxanthine and arginine were lower in non-survivors from LeoPARDS ($p_{Adj} < 0.05$) at TP0. All these metabolites except for cortisol had the same pattern in the alternate cohort but the results were not significant.

At TP1, some metabolites such as 1-methylnicotinamide and its metabolites (N1-methyl-2-pyridone-5-carboxamide and N1-methyl-2-pyridone-5-carboxamide), and N1-acetylspermidine were significantly raised in non-survivors ($p < 0.05$) whilst hypoxanthine was significantly reduced in non-surviving patients ($p_{Adj} < 0.05$) in LeoPARDS whilst in VANISH only N1-methyl-2-pyridone-5-carboxamide was significant elevated in patients who went on to die than those who survived ($p_{Adj} < 0.05$). In addition, several metabolites including, N2,N2- dimethylguansoine, 7-methylguanine, sucrose, carnitines (such as hydroxybutyrylcarnitine), urocanate, betaine, proline, lysine, N6, N6, N6-trimethyllysine, N,N-dimethylglycine, symmetric/asymmetric dimethylarginine, creatinine, cortisol, trigonelline were significantly present in higher concentrations in non-surviving patients from LeoPARDS after 24 h of ICU admission ($p_{Adj} < 0.05$) whilst arginine was significantly lower ($p = 3.70 \times 10^{-3}$) in patients who went on to die ($p_{Adj} < 0.05$). All these metabolites except for except for four metabolites (including betaine, 7-methylguanine, creatinine and symmetric/asymmetric dimethylarginine) followed the same pattern in non-survivors from VANISH.

Non-surviving patients from TP2 had significantly increased concentrations of seven metabolites including 1-methylnicotinamide and its metabolites (N1-methyl-2-pyridone-5-

carboxamide and N1-methyl-2-pyridone-5-carboxamide), N2,N2-dimethylguansoine, N1-acetylspermidine, urocanate, and N6, N6, N6-trimethyllysine in both cohorts ($p < 0.05$) and these seven metabolites remained significant after BH correction, but only in LeoPARDS ($p_{\text{Adj}} < 0.05$).

At TP3, only four metabolites including 1-methylnicotinamide, N2,N2-dimethylguansoine, N6, N6, N6-trimethyllysine and pseudouridine were significantly increased in non-survivors with respect to survivors in both cohorts ($p < 0.05$). All these metabolites remained significant after correction but only in LeoPARDS ($p_{\text{Adj}} < 0.05$).

Table 3.9: Welch two sample *t*-test results of HPOS ppR data for VANISH and LeoPARDS cohorts at TP0. Adjusted p-values were calculated with B-H correction. Adjusted p-values (p_{Adj}) and un-adjusted p-values (p) are highlighted in light green; Mean intensity values are highlighted for survivors and non-survivors (Yellow represents higher mean value; peach represents lower mean value for the two groups). Metabolites significant in the two cohorts are highlighted in different colours (green = In both cohorts; red = Only in VANISH; blue = Only in LeoPARDS; dark grey = None).

Metabolite	VANISH _{TP0} (N = 173, n = 127 & 46)				LeoPARDS _{TP0} (N = 459, n = 314 & 145)			
	Mean Intensity (a.u)		p	p_{Adj}	Mean Intensity (a.u)		p	p_{Adj}
	Survivors	Non-survivors			Survivors	Non-survivors		
N1-methyl-2-pyridone-5-carboxamide	12.27	13.11	1.77E-05	3.44E-04	12.04	12.78	4.09E-13	1.60E-11
1-Methylnicotinamide	10.70	11.74	3.43E-05	4.46E-04	11.45	12.3	2.26E-10	2.94E-09
N1-methyl-4-pyridone-3-carboxamide	10.36	11.12	1.26E-04	1.23E-03	10.57	11.31	1.53E-12	2.98E-11
N2,N2-Dimethylguanosine	8.21	8.45	0.032	0.060	8.89	9.22	1.52E-08	1.19E-07
Sucrose	11.21	11.89	0.048	0.079	10.79	11.81	4.29E-08	2.39E-07
Decanoylcarnitine (C10:0)	12.46	13.00	3.10E-04	2.14E-03	11.85	12.09	7.94E-05	2.58E-04
Hexadecenoylcarnitine (C16:1)	11.12	11.69	1.77E-03	6.92E-03	10.33	10.67	7.76E-05	2.58E-04
Stearoylcarnitine (C18:0)	10.93	11.37	3.28E-03	0.012	9.85	10.07	4.56E-03	8.25E-03
Palmitoylcarnitine (C16:0)	12.18	12.54	0.012	0.032	11.19	11.39	6.74E-03	0.011
Hydroxybutyrylcarnitine (C4:0-OH)	12.09	12.56	0.012	0.032	12.17	12.48	2.26E-03	4.65E-03
Tetradecenoylcarnitine (C14:1)	11.47	11.87	0.020	0.044	10.60	10.87	4.66E-03	8.25E-03
Oleoylcarnitine (C18:1)	12.92	13.25	0.022	0.045	11.68	11.86	0.011	0.017
N1-Acetylspermidine	11.59	12.23	9.06E-07	3.53E-05	11.86	12.30	1.85E-09	1.81E-08
Urocanate	9.80	10.30	0.043	0.076	10.66	11.38	3.98E-06	1.68E-05

Betaine	14.84	15.04	0.017	0.039	14.75	14.87	5.25E-03	8.89E-03
Proline	11.85	12.18	3.29E-04	2.14E-03	12.82	13.04	8.99E-05	2.70E-04
Pipecolate N-methyl proline	9.25	9.59	0.029	0.057	10.54	10.73	0.021	0.030
N6,N6,N6- Trimethyllysine	12.09	12.49	3.86E-04	2.15E-03	12.35	12.67	7.33E-08	3.57E-07
HPOS_Lysine	9.61	9.95	9.43E-04	4.60E-03	11.14	11.37	4.31E-06	1.68E-05
N,N-Dimethylglycine	8.58	8.94	1.33E-03	5.75E-03	9.53	9.77	3.14E-04	8.18E-04
Symmetric Asymmetric Dimethylarginine	12.90	13.16	7.68E-03	0.025	12.66	12.89	3.51E-08	2.28E-07
Carnitine	16.71	16.90	0.014	0.034	16.49	16.50	0.877	0.877
Trimethylaminoacetone	8.43	8.78	0.049	0.079	8.38	8.41	0.675	0.711
α - glycerophosphocholine	10.56	11.03	0.012	0.032	10.55	10.72	0.052	0.072
Hypoxanthine	10.95	10.37	0.111	0.161	11.16	10.52	7.17E-04	1.55E-03
Cortisol	10.45	10.41	0.915	0.939	10.66	11.00	2.07E-04	5.76E-04
Arginine	12.57	12.52	0.703	0.762	12.80	12.57	4.91E-04	1.20E-03
Pseudouridine	12.64	12.65	0.975	0.975	12.94	13.08	0.018	0.027
HPOS_Creatinine	13.07	13.12	0.436	0.500	13.39	13.46	6.58E-04	1.51E-03
Trigonelline	11.02	11.15	0.679	0.756	10.24	10.70	3.97E-03	7.75E-03
HPOS_Creatine	13.28	13.54	0.125	0.173	13.72	13.75	0.738	0.757
Niacinamide	8.98	8.61	0.146	0.196	9.26	9.43	0.190	0.212
Pantothenate	10.31	10.60	0.055	0.086	11.26	11.36	0.157	0.180
Taurine	9.32	9.64	0.081	0.121	10.68	10.82	0.074	0.093
7-Methylguanine	9.59	9.79	0.189	0.238	8.65	8.74	0.064	0.083
1-Methyladenosine	11.77	11.88	0.162	0.210	11.61	11.67	0.123	0.145
Acetaminophen	11.85	11.14	0.249	0.303	12.39	12.03	0.079	0.096
Proline Betaine	13.83	13.90	0.821	0.865	13.44	13.61	0.320	0.347
N-methyl pipecolic acid	8.19	8.41	0.275	0.324	11.13	11.31	0.058	0.078

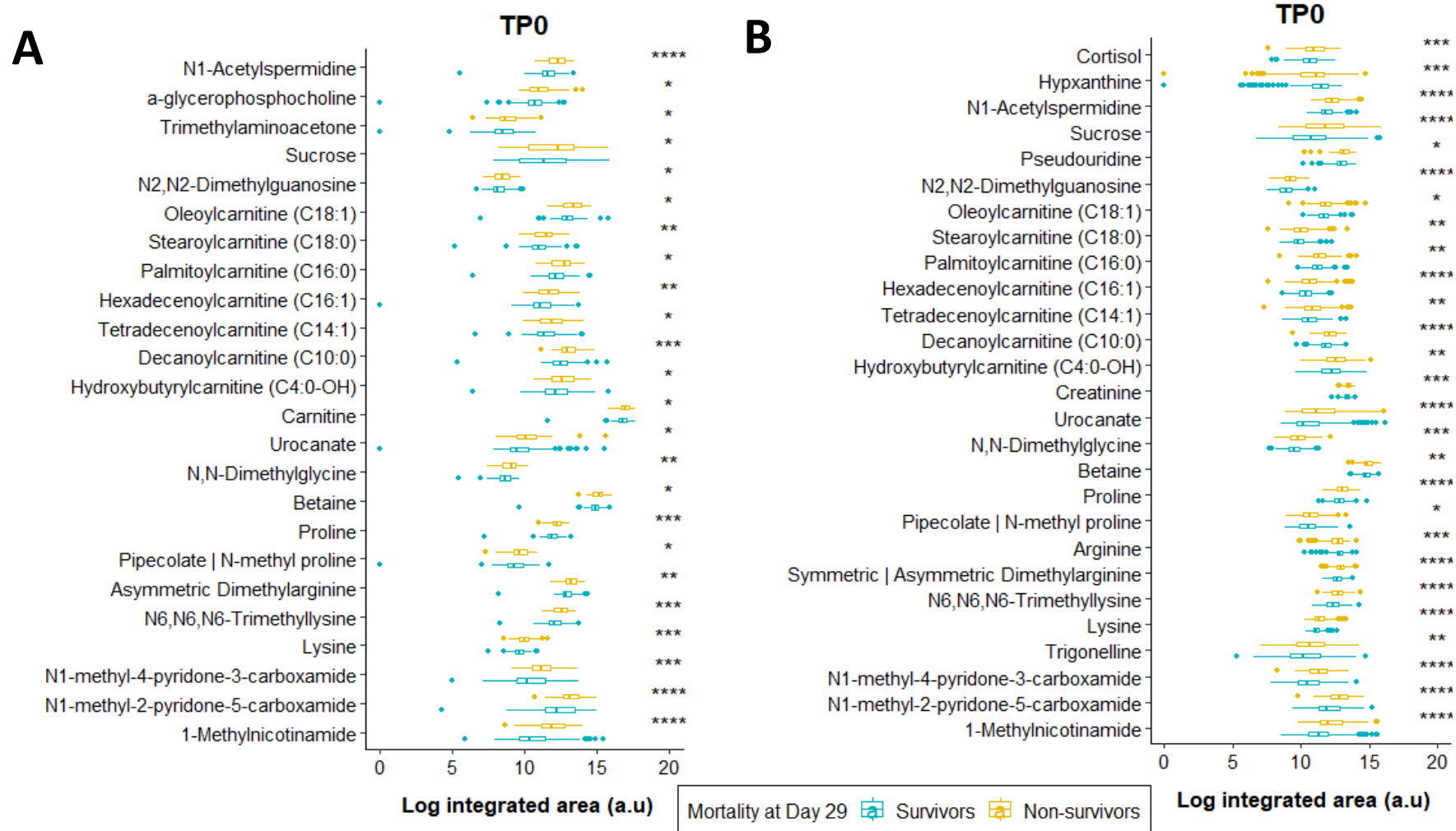


Table 3.10: Welch two sample t-test results of HPOS ppR data for VANISH and LeoPARDS cohorts at TP1. Adjusted p-values were calculated with B-H correction. Adjusted p-values (p_{Adj}) and un-adjusted p-values (p) <0.05 are highlighted in light green; Mean intensity values are highlighted for survivors and non-survivors (Yellow represents higher mean value; peach represents lower mean value and turquoise represent equal mean value). Metabolites significant in the two cohorts are highlighted in different colours (green = In both cohorts; blue = Only in LeoPARDS; dark grey = None).

Metabolite	VANISH _{TP1} (N = 137, n = 105 & 32)				LeoPARDS _{TP1} (N = 407, n = 298 & 109)			
	Mean Intensity (a.u)		p	p_{Adj}	Mean Intensity (a.u)		p	p_{Adj}
	Survivors	Non-survivors			Survivors	Non-survivors		
N1-methyl-2-pyridone-5-carboxamide	12.52	13.26	3.63E-04	0.014	12.15	12.87	9.74E-09	1.90E-07
N1-methyl-4-pyridone-3-carboxamide	10.56	11.24	5.18E-03	0.067	10.69	11.40	2.80E-08	3.64E-07
1-Methylnicotinamide	10.8	11.78	3.48E-03	0.067	11.68	12.64	5.62E-08	5.48E-07
N1-Acetylspermidine	11.66	12.04	0.022	0.177	11.69	12.06	1.20E-05	7.82E-05
Hypoxanthine	10.74	9.76	0.023	0.177	11.14	10.51	1.70E-04	5.53E-04
Niacinamide	8.81	8.74	0.749	0.973	9.24	9.55	0.032	0.057
Trigonelline	10.46	10.47	0.973	0.973	9.83	10.28	5.21E-03	0.011
Sucrose	11.28	11.31	0.935	0.973	10.90	11.69	8.19E-05	3.55E-04
7-Methylguanine	9.47	9.33	0.785	0.973	8.65	8.83	1.79E-03	4.36E-03
N2,N2-Dimethylguanosine	11.77	11.90	0.110	0.430	8.86	9.32	2.61E-10	1.02E-08
1-Methyladenosine	8.12	8.32	0.183	0.495	11.61	11.69	0.042	0.072
Decanoylcarnitine (C10:0)	12.51	12.82	0.090	0.424	11.77	11.94	0.027	0.050
Hydroxybutyrylcarnitine (C4:0-OH)	11.56	11.67	0.592	0.962	11.39	11.89	1.10E-04	4.31E-04
Urocanate	10.18	10.96	0.066	0.367	11.03	11.63	4.40E-03	9.53E-03
Betaine	14.85	14.83	0.852	0.973	14.65	14.82	1.58E-04	5.53E-04
Proline	11.96	12.11	0.200	0.495	12.66	12.96	6.88E-05	3.35E-04

N6,N6,N6-Trimethyllysine	12.26	12.51	0.098	0.424	12.51	12.77	2.20E-04	6.60E-04
HPOS_Lysine	9.83	9.98	0.203	0.495	11.20	11.42	6.46E-05	3.35E-04
N,N-Dimethylglycine	8.60	8.74	0.294	0.616	9.32	9.58	8.12E-04	2.26E-03
HPOS_Creatinine	13.14	13.09	0.401	0.745	13.34	13.40	8.53E-03	0.017
Symmetric Asymmetric Dimethylarginine	13.01	13.00	0.946	0.973	12.68	12.88	8.75E-06	6.83E-05
Arginine	12.84	12.63	0.177	0.495	12.94	12.76	3.70E-03	8.49E-03
Cortisol	10.60	11.06	0.060	0.367	10.63	10.98	1.70E-03	4.36E-03
Pseudouridine	12.63	12.44	0.300	0.616	12.95	13.01	0.377	0.525
Pantothenate	10.30	10.03	0.126	0.446	11.09	11.15	0.434	0.584
Taurine	8.82	8.74	0.631	0.962	10.29	10.40	0.198	0.296
Carnitine	16.74	16.72	0.877	0.973	16.39	16.38	0.789	0.879
Trimethylaminoacetone	8.20	8.42	0.229	0.526	8.26	8.26	0.970	0.970
a-glycerophosphocholine	10.49	10.29	0.180	0.495	10.32	10.41	0.329	0.475
Hexadecenoylcarnitine (C16:1)	11.22	11.30	0.649	0.962	10.29	10.42	0.089	0.139
Stearoylcarnitine (C18:0)	10.98	10.92	0.702	0.962	9.86	9.91	0.474	0.616
Palmitoylcarnitine (C16:0)	12.27	12.17	0.462	0.819	11.23	11.22	0.902	0.951
Tetradecenoylcarnitine (C14:1)	11.46	11.54	0.716	0.962	10.56	10.63	0.493	0.620
Oleoylcarnitine (C18:1)	13.00	13.01	0.949	0.973	11.67	11.70	0.721	0.828
Acetaminophen	11.50	11.26	0.686	0.962	11.78	11.69	0.705	0.828
Proline Betaine	13.58	13.36	0.578	0.962	13.09	13.10	0.937	0.962
Pipecolate N-methyl proline	9.43	9.45	0.883	0.973	10.37	10.54	0.070	0.113
N-methyl pipecolic acid	8.13	8.10	0.899	0.973	11.00	11.04	0.703	0.828
HPOS_Creatine	13.52	13.68	0.370	0.721	13.76	13.74	0.821	0.889

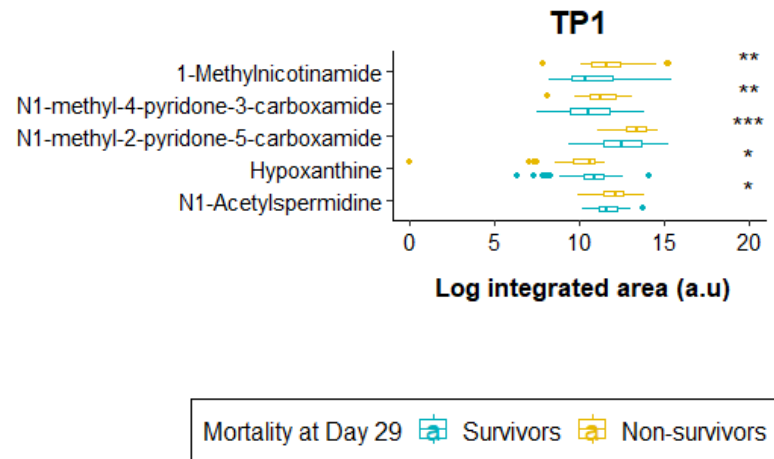
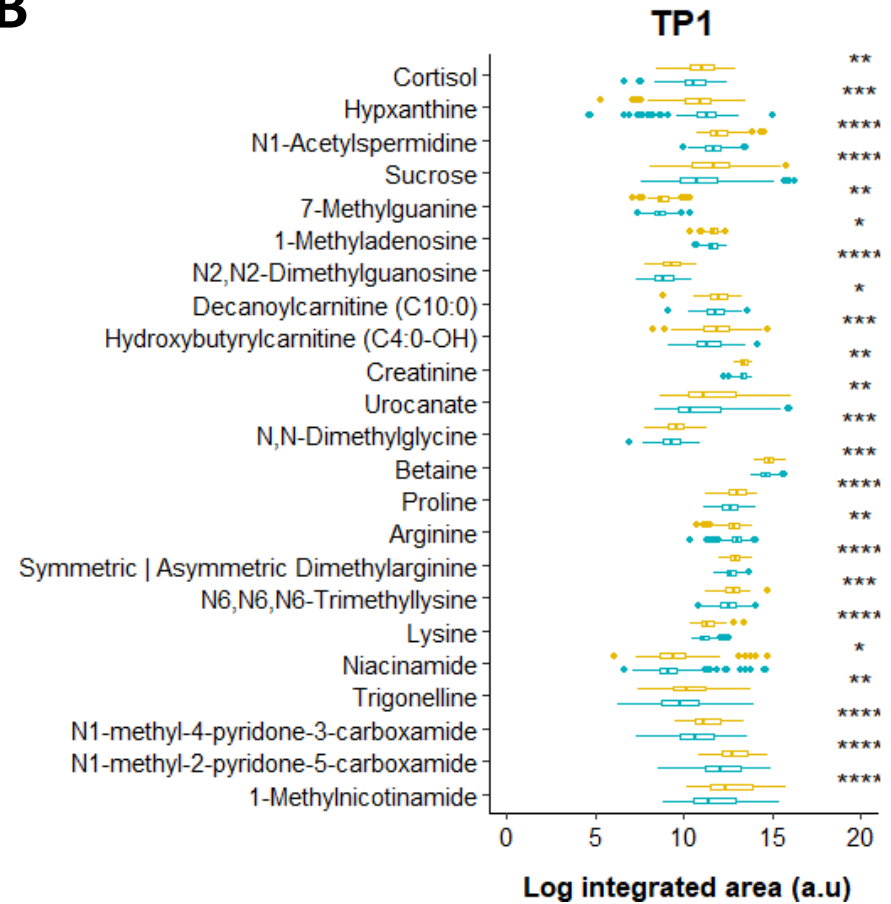
A**B**

Figure 3.15: Box blots for the metabolites which were significantly different between survivors and non-survivors at TP1 in VANISH (A) and LeoPARDS (B) HPOS ppR datasets.

Table 3.11: Welch two sample *t*-test results of HPOS ppR data for VANISH and LeoPARDS cohorts at TP2. Adjusted p-values were calculated with B-H correction. Adjusted p-values (p_{Adj}) and un-adjusted p-values (p) are highlighted in light green; Mean intensity values are highlighted for survivors and non-survivors (Yellow represents higher mean value; peach represents lower mean value and turquoise indicates equal mean value for the two groups). Metabolites significant in the two cohorts are highlighted in different colours (green = In both cohorts; red = Only in VANISH; blue = Only in LeoPARDS; dark grey = None).

Metabolite	VANISH _{TP2} (N = 103, n =80 & 23)				LeoPARDS _{TP2} (N = 319, n = 251 & 68)			
	Mean Intensity (a.u)		p	p_{Adj}	Mean Intensity (a.u)		p	p_{Adj}
	Survivors	Non-survivors			Survivors	Non-survivors		
1-Methylnicotinamide	11.00	12.04	0.014	0.165	11.77	12.55	1.29E-04	1.00E-03
N1-methyl-2-pyridone-5-carboxamide	12.63	13.24	0.022	0.165	12.13	12.66	1.42E-03	5.06E-03
N1-methyl-4-pyridone-3-carboxamide	10.67	11.28	0.042	0.225	10.67	11.18	2.47E-03	8.02E-03
N2,N2-Dimethylguanosine	8.04	8.42	0.019	0.165	8.79	9.26	3.81E-06	7.43E-05
N1-Acetylspermidine	11.60	12.17	1.32E-03	0.052	11.66	11.96	7.79E-04	3.80E-03
Urocanate	10.36	11.41	0.025	0.165	10.68	11.76	1.65E-04	1.07E-03
N6,N6,N6-Trimethyllysine	12.35	12.67	0.046	0.225	12.57	12.92	1.28E-05	1.67E-04
Decanoylcarnitine (C10:0)	12.56	13.01	0.020	0.165	11.59	11.65	0.579	0.664
7-Methylguanine	9.54	9.43	0.817	0.949	8.48	8.82	1.38E-06	5.37E-05
Trigonelline	10.08	10.24	0.687	0.837	9.35	9.78	0.014	0.041
1-Methyladenosine	11.76	11.89	0.136	0.443	11.41	11.61	1.82E-05	1.78E-04
Cortisol	10.45	10.97	0.077	0.335	10.55	11.01	2.84E-04	1.58E-03
HPOS_Creatinine	13.07	13.07	0.971	0.973	13.23	13.34	1.01E-03	4.38E-03
Symmetric Asymmetric Dimethylarginine	13	13.13	0.257	0.523	12.78	12.95	1.43E-03	5.06E-03
N,N-Dimethylglycine	8.68	8.56	0.543	0.706	9.50	9.74	0.019	0.054
Stearoylcarnitine (C18:0)	11.23	11.47	0.268	0.523	10.19	9.96	0.021	0.056

Palmitoylcarnitine (C16:0)	12.41	12.55	0.478	0.690	11.45	11.23	0.045	0.103
Betaine	14.89	14.89	0.973	0.973	14.65	14.81	0.030	0.073
Niacinamide	8.91	9.24	0.254	0.523	9.16	9.24	0.526	0.622
Pantothenate	10.23	10.10	0.418	0.679	11.13	11.12	0.879	0.902
Taurine	8.62	8.63	0.965	0.973	10.06	9.94	0.150	0.266
Sucrose	11.37	11.25	0.673	0.837	11.03	11.48	0.071	0.155
Carnitine	16.69	16.75	0.461	0.690	16.32	16.26	0.400	0.538
Hypoxanthine	11.04	10.38	0.250	0.523	11.06	10.85	0.12	0.246
Pseudouridine	12.60	12.47	0.522	0.706	12.84	12.93	0.364	0.507
Trimethylaminoacetone	8.41	8.43	0.915	0.973	8.28	8.35	0.465	0.584
α-glycerophosphocholine	10.63	10.68	0.827	0.949	10.56	10.41	0.146	0.266
Hexadecenoylcarnitine (C16:1)	11.34	11.70	0.136	0.443	10.52	10.44	0.525	0.622
Hydroxybutyrylcarnitine (C4:0-OH)	11.09	11.42	0.122	0.443	10.99	11.21	0.146	0.266
Tetradecenoylcarnitine (C14:1)	11.70	12.04	0.179	0.523	10.96	10.78	0.178	0.301
Oleoylecarnitine (C18:1)	13.14	13.38	0.232	0.523	11.89	11.77	0.222	0.347
Acetaminophen	11.47	10.47	0.250	0.523	11.44	11.06	0.295	0.426
Proline Betaine	13.11	13.03	0.879	0.973	12.46	12.48	0.956	0.956
Proline	12.06	12.16	0.409	0.679	13.05	13.10	0.437	0.568
Pipecolate N-methyl proline	9.35	9.45	0.536	0.706	10.38	10.42	0.657	0.712
N-methyl pipecolic acid	7.95	8.19	0.377	0.669	10.61	10.76	0.237	0.356
HPOS_Lysine	9.90	9.99	0.450	0.690	11.47	11.55	0.194	0.315
HPOS_Creatine	13.52	13.68	0.377	0.669	13.66	13.62	0.702	0.740
Arginine	12.97	12.76	0.193	0.523	13.14	13.11	0.626	0.698

Table 3.12: Welch two sample *t*-test results of HPOS ppR data for VANISH and LeoPARDS cohorts at TP3. Adjusted p-values were calculated with B-H correction. Adjusted p-values (p_{Adj}) and un-adjusted p-values (p) are highlighted in light green; Mean intensity values are highlighted for survivors and non-survivors (Yellow represents higher mean value; peach represents lower mean value). Metabolites significant in the two cohorts are highlighted in different colours (green = In both cohorts; red = Only in VANISH; blue = Only in LeoPARDS; dark grey = None).

Metabolite	VANISH _{TP3} (N = 75, n = 59 & 16)				LeoPARDS _{TP3} (N = 254, n = 205 & 49)			
	Mean Intensity (a.u)		p	p_{Adj}	Mean Intensity (a.u)		p	p_{Adj}
	Survivors	Non-survivors			Survivors	Non-survivors		
1-Methylnicotinamide	11.15	11.91	0.041	0.309	11.76	12.49	5.57E-04	3.10E-03
N2,N2-Dimethylguanosine	11.83	12.05	0.021	0.309	8.69	9.34	9.81E-09	3.82E-07
N6,N6,N6-Trimethyllysine	12.28	12.68	0.047	0.309	12.39	12.90	2.65E-08	5.17E-07
Pseudouridine	12.56	12.88	0.036	0.309	12.80	13.08	6.47E-03	0.018
Pipecolate N-methyl proline	9.26	9.66	0.020	0.309	10.35	10.51	0.076	0.129
N1-methyl-2-pyridone-5-carboxamide	12.72	13.24	0.055	0.309	12.32	12.92	1.51E-03	5.34E-03
N1-methyl-4-pyridone-3-carboxamide	10.80	11.25	0.120	0.467	10.84	11.44	2.31E-03	7.52E-03
Sucrose	11.35	11.74	0.417	0.677	11.03	12.02	7.54E-04	3.68E-03
1-Methyladenosine	8.11	8.38	0.200	0.571	11.38	11.59	9.74E-04	4.22E-03
α -glycerophosphocholine	10.80	10.66	0.620	0.780	10.87	10.51	6.34E-03	0.018
Decanoylcarnitine (C10:0)	12.49	12.72	0.273	0.626	11.51	11.74	0.017	0.039
Hydroxybutyrylcarnitine (C4:0-OH)	10.87	10.96	0.664	0.785	10.71	11.27	1.17E-04	9.12E-04
N1-Acetylspermidine	11.63	11.82	0.368	0.677	11.64	12.03	4.74E-04	3.08E-03
Urocanate	10.42	11.21	0.087	0.426	10.45	11.35	1.17E-03	4.57E-03
HPOS_Creatinine	13.06	13.16	0.151	0.534	13.23	13.33	6.81E-03	0.018

HPOS_Creatine	13.40	13.69	0.100	0.435	13.53	13.84	0.011	0.027
Symmetric Asymmetric Dimethylarginine	13.01	13.17	0.187	0.571	12.74	12.98	9.03E-07	1.17E-05
7-Methylguanine	9.47	9.28	0.770	0.883	8.38	8.73	6.47E-05	6.31E-04
HPOS_Lysine	9.89	9.90	0.925	0.949	11.39	11.52	0.042	0.091
Niacinamide	8.85	9.03	0.648	0.785	9.23	9.24	0.972	0.993
Trigonelline	9.73	10.03	0.397	0.677	9.02	9.44	0.072	0.129
Pantothenate	10.19	10.04	0.263	0.626	11.27	11.21	0.594	0.724
Taurine	8.52	8.73	0.299	0.649	10.18	10.12	0.552	0.694
Carnitine	16.64	16.69	0.605	0.780	16.41	16.45	0.483	0.628
Hypoxanthine	10.97	10.76	0.411	0.677	11.1	11.01	0.47	0.628
Trimethylaminoacetone	8.35	8.37	0.898	0.949	8.43	8.26	0.093	0.151
Hexadecenoylcarnitine (C16:1)	11.43	11.26	0.533	0.742	10.44	10.43	0.950	0.993
Stearoylcarnitine (C18:0)	11.31	11.25	0.802	0.894	10.14	9.93	0.061	0.125
Palmitoylcarnitine (C16:0)	12.46	12.27	0.439	0.684	11.37	11.16	0.064	0.125
Tetradecenoylcarnitine (C14:1)	11.76	11.73	0.924	0.949	11.01	10.97	0.797	0.863
Oleoylcarnitine (C18:1)	13.17	13.05	0.607	0.780	11.80	11.76	0.710	0.791
Cortisol	10.50	10.25	0.378	0.677	10.34	10.53	0.177	0.265
Acetaminophen	11.30	9.81	0.212	0.571	11.42	10.94	0.215	0.310
Betaine	14.88	14.82	0.531	0.742	14.76	14.80	0.446	0.621
Proline Betaine	12.68	12.67	0.989	0.989	12.01	12.00	0.993	0.993
Proline	12.09	12.18	0.376	0.677	13.06	13.17	0.075	0.129
N-methyl pipecolic acid	7.62	8.26	0.051	0.309	10.66	10.72	0.693	0.791
N,N-Dimethylglycine	8.62	8.76	0.456	0.684	9.76	9.94	0.115	0.179
Arginine	12.96	12.71	0.220	0.571	13.17	13.15	0.706	0.791

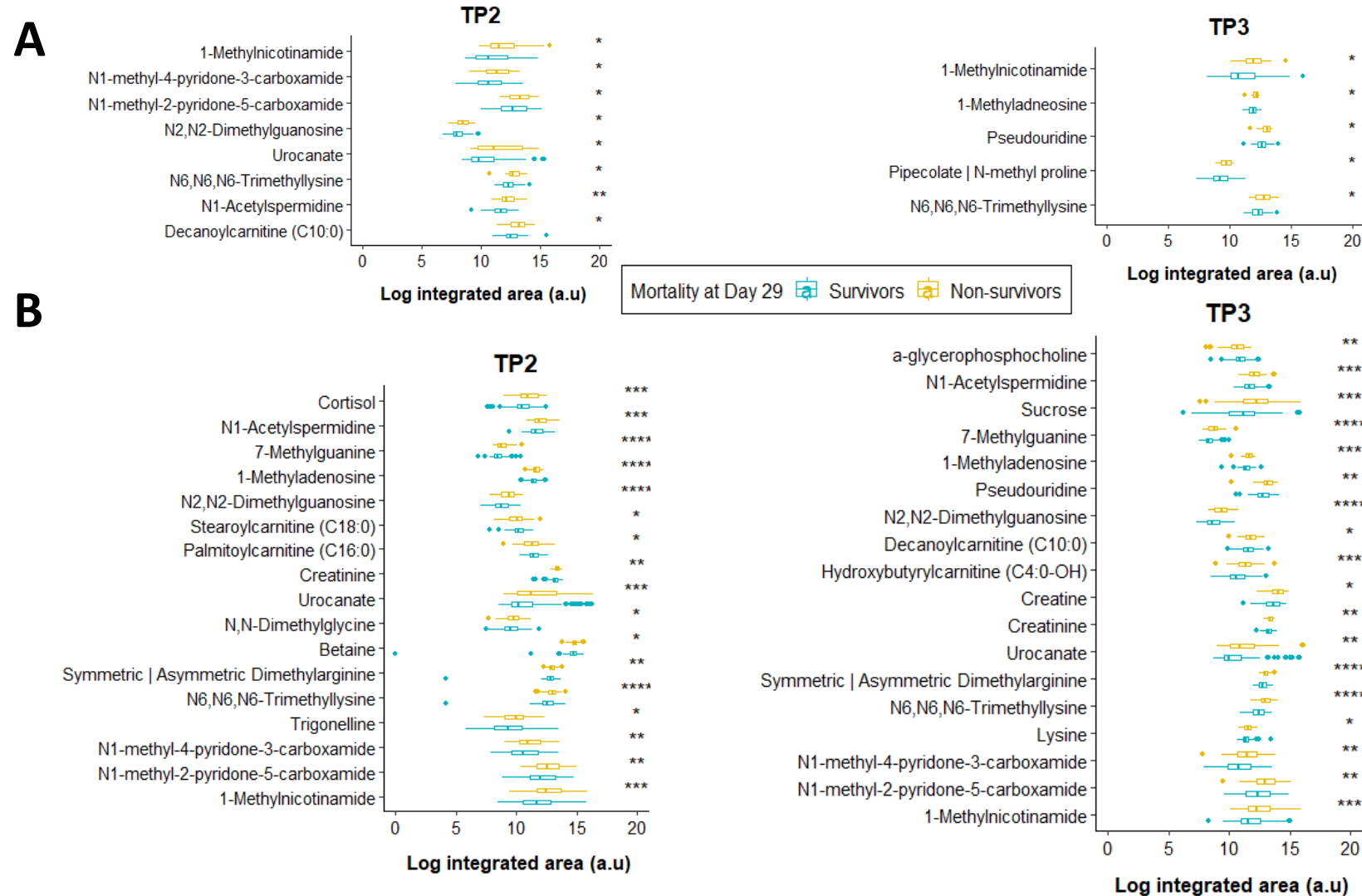


Figure 3.16: Box blots for the metabolites which were significantly different between survivors and non-survivors at TP2 & TP3 in VANISH (A) and LeoPARDS (B) HPOS ppR datasets.

3.3.5 Prediction of outcome based on metabolic changes in the first 24 h

Metabolic differences were found between survivors and non-survivors from the univariate analysis of small molecules obtained from the targeted data (both SMoESY-select and HPOS ppR). The aim was to validate these findings for both VANISH and LeoPARDS at ICU admission (i.e., TP0) and after 24 h of admission (i.e., TP1) to see whether the results from one cohort can be used to predict mortality if tested on another cohort and vice versa. Only samples from TP0 and TP1 were used for this analysis as detecting metabolic differences in the first 24 h will aid in earlier prognostication of patients who are likely to die or survive.

3.3.5.1 NMR targeted data (SMoESY-select)

The predictive models were performed on the SMoESY-select dataset from both cohorts at TP0 and TP1. The same number of samples were used in this analysis as the mortality analysis of the SMoESY-select data discussed previously at TP0 (N = 164 and N = 450 for VANISH and LeoPARDS, respectively) and TP1 (N = 134 and N = 393 for VANISH and LeoPARDS, respectively) (figure 3.7 & figure 3.8).

The performance metrics for the mortality prediction models of the SMoESY-select dataset for the two cohorts at TP0 and TP1 are listed in table 3.13. The validation models built in VANISH and LeoPARDS performed well at both timepoints.

The cross validated ROC curves for within cohort validation of VANISH test set at TP0 (VANISH TP0_{WC}) and validation of VANISH within LeoPARDS dataset at TP0 (VANISH TP0_{EXCO}) had an AUC of 0.66 and 0.72 (for 11 significant metabolites selected from the univariate mortality model of VANISH at TP0 and of those 10 were common in both cohorts as listed in table 3.5, figure 3.9A), respectively (figure 3.17). The predictive models for within cohort validation of VANISH at TP1 (VANISH TP1_{WC}) and validation of VANISH within LeoPARDS dataset at TP1 (VANISH TP1_{EXCO}) had an AUC of 0.73 and 0.69 (for 7 significant metabolites selected from the univariate mortality model of VANISH at TP1 and of those five were common in both cohorts as listed in table 3.6, figure 3.10A), respectively (figure 3.18).

Survivors and non-survivors were correctly classified by the predictive models with a balanced accuracy of 59.07% and 60.96% for VANISH TP0_{WC} and TP0_{EXCO}, respectively, whilst VANISH TP1_{WC} and TP1_{EXCO} had a balanced accuracy of 64.19% and 60.23% (table 3.13),

respectively indicating that the performance of the two pairs of classification models is almost the same at both TP0 and TP1.

Balanced accuracy is measured by taking the arithmetic mean of sensitivity and specificity. This parameter is useful in the case where the two classes in a dataset are imbalanced, as is the case in our data. Balanced accuracy helps to prevent misleading conclusions as it removes favouritism towards the majority class (in this case survivors), and is not sensitive to class skewness unlike parameters such as accuracy. (230)

The mortality prediction models for within cohort validation of LeoPARDS at TP0 (LeoPARDS TP0_{WC}) and validation of LeoPARDS within VANISH dataset at TP0 (LeoPARDS TP0_{EXCO}) had an AUC of 0.71 and 0.75 (for 15 significant metabolites of those 10 were common in both cohorts as listed in table 3.5, figure 3.9B) selected from the univariate mortality model of LeoPARDS at TP0), respectively (figure 3.17). The cross validated ROC curves for within cohort validation of LeoPARDS at TP1 (LeoPARDS TP1_{WC}) and validation of LeoPARDS within VANISH dataset at TP1 (LeoPARDS TP1_{EXCO}) had an AUC of 0.75 and 0.74 (for 16 significant metabolites of those five were common in both cohorts as listed in table 3.6, figure 3.10B) selected from the univariate mortality model of LeoPARDS at TP1), respectively (figure 3.18).

Survivors and non-survivors were correctly classified by the predictive models with a balanced accuracy of 61.86% and 70.55% for LeoPARDS TP0_{WC} and TP0_{EXCO}, respectively, whilst LeoPARDS TP1_{WC} and TP1_{EXCO} had a balanced accuracy of 63.62% and 63.22% (table 3.13), respectively indicating that the performance of the two pairs of classification models at TP0 and TP1 was better for the validation models (i.e., LeoPARDS TP0_{EXCO} and LeoPARDS TP1_{EXCO}) with the VANISH cohort in comparison with predictive models for mortality validation within cohort (i.e., LeoPARDS TP0_{WC} and TP1_{WC}).

Table 3.13: Performance metrics for mortality prediction models within cohort (WC) and with external cohort (EXCO) for both TP0 and TP1 sample from SMoESY-select.

Parameters	VANISH				LeoPARDS			
	TP0 _{WC}	TP0 _{EXCO}	TP1 _{WC}	TP1 _{EXCO}	TP0 _{WC}	TP0 _{EXCO}	TP1 _{WC}	TP1 _{EXCO}
Accuracy (%)	75.00	71.78	81.34	75.32	71.56	78.66	76.84	79.85
Balanced accuracy (%)	59.07	60.96	64.19	60.23	61.86	70.55	63.62	63.22
Sensitivity (%)	25.58	30.14	32.26	27.10	34.25	53.49	34.58	32.26
Specificity (%)	92.56	91.76	96.12	93.24	89.47	87.60	92.66	94.12
Positive predictive value (%)	55.00	63.77	71.43	60.42	60.98	60.53	63.79	62.50
Negative predictive value (%)	77.78	73.23	82.50	77.39	73.91	84.13	79.10	82.20
Prevalence of death (%)	26.22	32.44	23.13	27.22	32.44	14.02	27.23	23.13

Table 3.14: Confusion matrices for TP0 mortality predictive models for VANISH and LeoPARDS SMoIESY-select datasets. Note: TP0_{WC} = within cohort for TP0 and TP0_{EXCO} = with external cohort for TP0.

Validation of VANISH within cohort			
VANISH TP0 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	112	32
	Non-survivors	9	11
Validation of VANISH in LeoPARDS			
VANISH TP0 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	267	78
	Non-survivors	19	29
Validation of LeoPARDS within cohort			
LeoPARDS TP0 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	272	96
	Non-survivors	32	50
Validation of LeoPARDS in VANISH			
LeoPARDS TP0 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	97	21
	Non-survivors	6	10

Table 3.15: Confusion matrices for TP1 mortality predictive models for VANISH and LeoPARDS SMoIESY-select datasets. Note: TP1_{WC} = within cohort for TP1 and TP1_{EXCO} = with external cohort for TP1.

Validation of VANISH within cohort			
VANISH TP1 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	99	21
	Non-survivors	4	10
Validation of VANISH in LeoPARDS			
VANISH TP1 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	267	78
	Non-survivors	19	29
Validation of LeoPARDS within cohort			
LeoPARDS TP1 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	265	70
	Non-survivors	21	37
Validation of LeoPARDS in VANISH			
LeoPARDS TP1 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	97	21
	Non-survivors	6	10

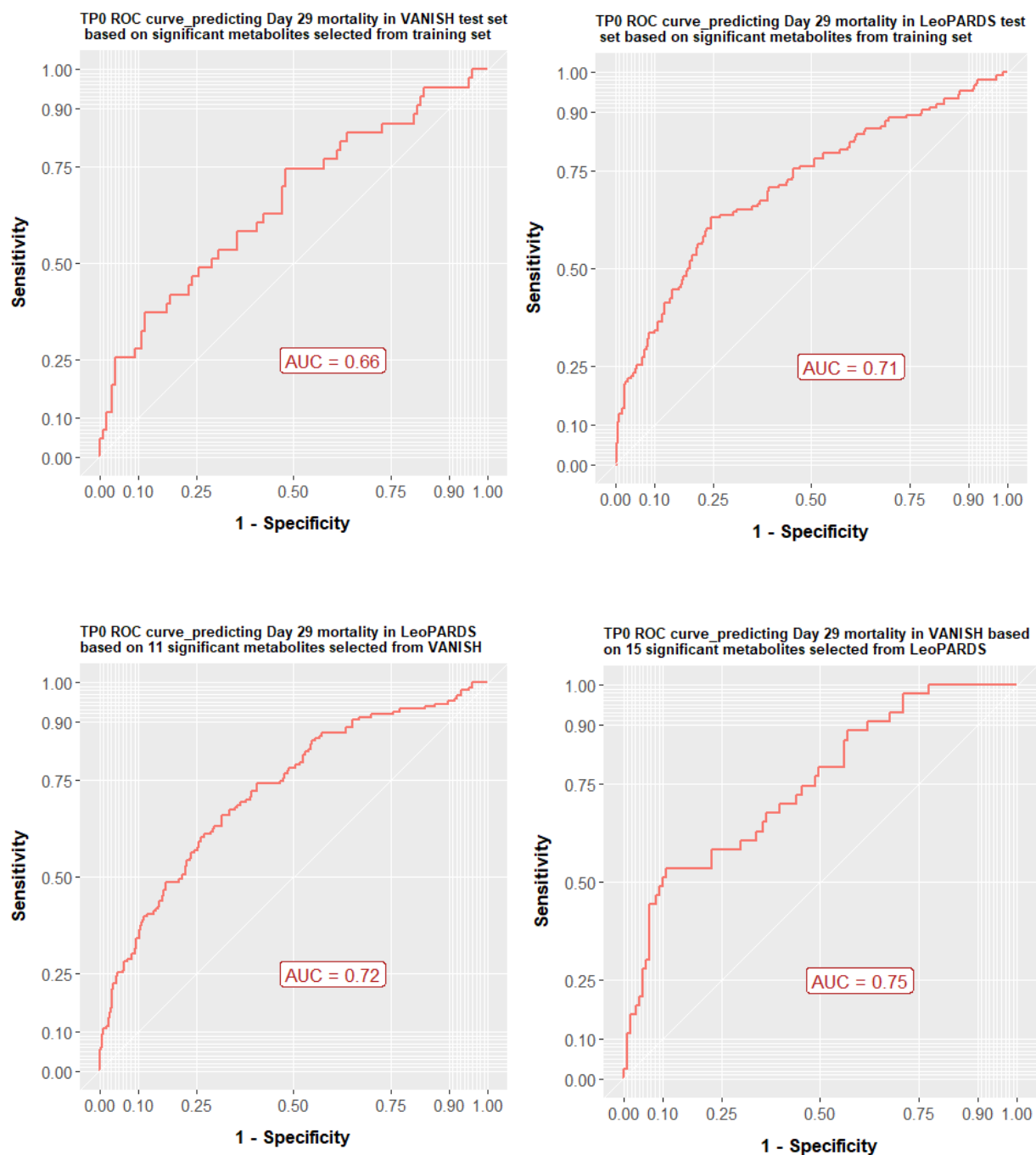


Figure 3.17: ROC curves for the predictive models of mortality on SMoIESY-select dataset for VANISH and LeoPARDS at TP0.

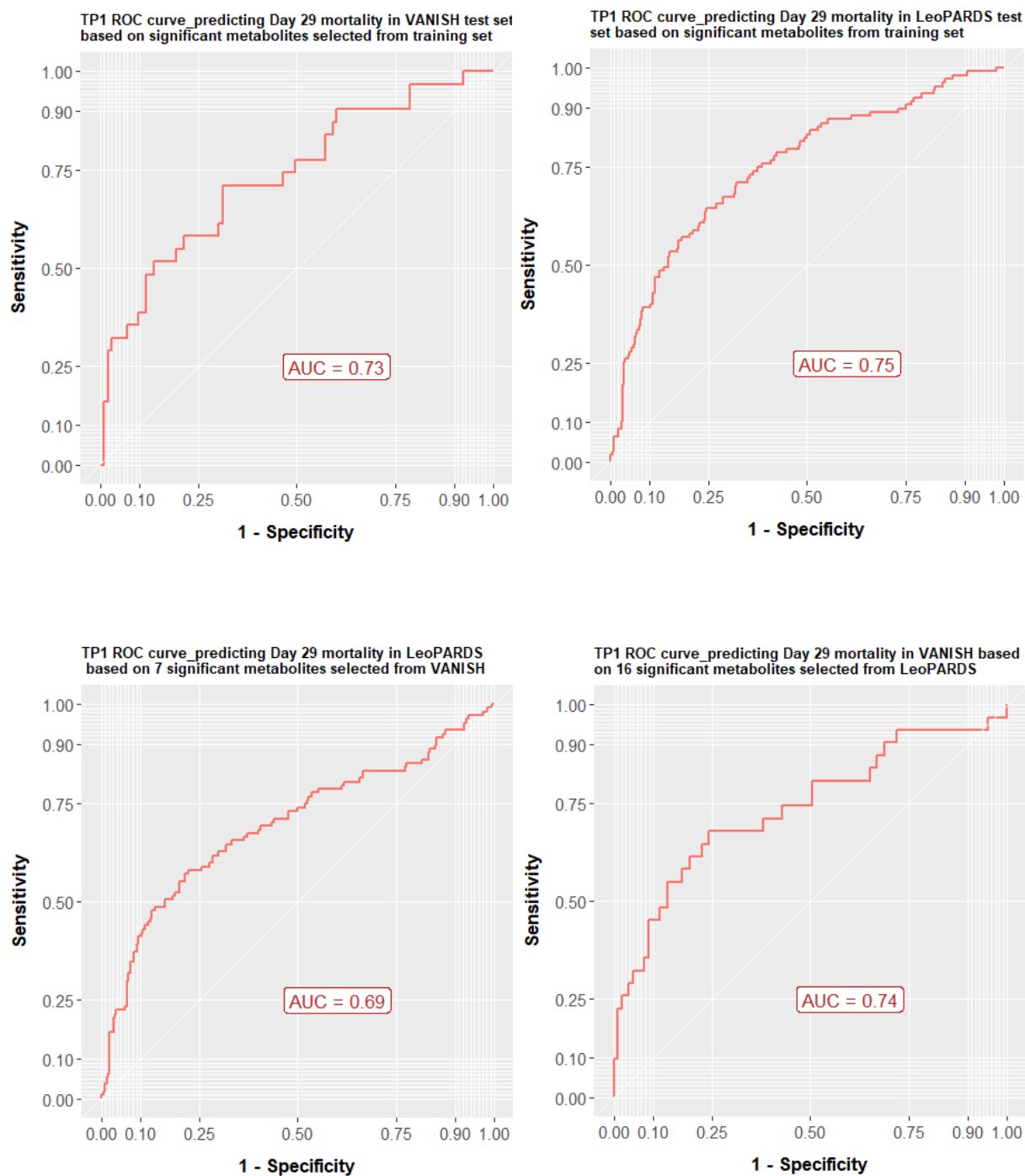


Figure 3.18: ROC curves for the predictive models of mortality on SMoIESY-Select dataset for VANISH and LeoPARDS at TP1.

3.3.5.2 UHPLC-MS targeted data (HPOS ppR)

The same number of samples were used to build predictive models as the mortality analysis of the HPOS ppR data discussed previously for both cohorts at TP0 (N = 173 and N = 459 for VANISH and LeoPARDS, respectively) and TP1 (N = 137 and N = 407 for VANISH and LeoPARDS, respectively) (figure 3.12 & figure 3.13).

The performance metrics for the mortality prediction models of the HPOS ppR dataset for the VANISH and LeoPARDS at TP0 and TP1 are listed in table 3.16. The cross validated ROC curves for within cohort validation of VANISH at TP0 (VANISH TP0_{WC}) and validation of VANISH within LeoPARDS dataset at TP0 (VANISH TP0_{EXCO}) had an AUC of 0.72 and 0.70 (for 24 significant metabolites of those 21 were common in both cohorts as listed in table 3.9, figure 3.14A) selected from the univariate mortality model of VANISH at TP0), respectively (figure 3.19). The predictive models for within cohort validation of VANISH at TP1 (VANISH TP1_{WC}) and validation of VANISH within LeoPARDS dataset at TP1 (VANISH TP1_{EXCO}) had an AUC of 0.66 and 0.71 (for 5 significant metabolites which were common in both cohorts as listed in table 3.10, figure 3.15A) selected from the univariate mortality model of VANISH at TP1), respectively (figure 3.20).

Survivors and non-survivors were correctly classified by the predictive models with a balanced accuracy of 62.08% and 60.99% for VANISH TP0_{WC} and TP0_{EXCO}, respectively, whilst VANISH TP1_{WC} and TP1_{EXCO} had a balanced accuracy of 55.57% and 52.08% (table 3.16), respectively indicating that the performance of the two pairs of classification models is roughly similar.

The mortality prediction models for within cohort validation of LeoPARDS at TP0 (LeoPARDS TP0_{WC}) and validation of LeoPARDS within VANISH dataset at TP0 (LeoPARDS TP0_{EXCO}) had an AUC of 0.75 and 0.76 (for 27 significant metabolites of those 21 were common in both cohorts as listed in table 3.9, figure 3.14B) selected from the univariate mortality model of LeoPARDS at TP0), respectively (figure 3.19). The cross validated ROC curves for within cohort validation of LeoPARDS at TP1 (LeoPARDS TP1_{WC}) and validation of LeoPARDS within VANISH dataset at TP1 (LeoPARDS TP1_{EXCO}) had an AUC of 0.73 and 0.69 (for 23 significant metabolites of those five were common in both cohorts as listed in table 3.10, figure 3.15B) selected from the univariate mortality model of LeoPARDS at TP1), respectively (figure

3.20). Survivors and non-survivors were correctly classified by the predictive models with a balanced accuracy of 64.27% and 59.89% for LeoPARDS TP0_{WC} and TP0_{EXCO}, respectively, whilst LeoPARDS TP1_{WC} and TP1_{EXCO} had a balanced accuracy of 61.78% and 54.21% (table 3.16), respectively indicating that the performance of the two pairs of classification models for LeoPARDS was better at TP0.

The performance of mortality prediction model, VANISH TP0_{EXCO} with 24 selected metabolites (AUC = 0.70 & sensitivity = 75.17%) was better than the predictive model LeoPARDS TP0_{EXCO} with 23 selected metabolites (AUC = 0.76 & sensitivity = 26.09%).

Table 3.16: Performance metrics for mortality prediction models within cohort (WC) and with external cohort (EXCO) for both TP0 and TP1 samples from HPOS ppR.

Parameters	VANISH				LeoPARDS			
	TP0 _{WC}	TP0 _{EXCO}	TP1 _{WC}	TP1 _{EXCO}	TP0 _{WC}	TP0 _{EXCO}	TP1 _{WC}	TP1 _{EXCO}
Accuracy (%)	72.83	55.77	75.18	73.71	73.20	75.72	74.69	78.10
Balanced accuracy (%)	62.08	60.99	55.57	52.08	64.27	59.89	61.78	54.21
Sensitivity (%)	39.13	75.17	18.75	5.51	40.00	26.09	33.95	9.38
Specificity (%)	85.04	46.82	92.38	98.66	88.54	93.7	89.60	99.04
Positive pred value (%)	48.65	80.33	42.86	60.00	61.70	60.00	54.41	75.00
Negative pred value (%)	79.41	80.33	78.86	74.06	76.16	77.78	78.76	78.20
Prevalence (%)	26.59	31.59	23.36	26.78	31.59	26.59	26.78	23.36

Table 3.17: Confusion matrices for TPO mortality predictive models for VANISH and LeoPARDS HPOS ppR datasets. Note: TPO_{WC} = within cohort for TPO and TPO_{EXCO} = with external cohort for TPO.

Validation of VANISH within cohort			
VANISH TPO _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	108	28
	Non-survivors	19	18
Validation of VANISH in LeoPARDS			
VANISH TPO _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	147	36
	Non-survivors	167	109
Validation of LeoPARDS within cohort			
LeoPARDS TPO _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	278	87
	Non-survivors	36	58
Validation of LeoPARDS in VANISH			
LeoPARDS TPO _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	119	34
	Non-survivors	8	12

Table 3.18: Confusion matrices for TP1 mortality predictive models for VANISH and LeoPARDS HPOS ppR datasets. Note: TP1_{WC} = within cohort for TP1 and TP1_{EXCO} = with external cohort for TP1.

Validation of VANISH within cohort			
VANISH TP1 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	97	26
	Non-survivors	8	6
Validation of VANISH in LeoPARDS			
VANISH TP1 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	294	103
	Non-survivors	4	6
Validation of LeoPARDS within cohort			
LeoPARDS TP1 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	267	72
	Non-survivors	31	37
Validation of LeoPARDS in VANISH			
LeoPARDS TP1 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	104	29
	Non-survivors	1	3

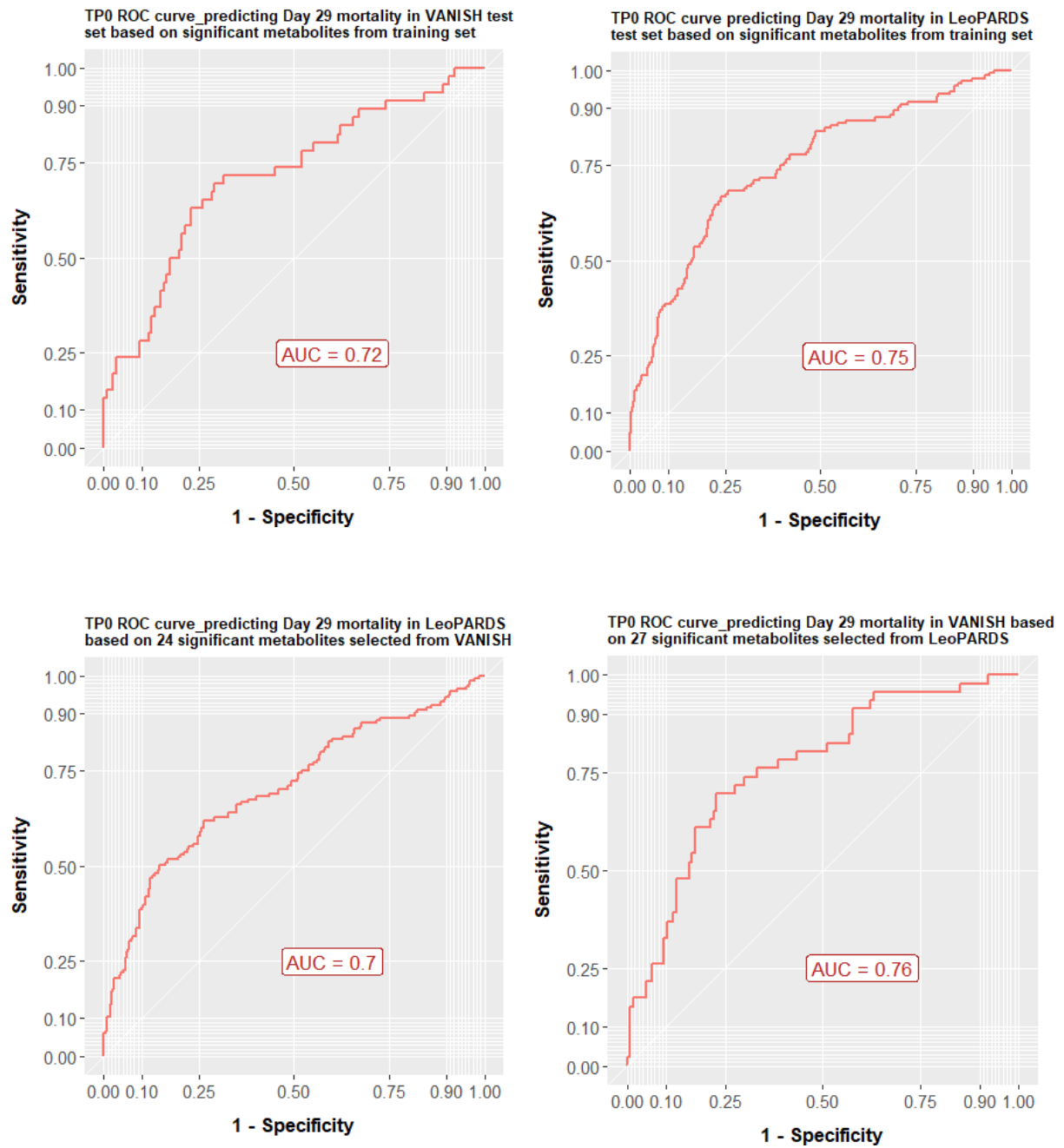


Figure 3.19: ROC curves for the predictive models of mortality on HPOS ppR dataset for VANISH and LeoPARDS at TP0.

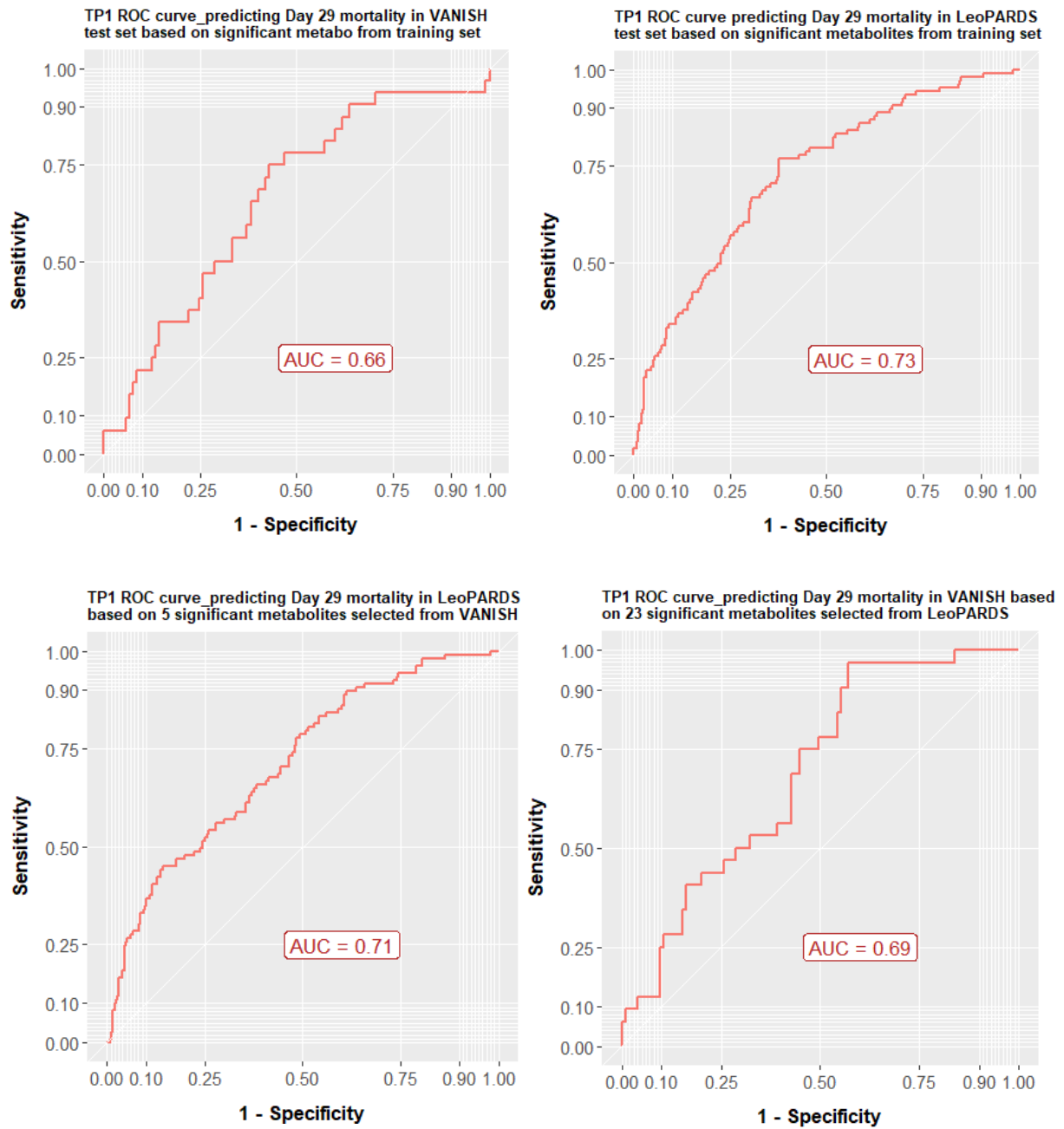


Figure 3.20: ROC curves for the predictive models of mortality on HPOS ppR dataset for VANISH and LeoPARDS at TP1.

3.3.6 Tyrosine analysis

Tyrosine was found to be one of the most significant metabolites driving the key differences between survivors and non-survivors in both VANISH and LeoPARDS. It is an important metabolite in the production of catecholamines (231) and is bound to serum albumin (232). To understand what could be driving tyrosine to be higher in non-survivors than survivors, further analysis was performed to find out if the tyrosine levels are influenced by the serum concentration of albumin and dose of noradrenaline being administered at randomisation. The results from the linear regression analysis of VANISH showed that there was no association between tyrosine and dose of noradrenaline for neither TP0 nor TP1 samples shown in figure 3.21C and figure 3.21D suggesting that the tyrosine levels in patients with septic shock are not influenced by the administered dose of noradrenaline. Moreover, there was no significant difference in the tyrosine levels between the patients who received noradrenaline in comparison to those who received vasopressin as the first study drug as seen in figure 3.21B. There was no association between serum albumin and tyrosine levels as seen in figures 3.22C and 3.22D.

Table 3.19: Welch two-sample t-test results of tyrosine and albumin regions for VANISH at TP0 and TP1. Yellow indicates higher mean levels whilst blue indicates lower mean levels.

Metabolite	Mean Intensity _{TP0} (a.u)		Mean Intensity _{TP1} (a.u)		p-value	
	Survivors	Non-Survivors	Survivors	Non-Survivors	TP0	TP1
Tyrosine (Peak 7.18)	3.02E+05	4.31E+05	3.27E+05	4.69E+05	1.60E-04	2.20E-06
Tyrosine (Peak 6.88)	3.59E+05	4.84E+05	3.85E+05	5.35E+05	1.50E-04	1.30E-06
Albumin (Region 1)	5.27E+07	5.09E+07	5.29E+07	5.28E+07	0.35	0.93
Albumin (Region 2)	2.77E+07	2.69E+07	2.80E+07	2.78E+07	0.4	0.87
Albumin (Region 3)	4.08E+07	4.00E+07	4.11E+07	4.14E+07	0.61	0.84

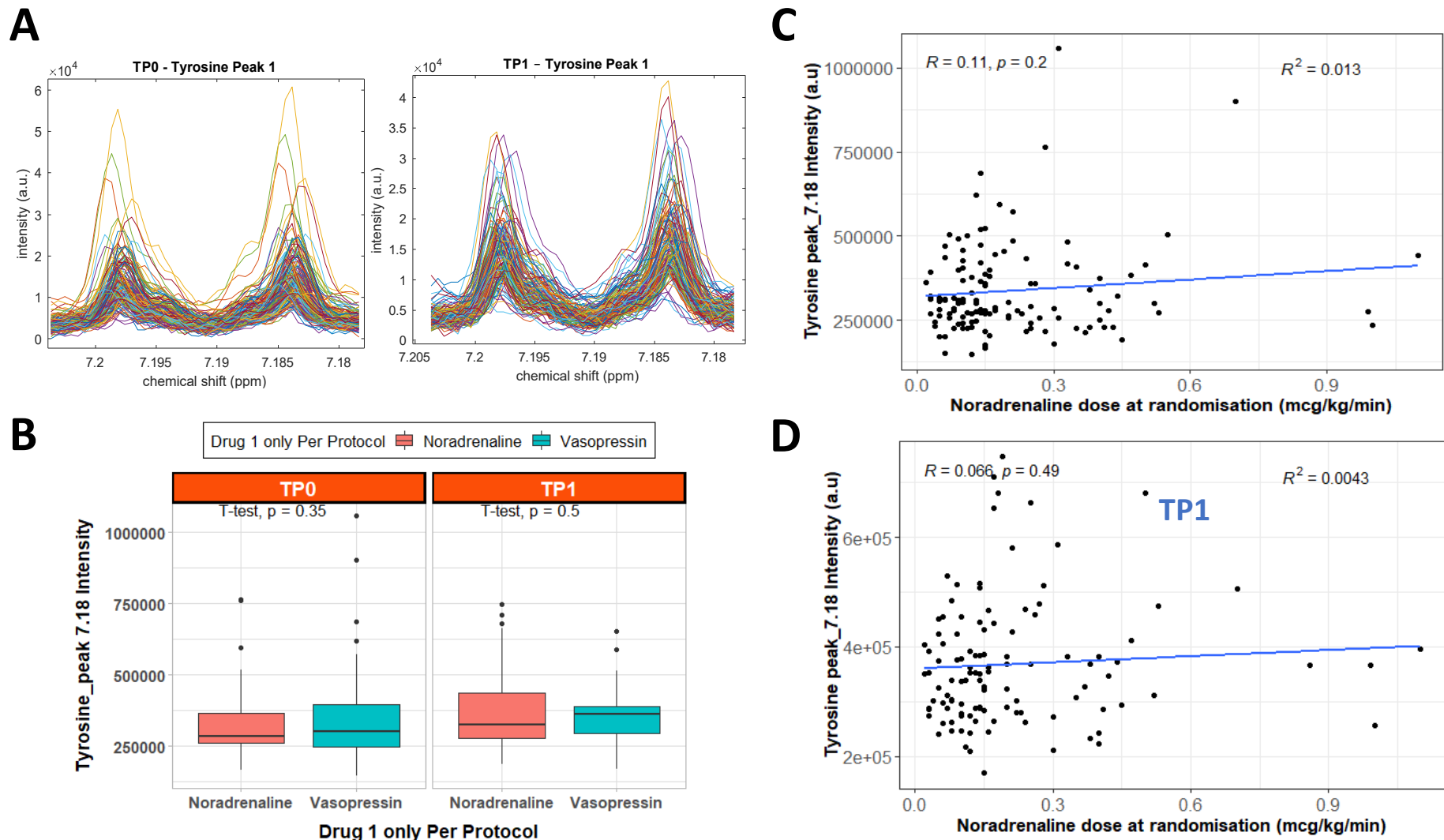


Figure 3.21: Association of tyrosine with study drug 1 and noradrenaline dose at randomisation. A) Tyrosine Peak 1 for TP0 & TP1 samples. B) Box plots for tyrosine levels in study drug 1. C) Scatter plot for the association between tyrosine and noradrenaline dose at randomisation for TP0. D) Scatter plot for the association between tyrosine and noradrenaline dose at randomisation for TP1.

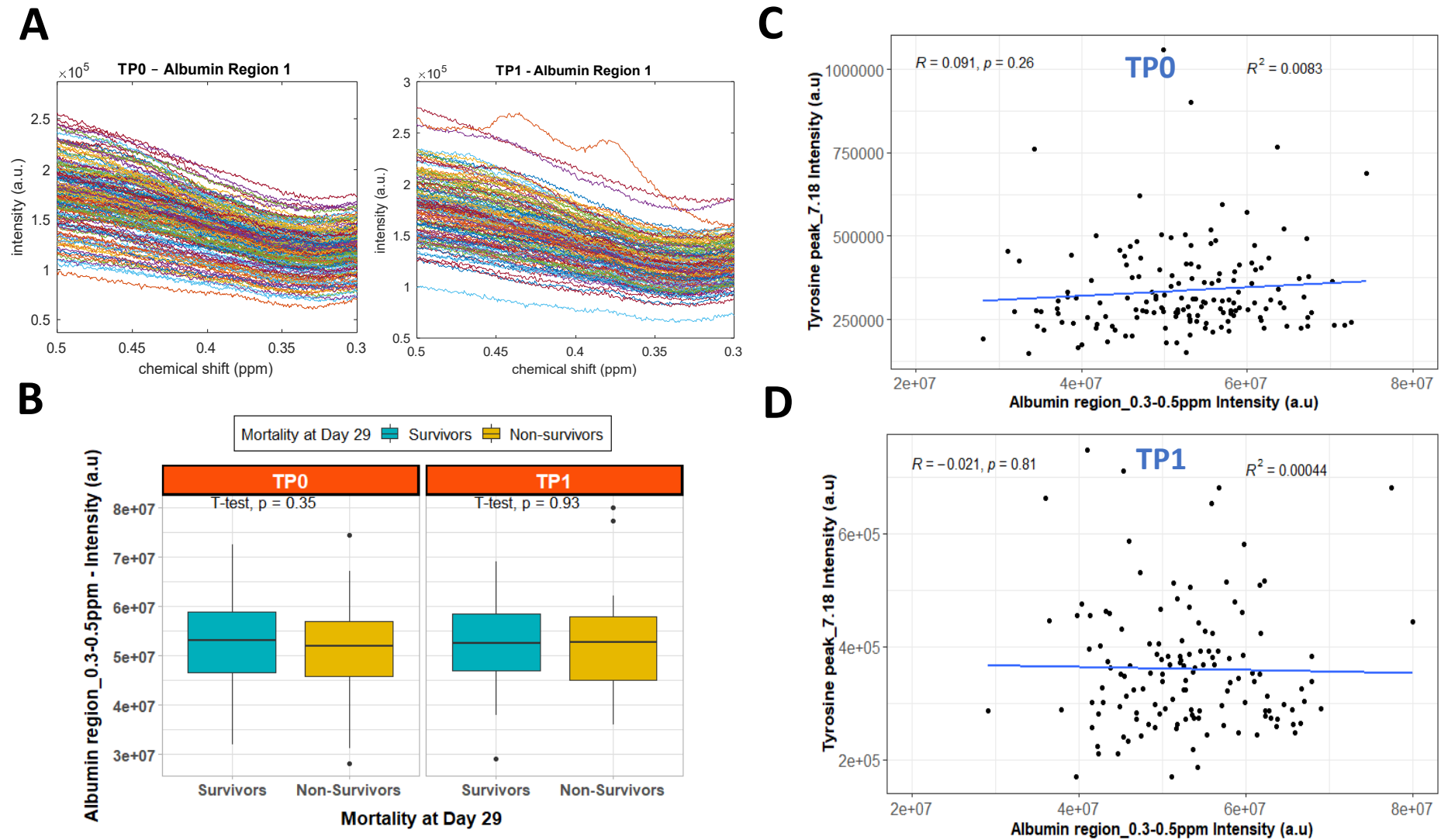


Figure 3.22: Mortality results of albumin and its association with tyrosine. A) Albumin region 1 for TP0 & TP1 samples. B) Box plots for albumin levels in survivors and non-survivors. C) Scatter plot for the association between tyrosine and albumin for TP0. D) Scatter plot for the association between tyrosine and albumin for TP1.

3.4 Discussion

Sepsis is a multifaceted condition with high morbidity and mortality and because of the heterogeneity of sepsis (1) and septic shock, prognostication is difficult and we are currently unable to target treatments to those who are most likely to benefit from them (66). Metabolomic techniques combined with bioinformatic analysis have great potential to address metabolite changes in relation to disease state.

In this chapter metabolomics was used to investigate the metabolic differences between septic shock survivors and non-survivors at ICU admission and after treatment using NMR and UHPLC-MS methodologies in the two cohorts and the findings were also validated using mortality prediction models on the metabolomic datasets from the two cohorts. This investigation revealed time related metabolic changes which differentiated septic shock survivors from non-survivors and these metabolic changes were consistent across both cohorts.

This study revealed that metabolites including amino acid and derivatives (tyrosine, phenylalanine, BCAAs, glycine, alanine, glutamine, histidine, creatine, creatinine, lysine, proline), glycolysis and TCA cycle metabolites (glucose, lactate, pyruvate, citric acid), fatty acyls (acylcarnitines), purine nucleoside (N²,N²-dimethylguansine), choline, urocanate, were accumulated in patients who went on to die in relation to those who survived whilst only hypoxanthine and arginine were reduced in these patients in both cohorts across timepoints.

In sepsis, patients have an overall catabolic state which causes breakdown of proteins, carbohydrates and lipids (233). Alterations in aromatic amino acids including tyrosine and phenylalanine in patients with sepsis are associated with oxidative stress which lead leads to overproduction of hydroxyl radicals converting L-phenylalanine enzymatically in the kidneys to para-tyrosine (234). TP0 and TP1 CPMG multivariate models showed upregulation of these two aromatic amino acids in non-survivors compared to survivors at ICU admission and after 24 h of treatment in both cohorts and these results were also in accordance with other studies (66, 147, 149, 151, 152, 154, 164-166, 229). The findings from this investigation were also supported by the univariate analysis of NMR targeted data which revealed that tyrosine was the only amino acid which was significantly elevated in non-

survivors compared to survivors at all four timepoints in LeoPARDS whilst in VANISH it was significantly elevated in non-survivors only at three timepoints (TP0-TP2) and notably, phenylalanine was the second most significant metabolite which accumulated in non-surviving patients in both cohorts at three timepoints (TP0-TP2). Accumulation of these aromatic amino acids in non-survivors might suggest increased muscle proteolysis (235) and these two metabolites could be converted into fumarate and enter the tricarboxylic acid (TCA) cycle (66) to compensate for the increased energy demand in patients who went on to die. The findings from the univariate and multivariate results also suggest that tyrosine, which takes part in the production of catecholamines (236), might be a key discriminatory metabolite in separating survivors from non-survivors and this was confirmed using STOCsY results for full set of TP0 and TP1 samples from CPMG, where tyrosine signal 7.185ppm was used as a driver peak. In STOCsY, tyrosine correlated with almost every metabolite which was found to differentiate between survivors and non-survivors at both timepoints (TP0 and TP1). The metabolites which correlated with tyrosine included alanine, phenylalanine, 3-methylhistidine, lactate, methionine, glycine, valine, leucine, isoleucine, lysine, pyruvate, glutamate, glutamine, 3-hydroxyisobutyrate, and creatinine. These results indicate that tyrosine and the correlated metabolites are all involved in similar metabolic pathways (143). Though, STOCsYs were also performed for other key discriminatory metabolites including lactate, alanine, phenylalanine, and pyruvate, but only STOCsY results for tyrosine showed correlation with key discriminatory metabolites driving the main differences between survivors and non-survivors. This indicates that tyrosine is one of the main metabolites driving the key differences between septic shock survivors and non-survivors. Due to this reason, additional analysis on tyrosine was carried to ensure that the results are not influenced by other factors including the albumin concentration and dose of noradrenaline administered at randomisation. This analysis revealed that there was no association between albumin concentration and tyrosine suggesting the relationship of tyrosine to outcome is not simply a consequence of hypoalbuminemia. The noradrenaline dosage administered to sepsis patients at randomisation for baseline samples was significantly higher in non-survivors with respect to survivors but there was no association of tyrosine with the noradrenaline dose providing reassurance that the relationship between tyrosine and outcome was not simply a consequence of treatment with exogenous noradrenaline.

This was further supported by the finding that there was no difference in tyrosine concentration between those patients randomised to receive noradrenaline compared to vasopressin as study drug 1.

This study revealed that patients who die might have an increased energy demand which will result in proteolysis to generate amino acid substrates for gluconeogenesis (237) and other processes (238). Glutamine, glycine and alanine were significantly higher in patients who went on to die than those who survived in both cohorts at baseline and after 24h of admission. These results suggest that sepsis patients who die might have increased muscle catabolism with these amino acids being used as substrates for generating energy to meet the increased energy demand seen in patients with more severe sepsis (239).

Glycine, an amino acid and a potent anti-oxidant with a cytoprotective role (240) is also involved in attenuating the immune response (241) and as non-survivors had higher concentrations of glycine which might also reflect increased inflammation in these patients and high levels of glycine are probably present to attenuate the immune response. Glycine was found to be raised in sepsis patients when compared to healthy controls (153, 157) and as our results indicate higher concentrations of glycine in non-survivors which suggests disease continuum in these patients. Glutamine also supports activated immune cells by taking part in the synthesis of cytokines and other amino acids (239, 242). Moreover, the serum concentrations of these three amino acids have been reported to be involved with hemolysis associated with sepsis. Hemolysis can be a consequence of sepsis that perpetuates inflammation, and it has been shown that free haemoglobin is associated with mortality in septic patients and there is also increasing evidence that in vivo hemolysis contributes to the pathogenesis and severity of sepsis (243).

Septic shock non-survivors at both TP0 and TP1 multivariate models showed increased levels of essential BCAAs, including leucine, isoleucine, and valine which are products of protein catabolism. These findings were also supported by the univariate analysis of NMR targeted data which showed that the BCAAs were significantly raised in non-survivors in at least one of the cohorts. Our results were also supported by findings from other metabolomic studies (147, 152, 158, 166) and imply a greater degree of catabolism in the non-survivors (165).

The results from the multivariate analysis of untargeted data and univariate analysis of NMR targeted data revealed that creatine and creatinine were present in higher concentrations in the serum of patients who die at baseline and after 24 h reflecting liver and kidney dysfunction as well as enhanced breakdown of proteins (244, 245). This metabolic finding is also supported by the higher rates of renal and liver failure seen at baseline in non-survivors in both datasets.

Other amino acids namely histidine and its post-translational metabolite (3-methylhistidine) were significantly higher in non-survivors when compared with survivors for TP0 and TP1 multivariate models, in accordance with other metabolomic studies (66, 147, 149, 151, 152, 229). These results were also validated from the univariate results of NMR targeted data which revealed that alanine was significantly higher in non-survivors in both cohorts at baseline and after 24 hrs.

Urocanate, which acts as an intermediate in the histidine metabolism was found to accumulate in non-survivors in both cohorts at baseline and at TP2 in both cohorts though the trend for this metabolite was in the same direction in VANISH as LeoPARDS. These results suggest that patients who die have increased histidine metabolism, to regulate increased levels of histidine.

Markers of oxidative stress such as symmetric/asymmetric dimethylarginine (SDMA/ADMA) are competitive inhibitors of nitric oxide (NO) synthase (246) and we found that these markers were significantly upregulated in non-survivors at all timepoints in LeoPARDS whilst in VANISH it was significantly elevated in non-survivors at baseline but with a similar trend at later timepoints. These findings have been supported by other studies (154, 155) and suggest that increased levels of SDMA/ADMA in non-survivors are probably present to counteract increased NO in the serum of patients who die. NO has various roles ranging from vasodilation of blood vessels because of relaxation of smooth muscles to acting as a crucial mediator in the immune response to infection where it inhibits cell proliferation of smooth muscle and their migration, causes reduction in platelet adhesion and aggregation as well as it also lowers the monocyte stickiness (155, 246). Interestingly, the amino acid lysine which takes part in regulating the production of NO (236) was found to be elevated in non-survivors from TP0 and TP1 multivariate models also supported by findings from Liu et

al study (66), suggesting that this pathway is dysregulated in patients who die. These findings were also validated with the results of univariate analysis of NMR targeted data which showed that patients who died had significantly raised levels of lysine in LeoPARDS at baseline and after 24 h of treatment whilst the results from the univariate analysis of UHPLC-MS targeted data (i.e., HPOS ppR) also revealed that the serum concentrations of lysine were significantly higher in patients who died.

The urea cycle metabolite, arginine plays an important role in the formation of creatine, polyamines and NO (155, 247) and it was found to be significantly reduced in non-survivors at baseline and after 24 h of ICU admission in LeoPARDS whilst in VANISH there was no significant difference between survivors and non-survivors however, arginine still had the same pattern in non-survivors as LeoPARDS at both timepoints (TP0 and TP1). These results suggest that there is a downregulation of urea cycle in non-survivors or increased conversion of arginine into SDMA/ADMA to counteract increased NO in these patients as discussed earlier. These findings were also supported by other metabolomic studies (159, 165).

Changes in metabolites related to oxidative stress including N2,N2-dimethylguanosine, and hypoxanthine were observed in patients with septic shock. N2,N2-dimethylguanosine was found to be significantly increased in non-survivors with respect to survivors across time, in accordance with other studies (145, 146). This reflects increased oxidative stress which leads to enhanced metabolism of transfer ribonucleic acid (tRNA) resulting in the release of this nucleoside (145) in the cytoplasm. Hypoxanthine, a purine base also associated with ROS which upon oxidation by xanthine oxidase leads to uric acid, an end product of purine metabolism (248) was reduced in non-survivors with respect to survivors and this might reflect increased conversion of hypoxanthine into uric acid in non-survivors.

Proline, a substrate for the synthesis of superoxide radicals was found to be elevated in non-survivors in the multivariate analysis and the univariate analysis of UHPLC-MS targeted data. These findings were also in accordance with the results from other studies (147, 149, 154). Our results indicate that patients who go on to die might have increased oxidative damage as a result of aggravated stress during inflammation (249) which might cause damage to kidneys and liver (160). Additionally, succinic acid was increased in non-survivors for only

TP0 multivariate model suggesting that reactive oxygen species (ROS) are causing damage to the endothelium due to hypoxia in tissues which leads to the accumulation of succinic acid in mitochondria in septic patients (250). Increased levels of myoinositol in non-survivors with respect to survivors in multivariate models might be indicative of hepatic failure and kidney injury (160). Mickiewicz et al. also reported elevated levels of myoinositol in septic shock patients compared to SIRS (160, 178) and as our results showed an increase in myoinositol, this suggests that there is a continuum in the disease severity. In addition, Stringer et al. reported that myoinositol has a positive association with acute physiology score i.e. disease severity in sepsis-induced lung injury (251).

The methyl donor, betaine was present in higher concentrations in non-surviving patients in both cohorts at baseline. Betaine plays a role in oxidative stress by improving the impaired metabolism of sulphur containing amino acids and it also causes inhibition of NF- κ B activity, regulates energy metabolism as well as plays a role in the mitigation of endoplasmic reticulum stress and apoptosis (252). Elevated levels of this methyl donor in non-survivors might be present to regulate energy metabolism or might reflect increased inflammation in patients who went on to die and higher concentrations are present probably to inhibit NF- κ B activity. Methionine an antioxidant derived from betaine (a choline derivative) was also found to be elevated in non-survivors when compared to survivors at both TP0 and TP1 and these were also consistent with findings from other studies (66, 164).

Our findings from the metabolomic analysis of the two cohorts revealed additional metabolites which could help us to have a better understanding of the pathways involved in the pathology of sepsis. The results from the univariate analysis of HPOS ppR targeted revealed that non-survivors had higher levels of short and medium-chain acylcarnitines (including hydroxybutyrylcarnitine, decanoylcarnitine) as well as long-chain acylcarnitines (including tetradecenoylcarnitine, hexadecenoylcarnitine, stearoylcarnitine, palmitoylcarnitine, oleoylcarnitine) with respect to non-survivors in both cohorts at ICU admission whilst carnitine was significantly raised in non-survivors only in VANISH at baseline. These results suggest that patients with septic shock who go on to die have enhanced incomplete fatty acid oxidation (i.e., β -oxidation) due to mitochondrial dysfunction (162, 253), in accordance with other metabolomic studies (146-149, 163).

In this study we found that cortisol was significantly higher in non-survivors at three timepoints (TP0-TP2) in LeoPARDS whilst the cortisol levels were not significantly different between survivors and non-survivors in VANISH at these timepoints, but the trend for cortisol was still in the same direction in VANISH except at baseline. These results were also supported by findings from other metabolomic studies (145, 146) and suggest that non-survivors had increased production of cortisol by the adrenal cortex via enhanced stimulation of ACTH due to the stress response. Cortisol exerts its effects by suppressing the expression of inflammatory cytokines, such as IL-1 β , TNF, and IL-6, as well as attenuating the release of ACTH and CRH by the anterior pituitary gland (90, 254).

Increased levels of 1-methylnicotinamide (MNA) and its metabolites, (N1-methyl-2-pyridone-5-carboxamide (2-Py) and N1-methyl-4-pyridone-3-carboxamide (4-Py)) were found in non-survivors at three timepoints (TP0-TP2) suggesting that patients who die might have an enhanced nicotinamide adenine dinucleotide (NAD⁺) degradation (255) which leads to the accumulation of these catabolic products (MNA and its metabolites) (256). These results were also supported by Seymour and co-workers who reported higher levels of 2-Py in non-survivors (145). Polyamine, N1-acetylspermidine was also significantly increased in non-survivors with respect to survivors at three timepoints (TP0-TP2) in both cohorts (i.e. VANISH and LeoPARDS) whilst at TP3, it was only significantly elevated in non-survivors from LeoPARDS and the accumulation of this polyamine in patients who go on to die might have increased apoptosis of macrophages in the lungs due the increased formation of ROS during polyamine metabolism (257).

Patients with sepsis have enhanced energy requirements due to increased metabolic and energetic failure and these energy demands are fulfilled by increased cellular respiration both in cytoplasm via glycolysis and in mitochondria via tricarboxylic acid cycle (TCA) (239). In sepsis, mitochondrial dysfunction could cause a build-up of metabolites associated with these two pathways including lactate, pyruvate, and citric acid (158). Lactate, a metabolite of anaerobic metabolism from glycolysis, is a well-known clinical biomarker widely used for the prognostication of patients with sepsis in the ICU (233) and it was found to be accumulated in sepsis patients who go on to die when compared to those who survive at ICU admission and after 24 hrs of treatments (from both multivariate and univariate analysis) in both cohorts. These results were also consistent with findings from other

metabonomic studies which reported higher levels of lactate (66, 146-149, 157, 158) in non-survivors with respect to survivors. High levels of lactate were also consistent with lower oxygen pressures ($\text{PaO}_2/\text{FiO}_2$ ratio) in non-survivors from both cohorts reflecting more severe tissue hypoxia in non-survivors following from the impaired organ perfusion (233) resulting in decreased aerobic respiration and abnormal energy synthesis (258). These results are also in accordance with the findings from Li and co-workers (66).

Our study revealed that pyruvate was higher in patients who went on to die with respect to those who survived in accordance with other studies (66, 148, 158). Accumulation of pyruvate and lactate in non-survivors might suggest an increased stimulation of glycolysis in non-survivors by endogenous epinephrine which leads to an increased generation of pyruvate in the cytoplasm, which is then metabolised in the mitochondria causing increased lactate formation (259) and lactic acidosis (260). Accumulation of citric acid in non-survivors with respect to survivors at baseline and after 24h of admission might reflect upregulation of the TCA cycle in non-survivors possibly to cope with the increased energy demands as well as enhanced formation of pyruvate as seen in non-survivors. These results were also consistent with other metabonomic studies (66, 147, 148, 157).

Glucose levels are commonly seen to fluctuate in critically ill patients and in general, sepsis have been linked with hypoglycaemia (261) as well as stress induced hyperglycaemia. Our results from the NMR targeted data revealed that glucose was reduced in patients who died relative to those who survive in LeoPARDS only at ICU admission whilst it followed the same pattern in the alternate cohort, but the results were not significant probably due to less statistical power. These results were not in accordance with other metabonomic studies (152, 159) which are relatively small studies, but our results were supported by the findings from Kushimoto and co-workers, who reported that hypoglycaemia (glucose $<70\text{mg/dL}$) was linked with higher mortality rate in patients with septic shock in comparison to the septic shock patients without hypoglycaemia (262). Our results suggest that hypoglycaemic state might develop in patient who go on to die probably due to the inhibition of gluconeogenesis in the liver stimulated by cytokines under circumstances when glycogen stores are depleted (263, 264) or it might be due to enhanced consumption of glucose into pyruvate to cope with the increased demand for energy. Surprisingly, sucrose which is a disaccharide was found to be elevated in non-survivors in both cohorts at baseline whilst at later timepoints

(i.e., TP1 and TP3) it was significantly higher in non-survivors but only in LeoPARDS though the trend for sucrose was in the same direction in VANISH as LeoPARDS and these results were also in accordance with other studies (152, 159). These results might suggest that glucose is being used up rapidly by the cells to meet increased energy demands but not much of the sucrose is being getting metabolised to glucose to replace the glucose levels in patients who went on to die.

Choline is an essential nutrient (265) with antioxidant and antiapoptotic roles (266). It reduces the levels of pro-inflammatory cytokines (including TNF- α , IL-1, IL-6, INE- γ , PGE₂, NO) and provides protection to the cell membrane from oxidative damage during sepsis and as well as in some other pathological conditions (266). It is also required for the synthesis of acetylcholine (a neurotransmitter), betaine, glycerophosphocholine, and phospholipids (including PCs, LPCs, SMs, and choline plasminogen which are integral part of cell membranes) (265, 266). Levels of choline were increased in non-survivors compared to survivors at baseline in both cohorts whilst at later timepoints (TP1-TP3), choline was significantly elevated in non-surviving patients but only in LeoPARDS though the trend for choline was in the same direction in VANISH except for at TP1 and that could be due to the technical reason discussed previously. Our results were also in accordance with the recent findings from Kumar and co-workers (229) and suggest that patients who went on to die have an increased oxidative stress and inflammation and higher levels of choline are present to attenuate the uncontrolled immune response to protect from further damage.

The validation of mortality results from VANISH and LeoPARDS revealed that no matter which way the validation was carried, the predictive models did not perform that well however, same metabolites were consistently increased or decreased in both cohorts across timepoints.

Some of the limitations of this study included, not being able to validate the results from drug analysis in LeoPARDS, as different drug intervention was used in LeoPARDS. The multivariate and trajectory analysis of untargeted data (i.e., CPMG) was only performed in VANISH not in LeoPARDS, as the metabolomic data for LeoPARDS became available towards the end of the PhD therefore, it was not feasible to carry out this analysis within the required timeframe.

3.5 Conclusion

Overall, we found that there are metabolic differences between survivors and non-survivors, specifically at baseline and after 24h of ICU admission. Metabolites related oxidative stress, energy supply, and protein breakdown were mainly responsible for differentiating the serum metabolome of survivors from non-survivors. These metabolic differences are likely to be due to the oxidative stress, pronounced inflammatory response, and organ dysfunction associated with septic shock patients which contribute towards poor outcomes. Knowledge of metabolites that distinguish survivors from non-survivors of septic shock, especially in the first 24h might help to better estimate disease severity whilst also allowing deeper understanding of the molecular biology of sepsis. To conclude, this study revealed baseline differences in the small molecule metabolites of septic shock survivors and non-survivors which will aid in understanding the metabolic trajectories of these two clinical groups later in the analysis.

Chapter 4 – Metabolic Trajectories of Small Molecule Metabolites in Septic Shock

4.1 Introduction

A metabolomic approach has been used in many studies to investigate the metabolic derangements in patients with sepsis and these studies have provided some understanding of the metabolic alterations which occur in sepsis patients based on mortality (144). These metabolic derangements have been previously described in chapter 3 and most of the studies focused on the metabolic differences between the two clinical groups (i.e., survivors and non-survivors) at static timepoints (66, 145-149, 151, 152, 154, 155, 157-160, 163-166, 229). Only one study has also investigated how the metabolic profiles evolve with time within each group. This study revealed that septic shock patients who go on to survive had decreased levels of amino acids (including glutamate, glutamine, phenylalanine, alanine), glycolysis metabolites (including lactate, pyruvate), citrate (a TCA cycle intermediate) after 24 h of ICU admission in comparison to baseline (i.e., ICU admission) conversely patients who go on to die had increased amino acids (including glutamate, glutamine, phenylalanine, alanine), citrate and creatinine after 24 hours of ICU admission whilst only N-acetyl glycoproteins were found to be reduced after 24 hours of ICU admission (66). This study had small sample size (N= 102; 32 paired survivors and 19 paired non-survivors), but it had an internal validation where the paired survivors and non-survivors were split into training and test set (66).

The present study uses metabolomics, from two different platforms, NMR and UHPLC-MS to investigate the trajectories of small molecules for survivors and non-survivors of septic shock in serum samples taken from VANISH (92) and LeoPARDS trial (88) with the purpose of understanding the metabolic pathways which change over time in sepsis. In future this might help clinicians in making clinical decisions as knowing how the small molecules change over time with outcome will allow clinicians to adjust the treatment depending on whether the patients get better or worse over time. In this study, metabolic trajectories of small molecules related to clinical patient outcomes i.e., mortality and survival will be explored in both cohorts and whether the trajectories are different among survivors and non-survivors. This will be taken a step further where the findings from one cohort will be validated in the other cohort and vice versa. To date this type of validation has not been previously done in other metabolic comparisons of survivors and non-survivors. There has not been any work performed on how the metabolites change with organ failure scores (i.e., SOFA scores) in

sepsis patients, and this study has also investigated the metabolic changes with SOFA scores with purpose of understanding the molecular biology of sepsis patients with organ dysfunction. Additionally, the metabolic changes and trajectories based on treatment were also investigated in this study, but only in VANISH and these findings were not validated in LeoPARDS as different drugs were used in the two clinical trials.

4.2 Aims

In this study we investigated whether the metabolic profiles of patients with septic shock evolve with time based on mortality and treatment and if there are any metabolic differences in septic shock patients based on organ dysfunction.

The aims of this chapter are as follows:

- 1) To determine whether there are differences in the metabolic trajectories of survivors and non-survivors and if these could be used to track patient course.
- 2) To assess whether the metabolic trajectories of septic shock patients from one cohort can validate mortality results in another cohort.
- 3) To determine if the metabolic signatures of septic shock patients vary with organ failure.
- 4) To determine if there are differences in the metabolic profiles and trajectories of septic shock patients in response to treatment.

4.3 Statistical Analysis

The complete methods for all the analyses related to trajectory analysis of targeted data are listed in chapter 2, section 2.3.5 whilst the methods for trajectory analysis of NMR untargeted data (CPMG) are listed in chapter 2 section 2.3.4.

4.4 Results

4.4.1 Metabolic trajectories focusing on outcome

4.4.1.1 Trajectory analysis of untargeted data

Trajectory analysis of CPMG data from VANISH was performed on a total of 103 pairs of survivors and 31 pairs of non-survivors shown in figure 4.1 (highlighted in blue). The PLS-DA model statistics (i.e., $R^2X=0.0014$; $R^2Y= 0.623$, $Q^2Y= -0.166$) did not satisfy the criteria of a

good PLS-DA model, but that could be due to the difference spectra being used in the analysis. However, this analysis allowed trends in the trajectories of metabolites for survivors and non-survivors to be identified. Samples were excluded as previously mentioned in chapter 2, section 2.2.1.3 and chapter 3, section 3.3.3.

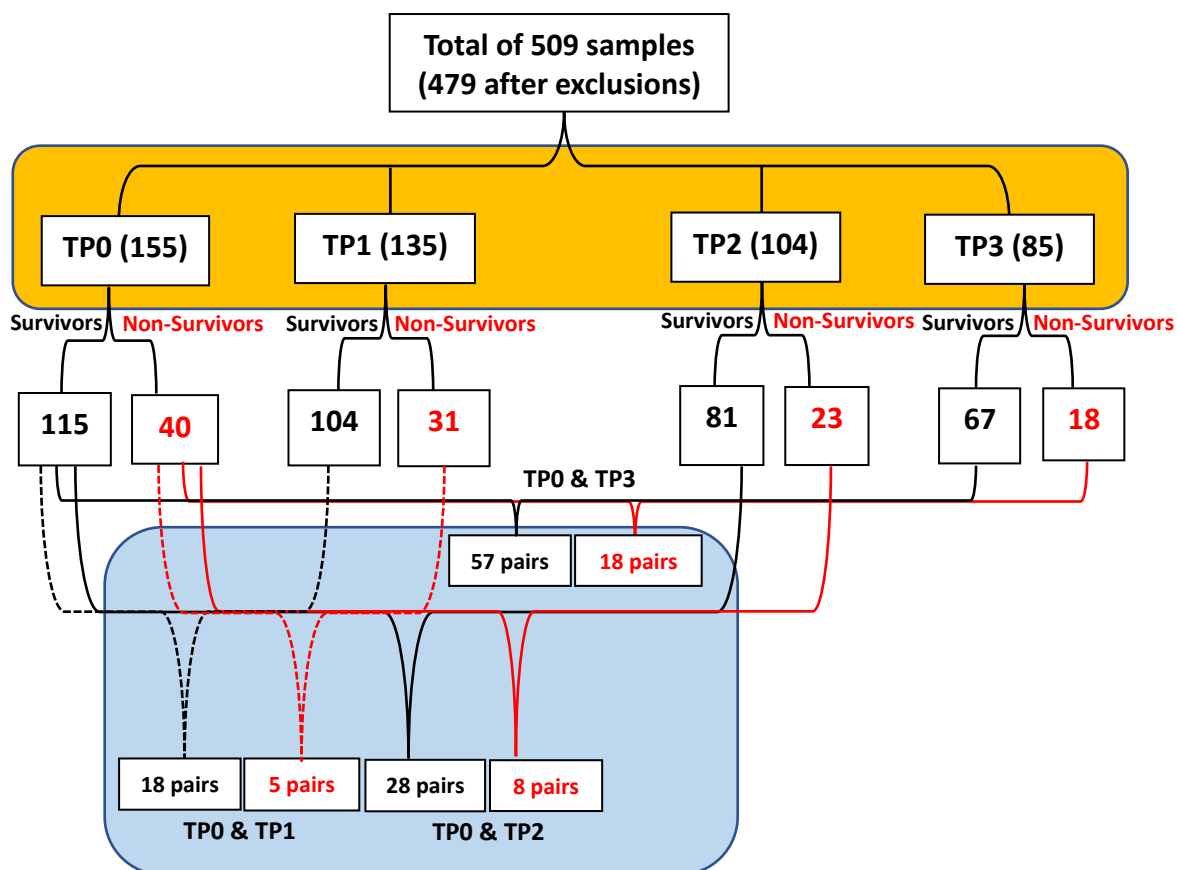


Figure 4.1: Flow chart representing pairs of survivors and non-survivors included in the mortality analysis of CPMG Data from VANISH.

Trajectories were determined by comparing the mean spectra shown in figure 4.2. Metabolites which were found to be changing in these two groups are listed in table 4.1. In all metabolites except for glucose the degree of change over time was smaller in non-survivors compared to survivors. Most of the metabolites increased in concentration over time however, some metabolites including lactate, 1-methylhistidine, glucose, acetone, acetate, and creatine showed a reduction over time.

Table 4.1: Metabolite trends observed in the mean spectra of difference spectra for survivors and non-survivors. Note: Δ Metabo refers to the magnitude of change in the levels of metabolites for survivors and non-survivors; $\uparrow$ denotes bigger and $\downarrow$ denotes smaller; s = singlet; d = doublet; q = quartet (represent peak multiplicity).

Metabolite	δ (ppm)	Survivors ΔMetabo	non-survivors ΔMetabo
<i>Tyrosine</i>	7.18 (d), 6.89 (d)	$\uparrow$	$\downarrow$
<i>1-MH</i>	7.05 (s), 7.78 (s)	$\uparrow$	$\downarrow$
<i>Glucose</i>	5.23 (d)	$\downarrow$	$\uparrow$
<i>Lactate</i>	4.10(q), 1.32 (d)	$\uparrow$	$\downarrow$
<i>Glycine</i>	3.56 (s)	$\uparrow$	$\downarrow$
<i>Alanine</i>	1.48(d)	$\uparrow$	$\downarrow$
<i>N-GlycA</i>	2.03(s)	$\uparrow$	$\downarrow$
<i>N-GlycB</i>	2.4 (S)	$\uparrow$	$\downarrow$
<i>Creatine</i>	3.04(s)	$\uparrow$	$\downarrow$
<i>Valine</i>	1.04(d), 0.99(d)	$\uparrow$	$\downarrow$
<i>Leucine</i>	1.01(d)	$\uparrow$	$\downarrow$
<i>Isoleucine</i>	0.97(d)	$\uparrow$	$\downarrow$
<i>Acetone</i>	2.22(s)	$\uparrow$	$\downarrow$
<i>Ethanol</i>	2.17(d)	$\downarrow$	$\uparrow$
<i>Formate</i>	8.45(s)	$\uparrow$	$\downarrow$
<i>CH₂ mixed lipoproteins</i>	1.21	$\uparrow$	$\downarrow$
<i>CH₃ mixed lipoproteins</i>	0.82	$\uparrow$	$\downarrow$

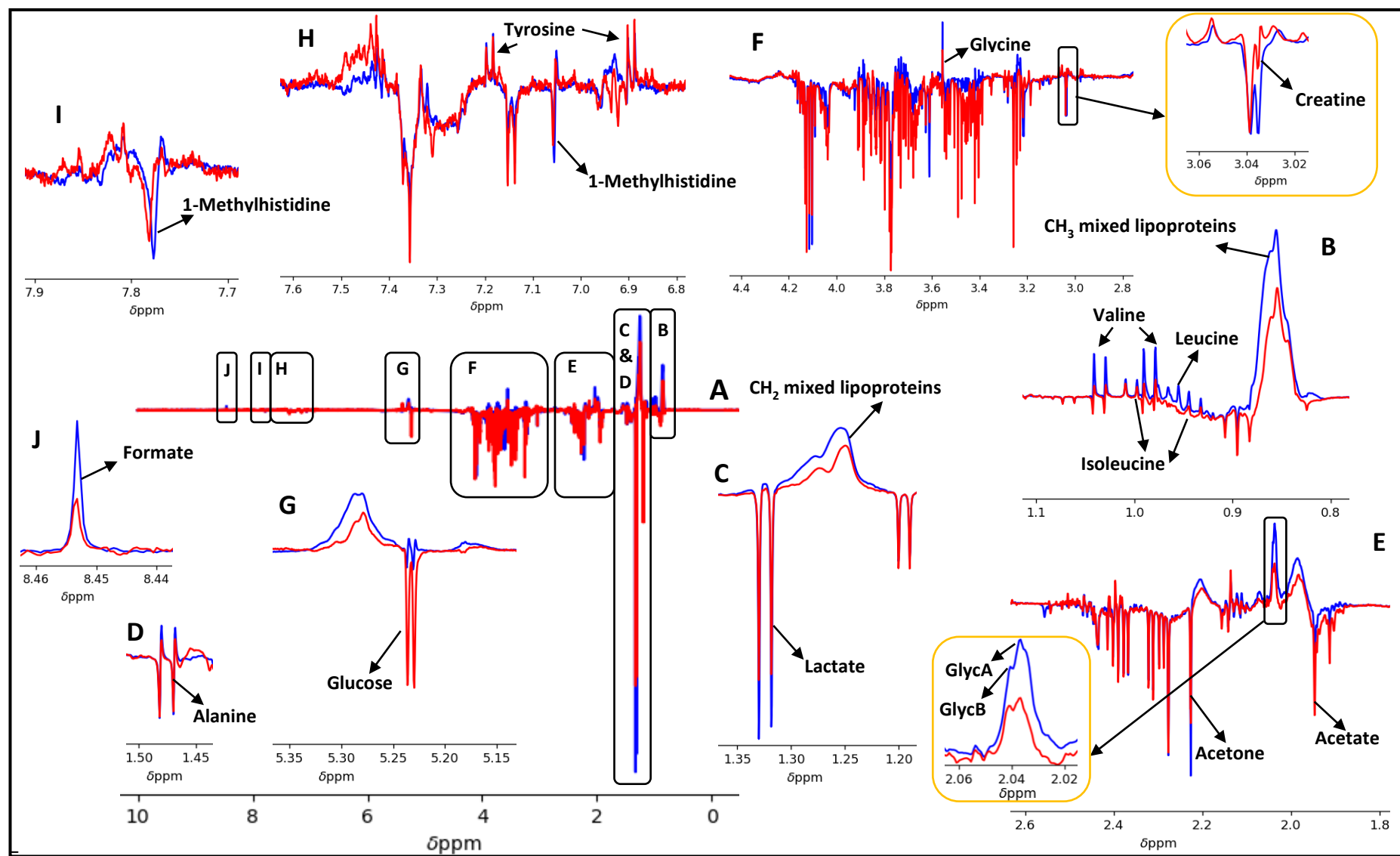


Figure 4.2: Overlay of mean spectra of difference spectra of survivors and non-survivors. Red spectrum = non-survivors; blue spectrum = survivors. Region expansions for the metabolites are shown in separate.

4.4.1.2 Trajectory analysis of Targeted data

NMR targeted data was used in conjunction with targeted extracted features from UHPLC-MS profiling data to determine the metabolic trajectories of small molecules for survivors and non-survivors from both cohorts. Initially, the trajectory analysis for mortality was performed on the combined SMoESY-select and HPOS ppR data using full set of metabolites (i.e., 72 small molecules) and the results for this analysis are shown in figure A1.1 (see appendix 1.3). Trajectory analysis was performed on a total of 472 samples from VANISH and 1404 samples from LeoPARDS, as shown in figures 4.3 and 4.4. In VANISH, the samples used for the trajectory analysis were from the combined SMoESY-select (N = 472) and HPOS ppR (N = 488) data and same samples were excluded from both cohorts as mentioned previously in chapter 2, (sections 2.2.1.3 and 2.2.2.3) and chapter 3 (sections 3.3.4.1 and 3.3.4.2).

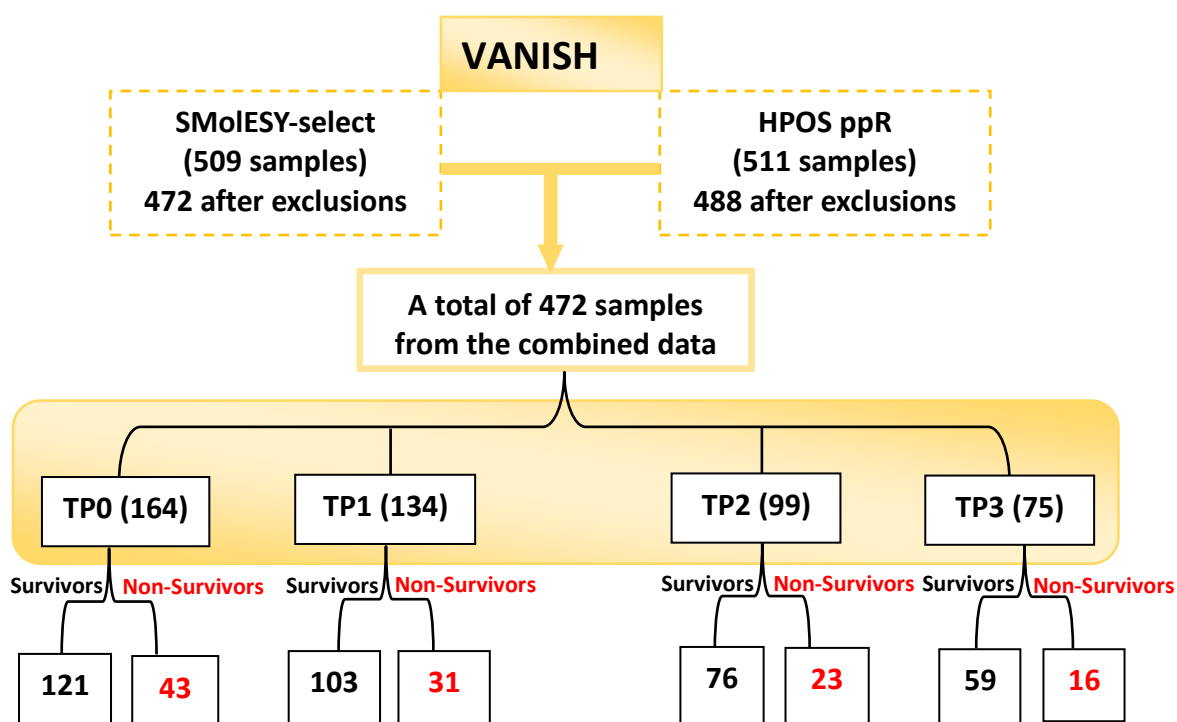


Figure 4.3: Flow chart for the samples included in the trajectory analysis of combined SMoESY-select and HPOS ppR data from VANISH.

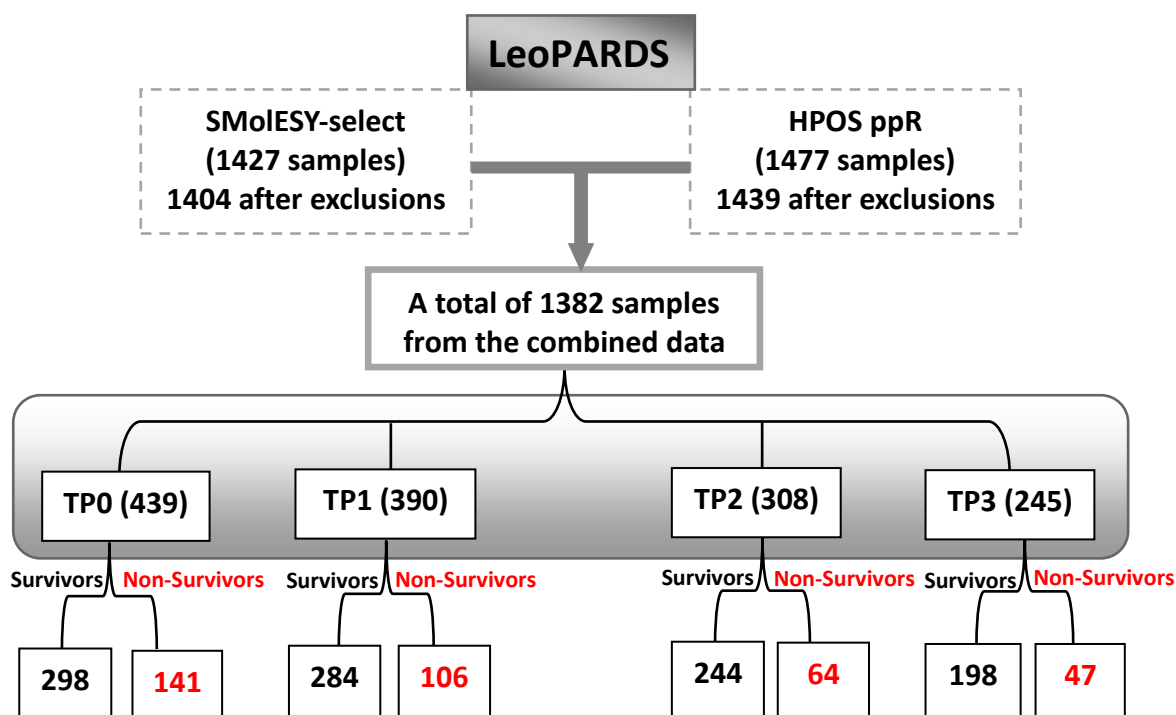


Figure 4.4 Flow chart for the samples included in the trajectory analysis of combined SMoESY-select and HPOS ppR data from LeoPARDS.

Trajectory analysis of small molecules for survivors and non-survivors was performed with and without BMI as a confounding factor in the LME models. Most of the results from the two LME models were the same with few exceptions which will be addressed shortly. A summary of the results for the trajectory analysis of 61 small molecules for survivors and non-survivors with and without BMI for both cohorts is presented in figure A1.2 (see appendix 1.3) and figure 4.5, respectively.

In LeoPARDS, the overall metabolic trajectories of 11 small molecules including glutamine, N6,N6,N6-trimethyllysine, acyl carnitines (hexadecenoylcarnitine, tetradecenoylcarnitine, stearoylcarnitine, palmitoylcarnitine), α -glycerophosphocholine, 1-methyladenosine, N2,N2-dimethylguanosine, 7-methylguanine, and hypoxanthine were significantly different between survivors and non-survivors (Trend estimate $p_{Adj} < 0.05$ shown by red asterisks) (figure 4.5) whilst in VANISH, the metabolic trajectories for only long-chain acylcarnitines including (hexadecenoylcarnitine, stearoylcarnitine, palmitoylcarnitine) were significantly different between the two groups, but not after B-H correction. This could reflect that LeoPARDS has greater statistical power than VANISH as it has almost three times more

samples. Three metabolites, hypoxanthine, tetradecenoylcarnitine and N2,N2-dimethylguanosine, increased over time in patients who went on to die in LeoPARDS and these metabolites except for tetradecenoylcarnitine had the same trend in non-survivors from the VANISH cohort. However, hypoxanthine and N2,N2-dimethylguanosine decreased over time in patients who went on to survive in LeoPARDS whilst in VANISH only N2,N2-dimethylguanosine followed this trend. Other long-chain acyl carnitines including hexadecenoylcarnitine, stearoylcarnitine, and palmitoylcarnitine showed a negative trend in non-survivors whilst these metabolites increased over time in survivors in both cohorts ($p < 0.05$). N6,N6,N6-trimethyllysine increased over time in both survivors and non-survivors from both cohorts and showed a steep increase in non-survivors compared to survivors in both cohorts. Two modified purine nucleosides including 7-methylguanine and 1-methyladenosine decreased over time in both survivors and non-survivors and showed a steeper decline in survivors over time in LeoPARDS whilst in VANISH only 7-methylguanine followed the same trend and non-survivors showed a steeper decline in the levels of 7-methylguanine over time in comparison to survivors. Glutamine and α -glycerophosphocholine had negative trend in patients who go on to die whilst patients who go on to survive had a positive trend in both cohorts.

The levels of small molecules change over time in patients with sepsis and it was found that in both cohorts a total of 24 metabolites were significantly different between survivors at baseline in comparison to day 4 ($p_{Adj} < 0.05$) (figure 4.5). Of those, 13 metabolites such as amino acids (arginine, valine, isoleucine, glycine, glutamine, HPOS_lysine, proline), long-chain acylcarnitines (hexadecenoylcarnitine, oleoylcarnitine, stearoylcarnitine, palmitoylcarnitine, tetradecenoylcarnitine), and formic acid had significantly higher EMMs at day 4 than baseline ($p_{Adj} < 0.05$) in survivors in both cohorts whilst EMMs of 11 small molecules including lactic acid, NMR_Creatinine, proline betaine, N-methyl pipecolic acid, hydroxybutyrylcarnitine, acetone, 3-hydroxybutyric acid, N2,N2-Dimethylguanosine, dimethyl sulfone, taurine and trigonelline were significantly higher in survivors at baseline than at day 4 ($p_{Adj} < 0.05$).

The metabolic trajectories of small molecules for non-survivors were also determined and it was found that there was a significant difference in the EMMs of 11 metabolites in non-survivors at baseline and at day 4 in both cohorts (figure 4.5). All these metabolites

(NMR_Creatinine, proline betaine, histidine, N1-Acetylspermidine, lactic acid, hydroxybutyrylcarnitine, acetone, 3-hydroxybutyric acid, dimethyl sulfone, taurine and trigonelline) were significantly higher in non-survivors at baseline ($p_{Adj} < 0.05$) than at day 4 in both cohorts.

In addition to the metabolic trajectories, the metabolic differences between survivors and non-survivors at day 0 and at day 4 were also determined using the LME models. At day 0, 18 small molecules including alanine, phenylalanine, glycine, N,N-dimethylglycine, HPOS_lysine, N6,N6,N6-trimethyllysine, proline, histidine, N1-acetylspermidine, lactic acid, acetic acid, hexadecenoylcarnitine, stearyl carnitine, decanoylcarnitine, choline, 1-methylnicotinamide, N1-methyl-2-pyridone-5-carboxamide and N1-methyl-4-pyridone-3-carboxamide had significantly higher EMMs in non-survivors ($p_{Adj} < 0.05$) with respect to survivors in both cohorts whilst hypoxanthine was lower in non-survivors than survivors at day 0 in both cohorts (figure 4.5). These results were consistent with our findings from the univariate analysis of targeted data (chapter 3).

At day 4, none of the metabolites were common between the two cohorts in differentiating survivors from non-survivors based on the adjusted p-values (figure 4.5).

The trajectory model of mortality with BMI also showed the same results as the model without BMI, but four metabolites based on the overall slope for survivors and non-survivors (N6,N6,N6-trimethyllysine, hexadecenoylcarnitine, tetradecenoylcarnitine, and hypoxanthine) did not remain significant after B-H correction in LeoPARDS in the model with BMI (figure A1.2, appendix 1.3) whilst they remained significant after correction in the LME model without BMI (figure 4.5). However, the trend for all these metabolites was in the same direction for survivors and non-survivors in both LME models with and without BMI for LeoPARDS.

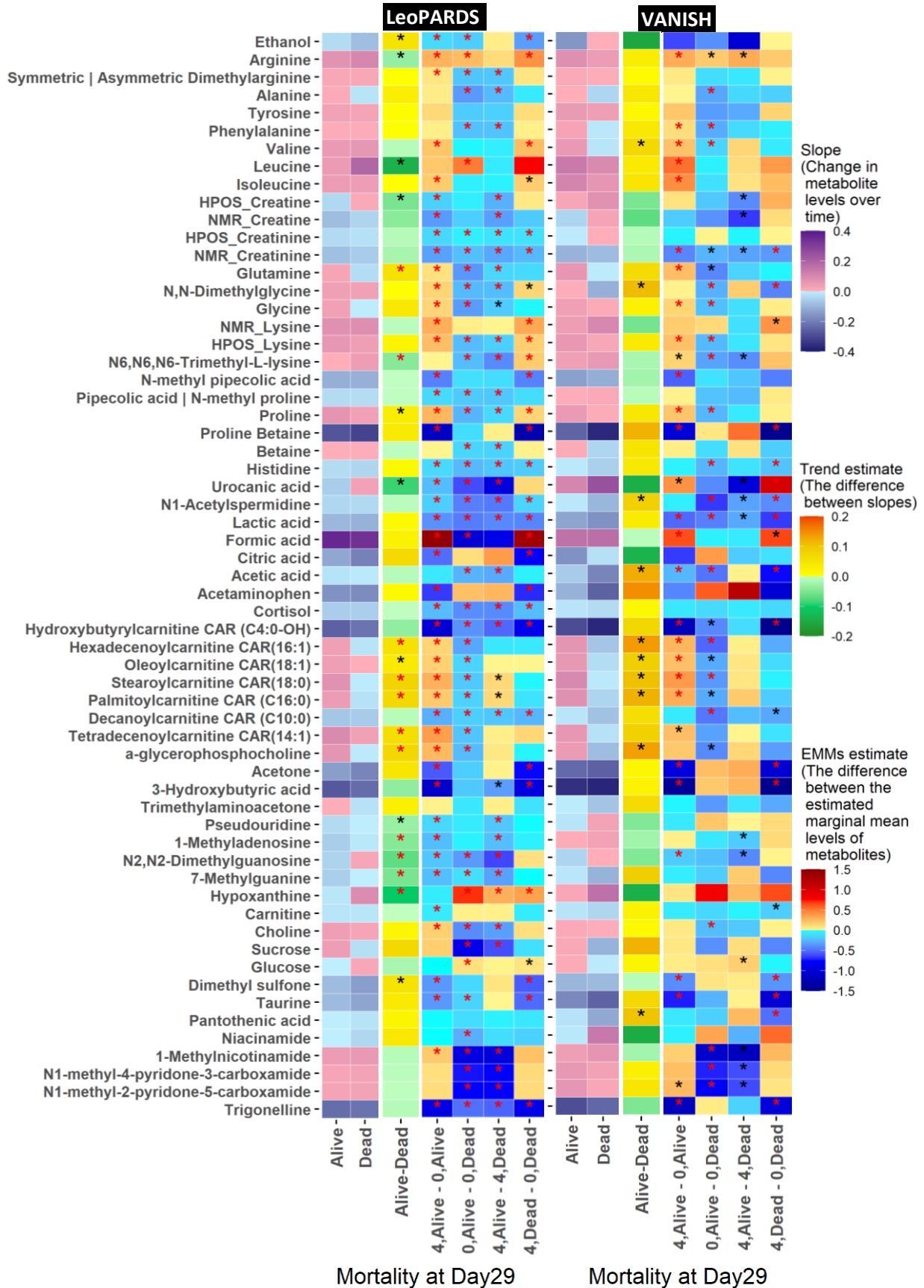


Figure 4.5: The results of LME models focusing on mortality for combined SMoESY-select and HPOS ppR log transformed data with age and sex as confounders for LeoPARDS and VANISH. Various contrasts were performed during the trajectory analysis and their outcomes are shown in this figure. LME model outputs

including slope (i.e., change in metabolite levels over time), trend estimate (i.e., the difference between slopes) and EMMs estimate (i.e., the difference between the estimated marginal mean levels of metabolites) are presented in different colour scales. Metabolites which are significantly different between groups from various pairwise comparisons are highlighted in black asterisk whilst those that remained significant after correction are shown in red. The slopes for alive (i.e., survivors) and dead (i.e., non-survivors) are shown as well as the difference between their slopes and whether this difference in the overall slopes is significant between the two groups. The scale for slope goes from positive to negative. Positive values for slope indicate that the metabolites increase with time whilst the negative values indicate that the metabolites decrease over time and the stronger the colour the bigger the magnitude for both positive and negative slopes. The difference between the slopes for alive and dead is presented in different colour scale (i.e., Trend estimate). Estimated marginal means were also calculated for each group at days 0 and day 4 using the LME model and the difference between the EMMs for four pairwise comparisons are shown in this figure. The four pairwise comparisons include: 4,Alive-0,Alive = Difference between the EMMs of alive at day 4 and day 0 based on the LME model; 4,Dead-0,Dead = Difference between the EMMs of dead at day 4 and day 0 based on the LME model; 0,Alive-0,Dead = Difference between the EMMs of alive and dead at day 0 based on the LME model; 4,Alive-4,Dead = Difference between the EMMs of alive and dead at day 4 based on the LME model.

4.4.1.3 Prediction of outcome based on metabolic changes from baseline

The differences between the trajectories of small molecules for survivors and non-survivors were found from the trajectory analysis of combined targeted data (both SMoLESY-select and HPOS ppR). The aim was to validate these findings for both VANISH and LeoPARDS and determine whether the results from one cohort can be used to predict outcome in the other.

The predictive models were not only performed on paired samples of TP0 and TP1 with fold changes (TP0TP1) (N = 126 and N = 368 for VANISH and LeoPARDS, respectively) but also on paired samples of TP0 and TP2 with fold changes (i.e., TP0TP2) (N = 92 and N = 286 for VANISH and LeoPARDS, respectively) from SMoLESY-select and HPOS ppR data from both cohorts as shown in figure 4.6 and figure 4.7. The same number of samples were excluded from all the datasets (SMoLESY-select and HPOS ppR) as the trajectory analysis for outcome discussed previously in this chapter. In this analysis, TP0TP1 and TP0TP2 matched samples with fold-changes were used to see whether the small molecules with significantly different trajectories for survivors and non-survivors could be used to aid earlier identification patients who were likely to die or survive. In the predictive models for validation within the study cohort, LME models were built on samples which had matching patient IDs with TP0TP1 and TP0TP2 samples with fold changes from VANISH (N = 342 and N = 277 for TP0TP1 and TP0TP2 matched samples with fold changes, respectively) and LeoPARDS (N = 1040 and N = 891 for TP0TP1 and TP0TP2 matched samples with fold-changes, respectively).

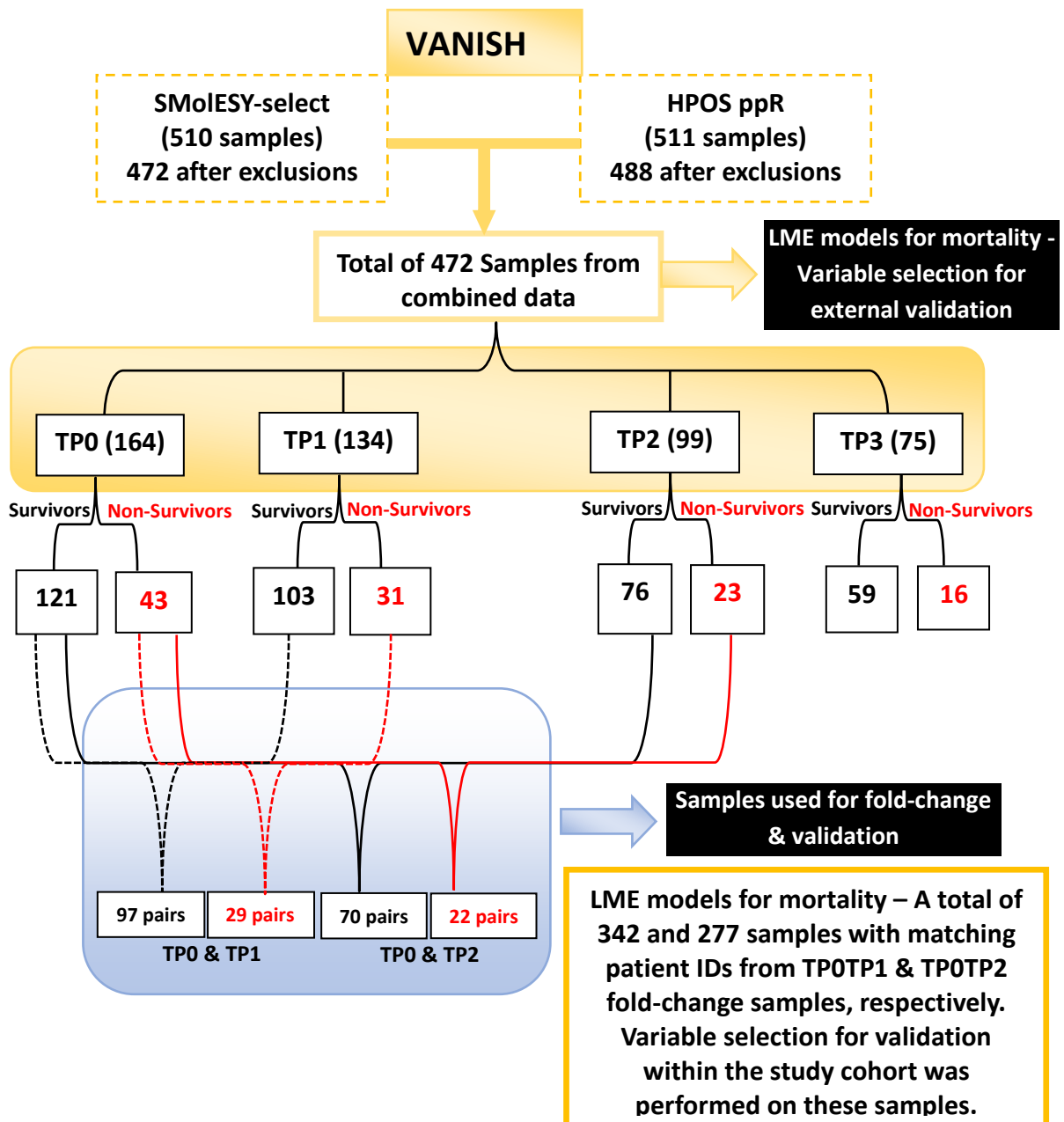


Figure 4.6: Flow chart representing samples included in the validation of mortality results from the trajectory analysis of combined SMoIESY-select and HPOS pp Data from VANISH.

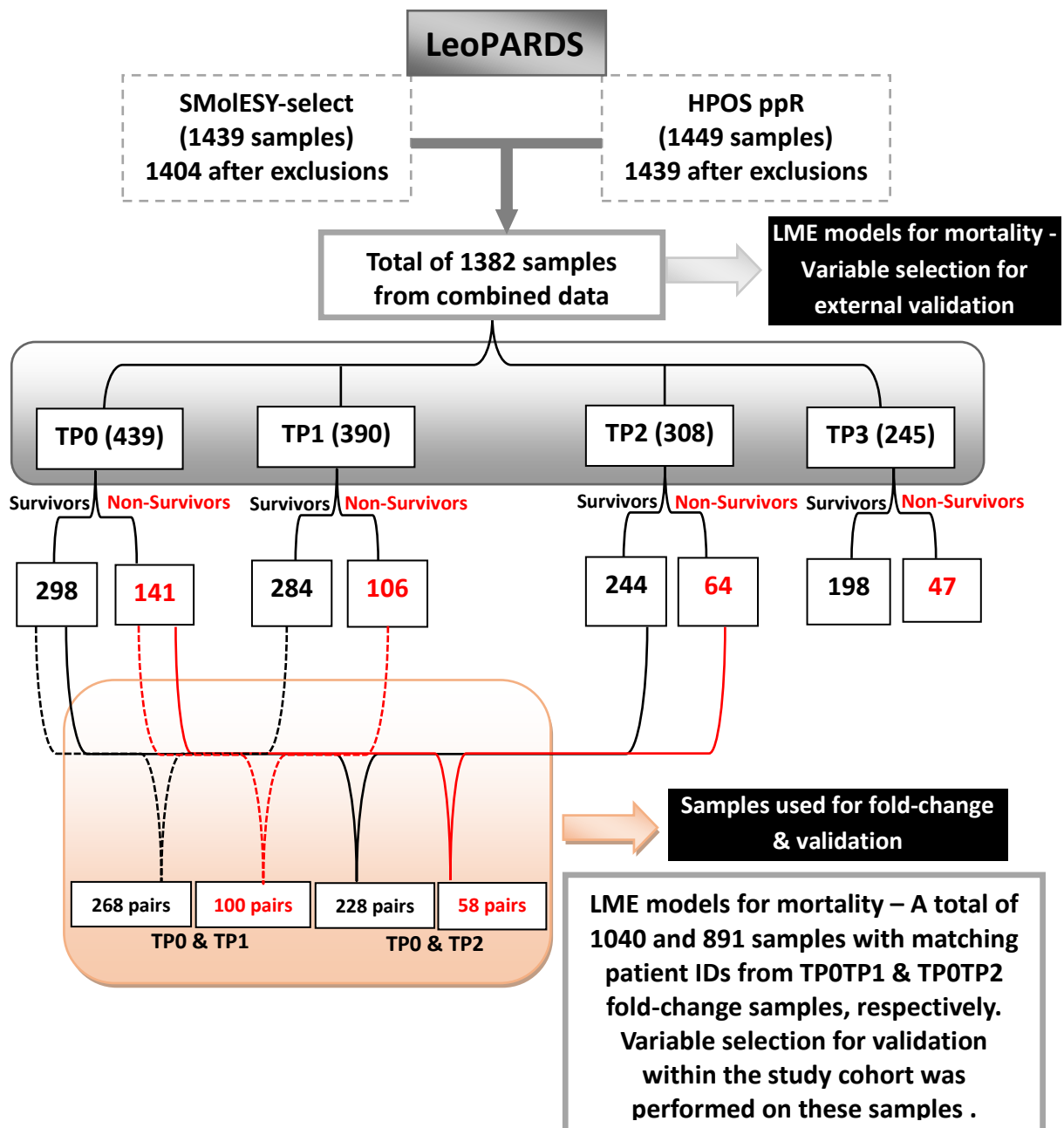


Figure 4.7: Flow chart representing samples included in the validation of mortality results from the trajectory analysis of combined SMoIESY-select and HPOS pp Data from LeoPARDS.

The performance metrics for the mortality prediction models of the combined targeted data for the two cohorts for TP0TP1 and TP0TP2 samples with fold-changes are listed in table 4.2. The predictive models for TP0TP1 samples with fold changes for both cohorts performed slightly better than the predictive models for TP0TP2 samples with fold changes (figure A1.3, see appendix 1.3) but still the models did not perform well. In brief, the cross validated ROC curve for within cohort validation of VANISH for TP0TP1 samples with fold-changes (VANISH

TPOTP1_{WC}) for VANISH test set and validation of VANISH within LeoPARDS dataset for TPOTP1 samples with fold-changes (VANISH TPOTP1_{EXCO}) had an area under ROC curve (AUC) of 0.56 and 0.53, respectively (figure 4.8) (for 10 significant metabolites (5 metabolites including four acyl carnitines and α -glycerophosphocholine were common in both cohorts) selected from LME model based on mortality for VANISH with age and sex as confounders, figure 4.5). Survivors and non-survivors were correctly classified by the predictive models with a balanced accuracy of 51.05% and 51.44% for VANISH TPOTP1_{WC} and TPOTP1_{EXCO}, respectively.

The cross validated ROC curves for within cohort validation of LeoPARDS for TPOTP1 samples with fold-changes (LeoPARDS TPOTP1_{WC}) from LeoPARDS test set and validation of LeoPARDS within VANISH dataset for TPOTP1 samples with fold-changes (LeoPARDS TPOTP1_{EXCO}) had an area under ROC curve (AUC) of 0.64 and 0.60, respectively (figure 4.8) (for 20 significant metabolites (5 metabolites were common in both cohorts same as the previous model) selected from LME model based on mortality for LeoPARDS with age and sex as confounders figure 4.5). Both of our validation models did not work as there were only five common metabolites between the two cohorts which had significant different overall trajectories for survivors and non-survivors. Survivors and non-survivors were correctly classified by the predictive models with a balanced accuracy of 54.71% and 58.99% for LeoPARDS TPOTP1_{WC} and TPOTP1_{EXCO}, respectively. The predictive models with LeoPARDS performed slightly better than predictive models with VANISH.

Table 4.2: Performance metrics for mortality prediction models within cohort (WC) and with external cohort (EXCO) for TP0TP1 and TP0TP2 samples with fold changes from combined SMoIESY-select and HPOS ppR data. Abbreviations: TP0TP1_{WC} = within cohort for TP0TP1 samples with fold changes, TP0TP1_{EXCOL} = with external cohort for TP0TP1 samples with fold changes; TP0TP2_{WC} = within cohort for TP0TP2 samples with fold changes; TP0TP2_{EXCOL} = with external cohort for TP0TP2 samples with fold changes.

Parameters	VANISH				LeoPARDS			
	TP0TP1 _{WC}	TP0TP1 _{EXCO}	TP0TP2 _{WC}	TP0TP2 _{EXCO}	TP0TP1 _{WC}	TP0TP1 _{EXCO}	TP0TP2 _{WC}	TP0TP2 _{EXCO}
Accuracy (%)	73.02	71.74	69.57	75.87	71.47	72.22	78.67	68.48
Balanced accuracy (%)	51.05	51.44	48.83	48.87	54.71	58.99	55.13	51.23
Sensitivity (%)	10.35	7.00	9.09	3.45	18.00	34.48	15.52	18.18
Specificity (%)	91.75	95.90	88.57	94.30	91.42	83.51	94.74	84.27
Positive predicted value (%)	27.27	38.89	20.00	13.33	43.90	38.46	42.86	26.67
Negative predicted value (%)	77.39	73.43	75.61	79.34	74.92	81.00	81.51	77.62
Prevalence of death (%)	23.02	27.17	10.87	20.28	27.17	23.02	20.28	23.91

Table 4.3: Confusion matrices for TP0TP1 fold change mortality predictive models for VANISH and LeoPARDS SMOESY-select datasets. Abbreviations: TP0TP1_{WC} = within cohort for TP0TP1 and TP0TP1_{EXCO} = with external cohort for TP0TP1.

Validation of VANISH within cohort			
VANISH TP0TP1 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	89	26
	Non-survivors	8	3
Validation of VANISH in LeoPARDS			
VANISH TP0TP1 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	257	93
	Non-survivors	11	7
Validation of LeoPARDS within cohort			
LeoPARDS TP0TP1 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	245	82
	Non-survivors	23	18
Validation of LeoPARDS in VANISH			
LeoPARDS TP0TP1 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	81	19
	Non-survivors	16	10

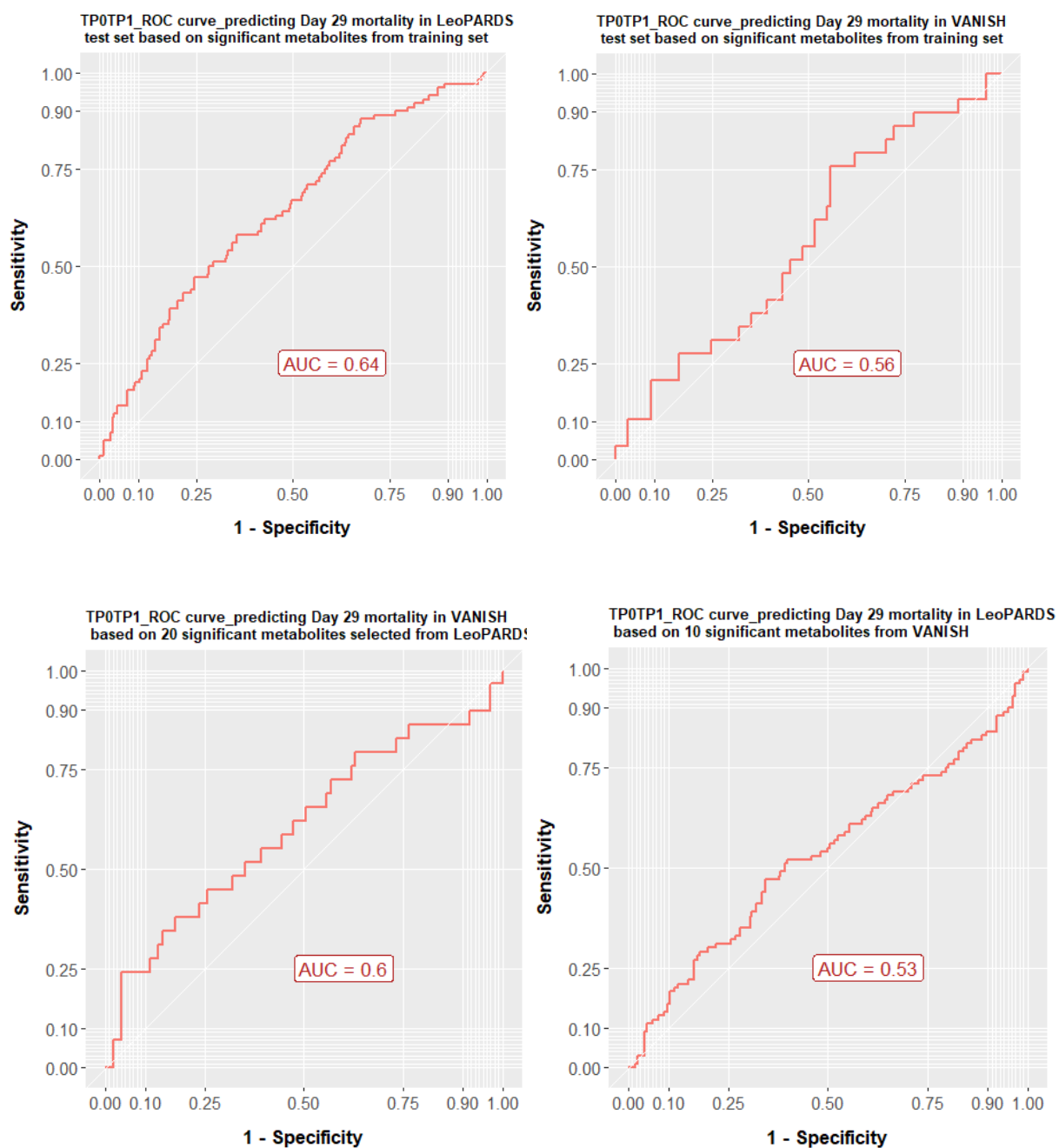


Figure 4.8: ROC curves for the predictive models of mortality on combined SMolESY-select and HPOS ppR dataset for VANISH and LeoPARDS for TP0TP1 samples with fold-changes.

4.5.1 Metabolic changes and trajectories focusing on treatment

4.5.1.1 Metabolic trajectories focusing on Study drug1

NMR targeted data (i.e., 24 small molecules from SMoESY-select) was used in conjunction with targeted extracted features from UHPLC-MS profiling data (i.e., 50 metabolites from HPOS ppR) to determine the metabolic trajectories of small molecules based on study drug 1. Trajectory analysis for study drug 1 was performed on a total of 469 samples from VANISH as shown in figure 4.9. Same samples were excluded from VANISH as discussed previously in chapter 2 (sections 2.2.1.3 and 2.2.2.3) and chapter 3 (sections 3.3.4.1 and 3.3.4.2). An additional, four samples were excluded from this analysis as study drug1 was not given because they were not sick enough to receive it.

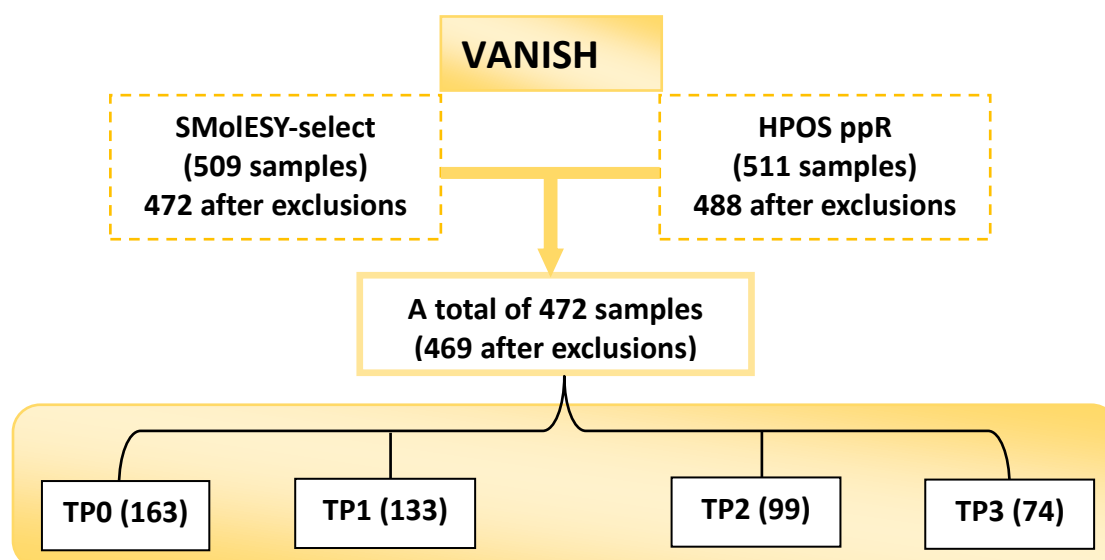


Figure 4.9: Flow chart representing samples included in the drug1 analysis of combined SMoESY-select and HPOS pp Data from VANISH to determine the metabolic trajectories based on drug1 treatment.

The results from the LME models of drug1 revealed that the metabolic trajectories of six small molecules (5'-methylthioadenosine, betaine, hydroxybutyrylcarnitine, tetradecenoylcarnitine, acetone, and 3-hydroxybutyric acid) were significantly different between patients who received noradrenaline and those who received vasopressin ($p < 0.05$), but these did not remain significant after B-H correction as shown in figure 4.10.

Trajectory analysis of patients who had noradrenaline and those with vasopressin revealed that the levels of six small molecules including proline betaine, N1-acetylsepermidine, lactic acid, hydroxybutyrylcarnitine and paraxanthine were significantly higher on days 0 and 1 in

comparison to day 4 (figure 4.10). Additionally, patients who received vasopressin also had significantly lower levels of NMR_Creatinine, N1-acetylputrescine, acetic acid, acetone, 3-hydroxybutyric acid, trimethylamine N-oxide, and trigonelline on days 0 and day 1 in comparison to day 4 ($p_{Adj} < 0.05$) whilst the levels of hypoxanthine were higher in patients on vasopressin at day 4 in comparison to day 0 and day 1 ($p_{Adj} < 0.05$). Overall, neither of these trial drugs influenced the serum metabolome of sepsis patients.

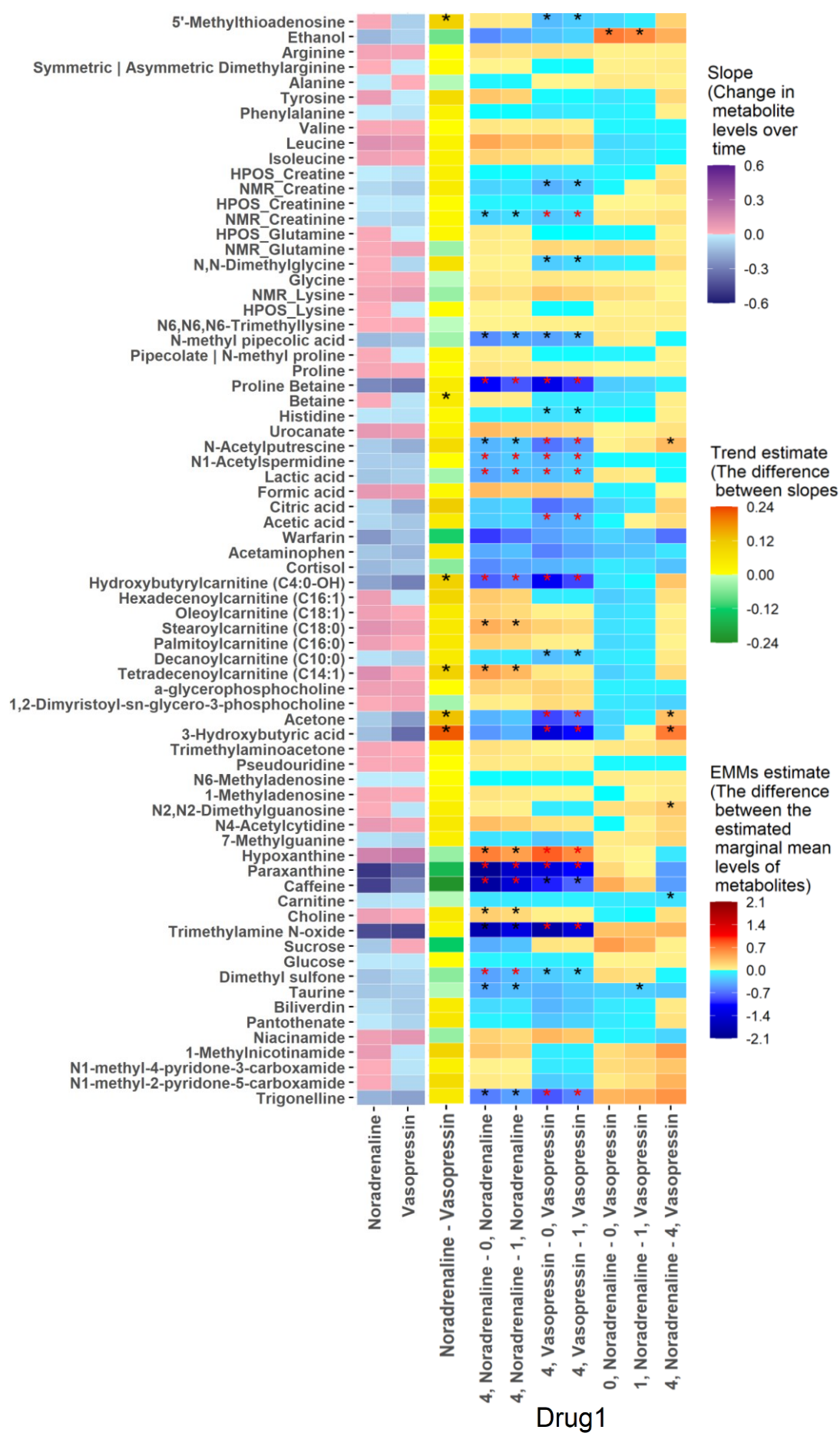


Figure 4.10: The results of LME models based on drug1 for combined SMoESY-select and HPOS ppR log transformed data with age and sex as confounders for VANISH. LME model outputs including slope (i.e., Change in metabolite levels over time), trend estimate (i.e., the difference between slopes) and EMMs estimate (i.e., the difference between the estimated marginal mean levels of metabolites) are presented in different colour scales. Metabolites which are significantly different between groups from various pairwise comparisons are highlighted in black asterisk whilst those that remained significant after correction are shown in red. The slopes for drug1 (Noradrenaline and Vasopressin) are shown as well as the difference between their slopes and whether this difference in the overall slopes is significant between the two groups. The scale for slope goes from positive to negative. The difference between the slopes for noradrenaline and vasopressin is presented in different colour scale (i.e., Trend estimate). The difference between the EMMs for seven pairwise comparisons are shown here as an EMMs estimate. The seven pairwise comparisons include: 4,Noradrenaline-0,Noradrenaline = Difference between the EMMs of patients who received noradrenaline at day 4 and day 0 based on the LME model; 4,Noradrenaline-1,Noradrenaline = Difference between the EMMs of noradrenaline at day 4 and day 1 based on the LME model; 4,Vasopressin-0,Vasopressin = Difference between the EMMs of patients who received vasopressin at day 4 and day 0 based on the LME model; 4,Vasopressin-1,Vasopressin = Difference between the EMMs of patients who received vasopressin at day 4 and day 1 based on the LME model; 0,Noradrenaline-0,Vasopressin = Difference between the EMMs of patients who received noradrenaline at day 0 and vasopressin at day 0 based on the LME model; 1,Noradrenaline-1,Vasopressin = Difference between the EMMs of patients who received noradrenaline at day 1 and vasopressin at day 1 based on the LME model; 4,Noradrenaline-4,Vasopressin = Difference between the EMMs of patients who received noradrenaline at day 0 and vasopressin at day 4 based on the LME model.

4.5.1.2 Metabolic trajectories focusing on Study drug2

NMR targeted data was used in conjunction with targeted extracted features from UHPLC-MS profiling data to determine the metabolic trajectories of small molecules based on study drug 2, hydrocortisone vs placebo. Trajectory analysis for study drug 2 was performed on a total of 316 samples from VANISH as shown in figure 4.11. Same samples were excluded from VANISH as discussed previously in chapter 2 (sections 2.2.1.3 and 2.2.2.3) and chapter 3 (sections 3.3.4.1 and 3.3.4.2). An additional, 156 samples were excluded from this analysis as study drug 2 was only received by those patients who required a threshold dose of the first study drug.

Two types of LME models were built on these samples, one which focuses on whether the metabolome changes with the study drug2 (Drug2_model1) and a second Drug2_model2 focusses on how the small molecules change over time in patients who receive study drug 2 i.e., the overall trend (Drug2_model2) as shown in figure 4.12.

The results from Drug2_model1 revealed that only cortisol levels were significantly higher in patients who received hydrocortisone ($p_{Adj} < 0.05$) in comparison to those who received placebo and those who did not receive drug2 yet but will go on to receive it later (i.e., pre samples). Additionally, six metabolites (phenylalanine, isoleucine, HPOS_Creatine,

NMR_Creatine, N1-methyl-2-pyridone-5-carboximide, N1-methyl-4-pyridone-3-carboximide) were significantly higher in both hydrocortisone and placebo groups in comparison with pre samples ($p_{Adj} < 0.05$) whilst NMR_Creatinine, hydroxybutyrylcarnitine, α -glycerophosphocholine, 1,2-dimyristoyl-sn-glycero-3-phosphocholine, acetone, 3-hydroxybutyric acid, trimethylaminoacetone, hypoxanthine, trimethylamine-N-oxide, taurine, biliverdin, pantothenate, trigonelline were significantly lower in both hydrocortisone and placebo groups with respect to those who had not yet received drug2 ($p_{Adj} < 0.05$). Additionally, HPOS_Lysine and N6,N6,N6-trimethyllysine were only higher in patients who received hydrocortisone than those who had not yet received drug2 ($p_{Adj} < 0.05$) whilst N1-acetylputrescine and pseudouridine were lower in patients with hydrocortisone than those who had not yet received drug 2 ($p_{Adj} < 0.05$).

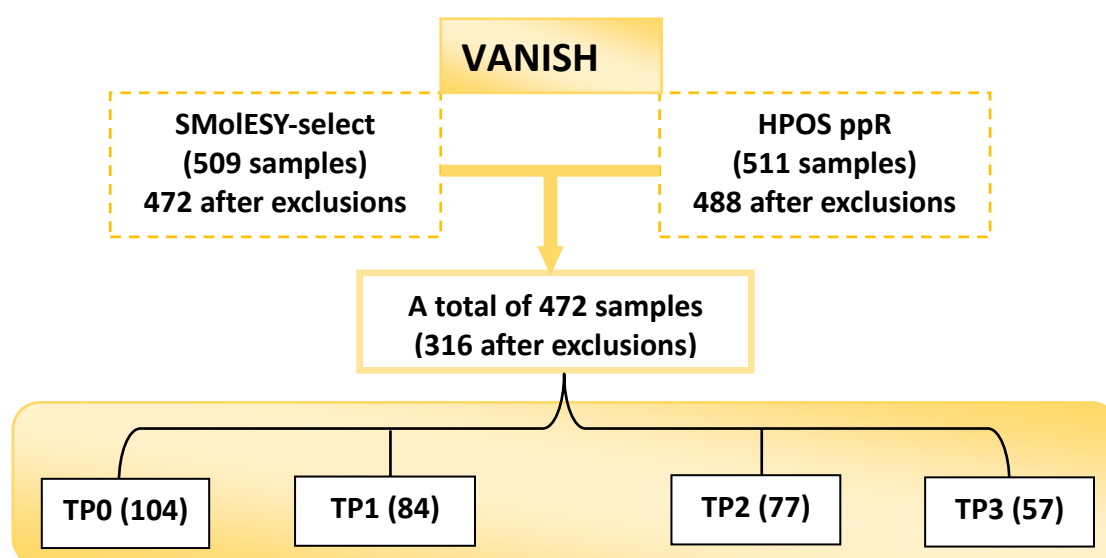


Figure 4.11: Flow chart representing samples included in the drug2 analysis of combined SMoIESY-select and HPOS pp Data from VANISH to determine the metabolic trajectories based on treatment.

The results from Drug2_model2 showed that the slope (i.e., change in metabolite levels over time) for none of the metabolites was significantly different between patients who received hydrocortisone and those who got placebo ($p_{Adj} < 0.05$). However, when comparing the EMMs of small molecules for patients who had received hydrocortisone and those with placebo from the results of Drug2_model2 (figure 4.12), it was found that only cortisol, was significantly higher in patients who had received hydrocortisone with respect to those who had placebo ($p_{Adj} < 0.05$).

Figure 4.12: The results of LME models based on drug2 for combined SMoIESY-select and HPOS ppR targeted data with age and sex as confounders for VANISH. The results from two LME models are shown here. Various contrasts were performed during this analysis and their outcomes are shown in this figure. LME model outputs including slope (i.e., change in metabolite levels over time), trend estimate (i.e., the difference between slopes) and EMMs estimate (i.e., the difference between the estimated marginal mean levels of metabolites) are presented in different colour scales. Metabolites which are significantly different between groups from various pairwise comparisons are highlighted in black asterisk whilst those that remained significant after correction are shown in red. The slopes for patients who received hydrocortisone, placebo and those patients who had yet not received drug2 (i.e., Pre drug2) are shown as well as the difference between their slopes and whether this difference in the overall slopes is significant between the groups. The difference between the slopes for three different comparisons is presented in different colour scale (i.e., Trend estimate). Estimated marginal means for both Drug2_model1 and Drug2_model2 for each group and the difference between the EMMs for three pairwise comparisons (including Hydrocortisone – placebo, Hydrocortisone -Pre drug2, and Placebo – Pre drug2) are shown.

4.5.1.3 Metabolic trajectories focusing on Study drug2 and outcome

NMR targeted data was used to determine the metabolic trajectories of small molecules based on day29 mortality with study drug 2 allocation. Trajectory analysis for study drug 2 was performed on a total of 316 samples from VANISH as shown in figure 4.13. Same samples were excluded from VANISH as discussed previously in chapter 2 (sections 2.2.1.3 and 2.2.2.3) and chapter 3 (sections 3.3.4.1 and 3.3.4.2). An additional, 156 samples were excluded from this analysis as study drug 2 was only received by those patients who required a threshold dose of the first study drug.

The results from the LME models revealed that the metabolic trajectories of nine small molecules including 5'-methyladenosine, arginine, phenylalanine, proline, N1-acetylspermidine, N6-methyladenosine, hypoxanthine, biliverdin, and pantothenate were significantly different between survivors and non-survivors who had received hydrocortisone ($p < 0.05$) as shown in figure 4.14. The levels of 5'-methyladenosine, phenylalanine, and N1-acetylspermidine decreased with time in both survivors and non-survivors who had hydrocortisone and the decline was steeper in patients who went on to die in comparison to those who survived. Biliverdin and pantothenate decreased over time in non-survivors who had received hydrocortisone whilst these two metabolites increase over time in survivors who had received hydrocortisone. Hypoxanthine levels increased over time in both survivors and non-survivors who had hydrocortisone. When comparing the EMMs of metabolites for survivors and non-survivors who had hydrocortisone at day 2, it was found that arginine, pseudouridine and dimethylsulfone were lower in patients who go on to die with respect to those who survive ($p < 0.05$) whilst 10 small molecules including

alanine, phenylalanine, NMR_Creatinine, histidine, N1-acetylspermidine, lactic acid, α -glycerophosphocholine, trimethylaminoacetone, taurine and 1-methylnicotinamide were higher in patients who go on to die in relation to those who survive ($p < 0.05$).

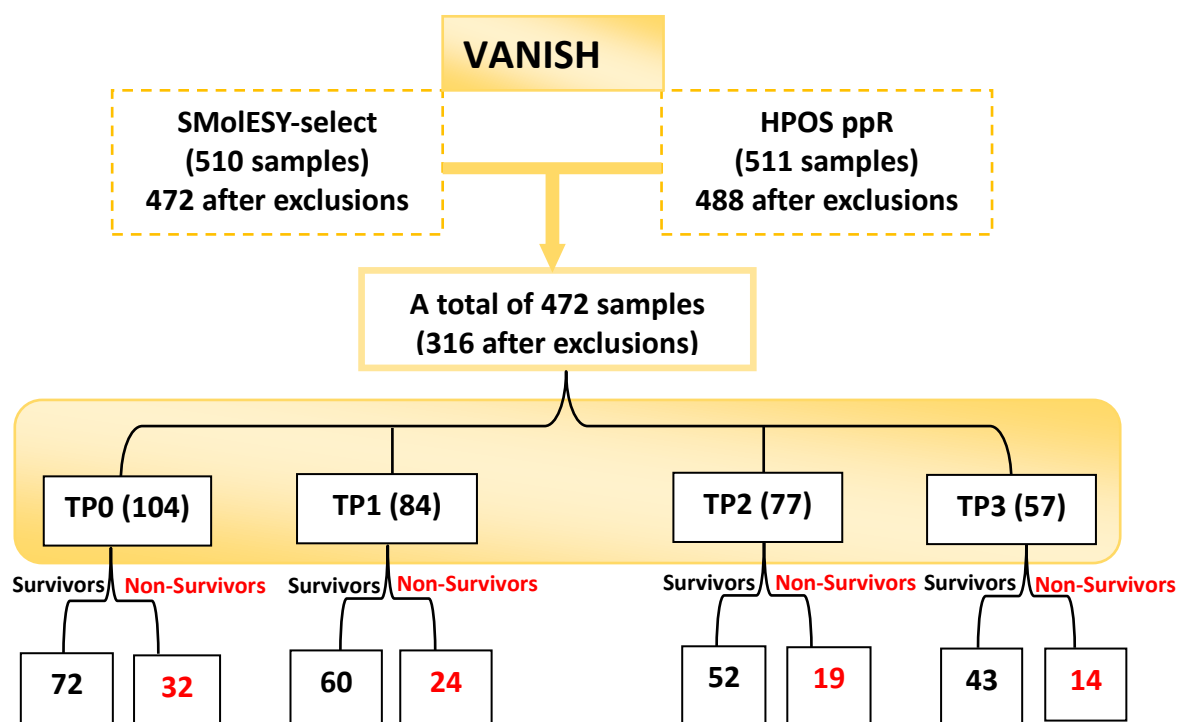


Figure 4.13: Flow chart representing samples included in the drug2 analysis of combined SMolESY-select and HPOS pp Data from VANISH to determine the metabolic trajectories based on drug2 treatment.

The metabolic trajectories of seven small molecules including N,N-dimethylglycine, urocanate, cortisol, α -glycerophosphocholine, trimethylaminoacetone, pseudouridine, and glucose were significantly different between survivors and non-survivors who had received placebo ($p < 0.05$), but none of these metabolites remained significant after correction. However, when comparing the EMMs of metabolites at day 2 for survivors and non-survivors who had placebo, it was found that 4 metabolites (phenylalanine, NMR_Glutamine, N1-acetylspermidine, 1-methylnicotinamide) were significantly higher in patients who went on to die with respect to those who survived ($p_{Adj} < 0.05$) whilst hypoxanthine was lower in patients who went on to die in relation to those who survived ($p_{Adj} < 0.05$) (figure 4.14).

The metabolic trajectories of four small molecules including NMR_Creatine, HPOS_Lysine, carnitine and pantothenate were significantly different in survivors who had received

hydrocortisone than those who had placebo ($p < 0.05$) (figure 4.14) but none of these metabolites remained significant after correction. However, when comparing the EMMs of metabolites at day 2 for survivors who had placebo and those who received hydrocortisone, it was found that only cortisol was significantly higher in surviving patients who had received hydrocortisone with respect to those who had placebo ($p_{\text{Adj}} < 0.05$).

Non-surviving patients with hydrocortisone and those with placebo had different metabolic trajectories for 7 small molecules including 5'-methylthioadenosine, urocanate, cortisol, α -glycerophosphocholine, pseudouridine, dimethyl sulfone and taurine ($p < 0.05$) but none of these metabolites remained significant after correction (figure 4.14). When comparing the EMMs of metabolites at day 2 for non-survivors with placebo and those with hydrocortisone, it was found that only cortisol was significantly higher in non-survivors who had received hydrocortisone with respect to those who had placebo ($p_{\text{Adj}} < 0.05$).

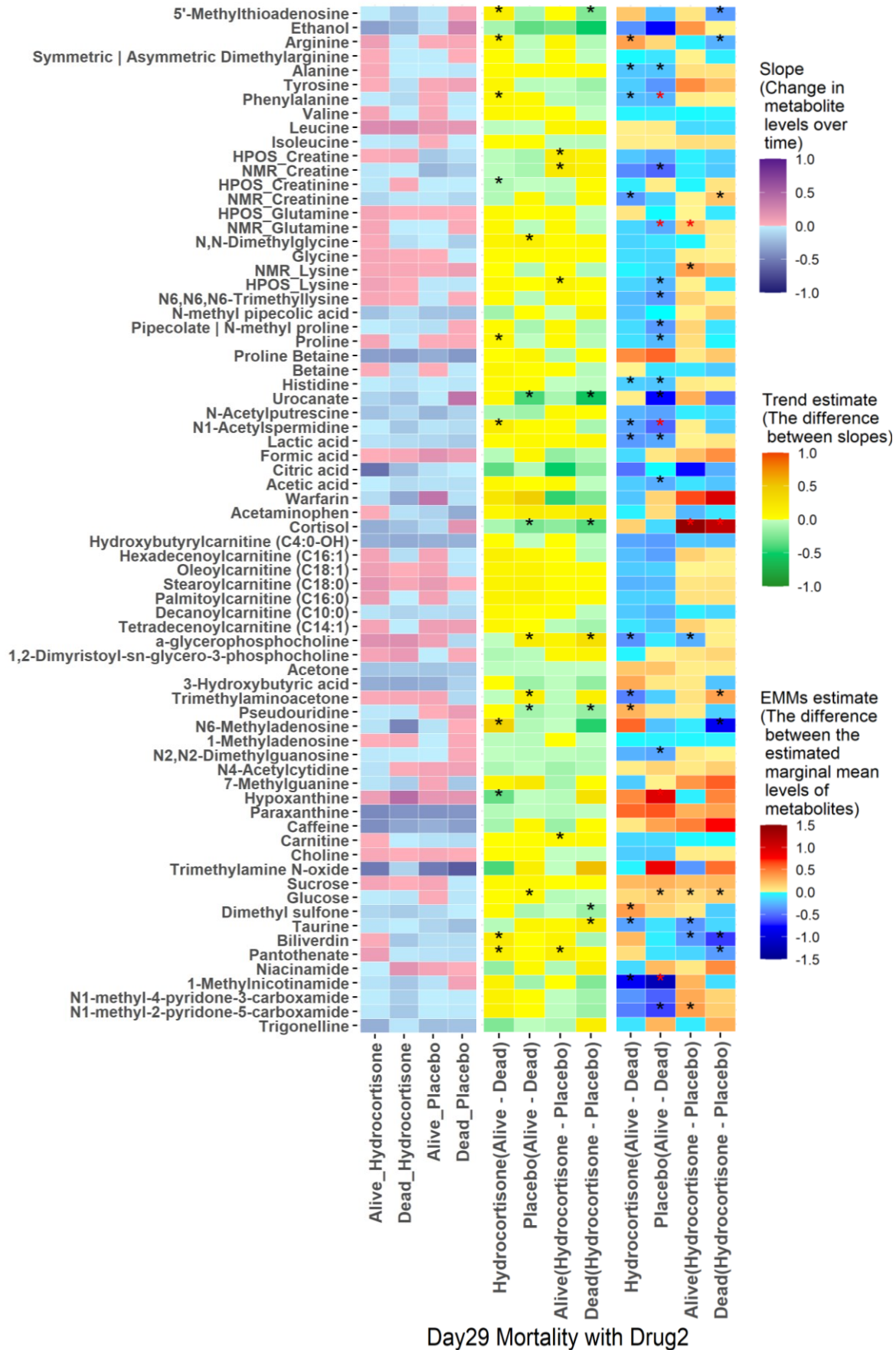


Figure 4.14: The results of LME models based on day29 mortality with drug2 for combined SMoIESY-select and HPOS ppR log transformed data with age and sex as confounders for VANISH. Various contrasts were

performed during the trajectory analysis and their outcomes are shown here. LME model outputs including slope (i.e., change in metabolite levels over time), trend estimate (i.e., the difference between slopes) and EMMs estimate (i.e., the difference between the estimated marginal mean levels of metabolites) are presented in different colour scales. Metabolites which are significantly different between groups from various pairwise comparisons are highlighted in black asterisk whilst those that remained significant after correction are shown in red. The slopes for alive (i.e., survivors) and dead (i.e., non-survivors) groups for both hydrocortisone and placebo are shown as well as the difference between their slopes and whether this difference in the overall slopes is significant between the four different comparison groups. The difference between the slopes for four pairwise comparisons for mortality within drug2 and between the two drugs is presented in different colour scale (i.e., Trend estimate). Estimated marginal means were also calculated for each group at day 2 using the LME model and the difference between the EMMs for four pairwise comparisons are shown in different colour scale. The four pairwise comparisons include: Hydrocortisone (Alive -Dead) = Difference between the EMMs of alive and dead in patients who received hydrocortisone at day 2 based on the LME model; Placebo (Alive -Dead) = Difference between the EMMs of alive and dead in patients who received placebo at day 2 based on the LME model; Alive (Hydrocortisone – Placebo) = Difference between the EMMs of patients who received hydrocortisone and those who received placebo at day 2 in the alive group; Dead (Hydrocortisone – Placebo) = Difference between the EMMs of patients who received hydrocortisone and those who received placebo at day 2 in the dead group.

4.6.1 Metabolic changes focusing on organ failure

NMR targeted data was used in conjunction with targeted extracted features from UHPLC-MS profiling data to determine how the metabolic profiles of patients with septic shock vary with organ failure. Initially, this analysis was performed on the combined data using full set of metabolites (i.e., 72 small molecules from both SMoIESY-select and HPOS ppR) for VANISH and the results for this analysis are shown in figure A1.4 (see appendix 1.4). The analysis of small molecules focusing on organ failure scores was performed on the combined data (SMoIESY-select and HPOS ppR).

The metabolic changes with SOFA scores were determined by generating LME models on a total of 456 samples from VANISH and 1349 samples from LeoPARDS, as shown in figure 4.15 and figure 4.16. Sample exclusions from both cohorts were performed as mentioned previously in chapter 2, (section 2.2.1.3 and 2.2.2.3). In VANISH, an additional 37 samples from SMoIESY-select (35 samples had no sample timings, and 2 samples belonged to the patients which had high glucose and the other which had different metabolic profile to the rest like the exclusions from the univariate analysis of targeted data as discussed previously in chapter 3, section 3.3.4.1) and 19 samples which had no sample timings from HPOS ppR were excluded. At the end, 4 additional samples from the matching 460 samples were excluded as the sofa scores for those samples were not recorded).

In LeoPARDS, an additional 34 samples from SMoIESY-select and 41 samples from HPOS ppR were excluded as these samples had no sample timings recorded. The LME models for this

analysis were built with and without BMI as a confounder and the results for both models were almost the same except for few small differences which will be addressed later.

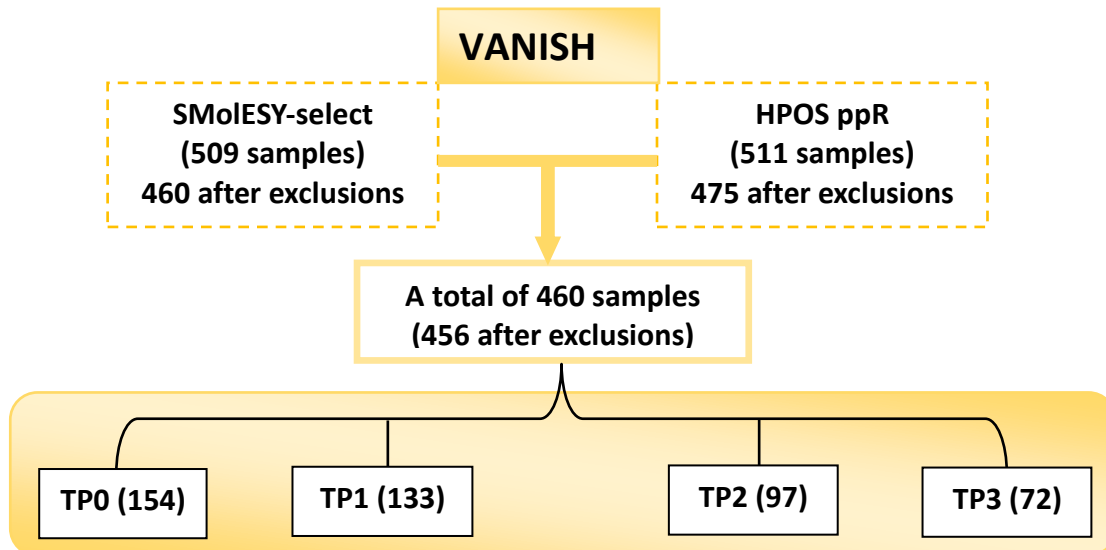


Figure 4.15: Flow chart representing samples included in the trajectory analysis of combined SMolESY-select and HPOS ppR Data from VANISH to determine the metabolic trajectories based on organ failure scores.

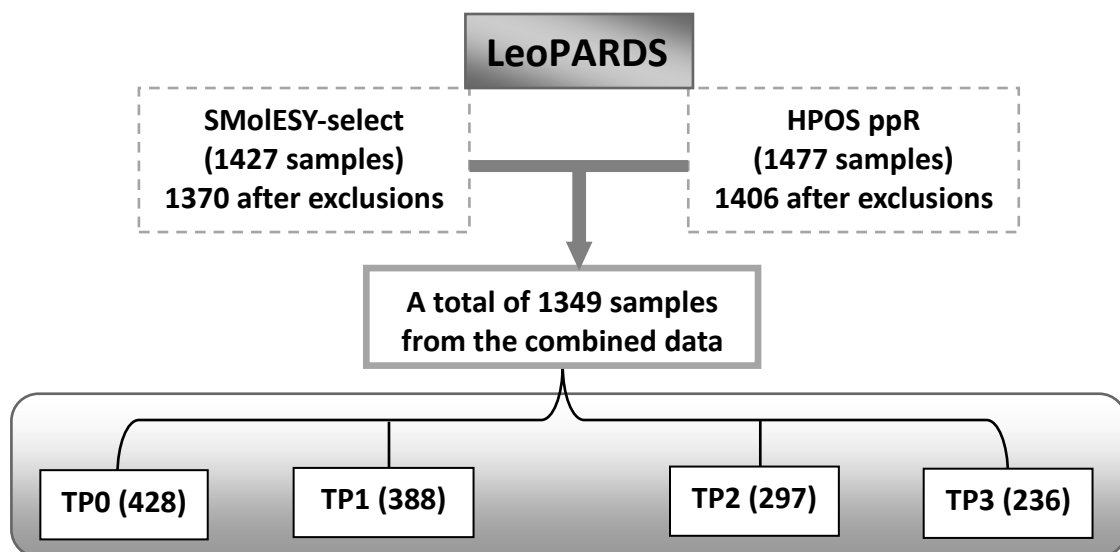


Figure 4.16: Flow chart representing samples included in the trajectory analysis of combined SMolESY-select and HPOS pp Data from LeoPARDS to determine the metabolic trajectories based on organ failure scores.

The results from the LME models of SOFA scores without BMI (figure 4.17) as a confounder revealed that the higher the total SOFA score the higher many of the metabolites (including symmetric/asymmetric dimethylarginine, alanine, phenylalanine, (NMR and HPOS_)creatinine and creatinine, glycine, HPOS_Lysine, N6,N6,N6-trimethyllysine, proline, histidine, urocanate, N1-acetylspermidine, lactic acid, acetic acid, hydroxybutyrylcarnitine, decanoylcarnitine, 1-methyladenosine, N2,N2-dimethylguanosine, 7-methylguanine, choline, 1-methylnicotinamide and its metabolites in patients with sepsis from both cohorts ($p_{Adj} < 0.05$). In LeoPARDS, some metabolites such as arginine, stearoylcarnitine, hypoxanthine decrease as the total SOFA score increases ($p_{Adj} < 0.05$), but these metabolites did not remain significant after B-H correction in VANISH.

Changes in the small molecules linked with the cardio SOFA score (figure 4.17) were observed in both cohorts with five metabolites, 1-methyladenosine, N2,N2-dimethylguanosine, 1-methylnicotinamide, N1-methyl-4-pyridone-3-carboxamide and N1-methyl-2-pyridone-5-carboxamide increasing as the cardio SOFA score increases ($p_{Adj} < 0.05$) whilst three metabolites including arginine, stearoylcarnitine and palmitoylcarnitine decreased in patients who progress towards cardiac failure in both cohorts ($p_{Adj} < 0.05$).

As the coagulation SOFA score increases, 11 small molecules including symmetric/asymmetric dimethylarginine, phenylalanine, NMR_Creatine, glycine, HPOS_Lysine, N6,N6,N6-trimethyllysine, proline, histidine, lactic acid, 7-methylguanine, 1-methylnicotinamide also increase in both cohorts ($p_{Adj} < 0.05$) whilst the levels of stearoylcarnitine, tetradecenoylcarnitine, α -glycerophosphocholine and hypoxanthine decrease with the coagulation SOFA score in both cohorts ($p_{Adj} < 0.05$) (figure 4.17).

The results from this analysis also revealed that higher the renal SOFA score the higher the levels of 26 small molecules ($p_{Adj} < 0.05$) (including symmetric/asymmetric dimethylarginine, phenylalanine, NMR_Creatine, NMR and HPOS_Creatinine, N6,N6,N6-trimethyllysine, N-methyl pipecolic acid, proline, histidine, urocanate, N1-acetylspermidine, lactic acid, acetic acid, hydroxybutyrylcarnitine, decanoylcarnitine, trimethylaminoacetone, pseudouridine, 1-methyladenosine, N2,N2-dimethylguanosine, 7-methylguanine, choline, sucrose, taurine, 1-methylnicotinamide, N1-methyl-4-pyridone-3-carboxamide, N1-methyl-2-pyridone-5-carboxamide, and trigonelline) in both cohorts.

Alterations in small molecules related to respiratory SOFA score were also observed, and it was found that the levels of five small molecules including alanine, histidine, N1-acetylspermidine, 1-methyladenosine, and 1-methylnicotinamide increase as the respiratory SOFA score increases ($p_{Adj} < 0.05$) in both cohorts.

The changes in the small molecules focusing on the Liver SOFA score were also observed and it was found that the levels of 15 metabolites including symmetric/asymmetric dimethylarginine, alanine, phenylalanine, HPOS_Lysine, pipecolate/N-methyl proline, proline, histidine, N1-acetylspermidine, lactic acid, hydroxybutyrylcarnitine, decanoylcarnitine, N2,N2-dimethylguanosine, 7-methylguanine, choline and 1-methylnicotinamide increase as the Liver SOFA score increases ($p_{Adj} < 0.05$) in both cohorts whilst α -glycerophosphocholine decreases with the increase in the Liver SOFA score ($p_{Adj} < 0.05$) in both cohorts.

All changes in the small molecules from LME model with (figure A1.5, see appendix 1.4) and without BMI (figure 4.17) were the same for the metabolites discussed earlier except for some small differences.

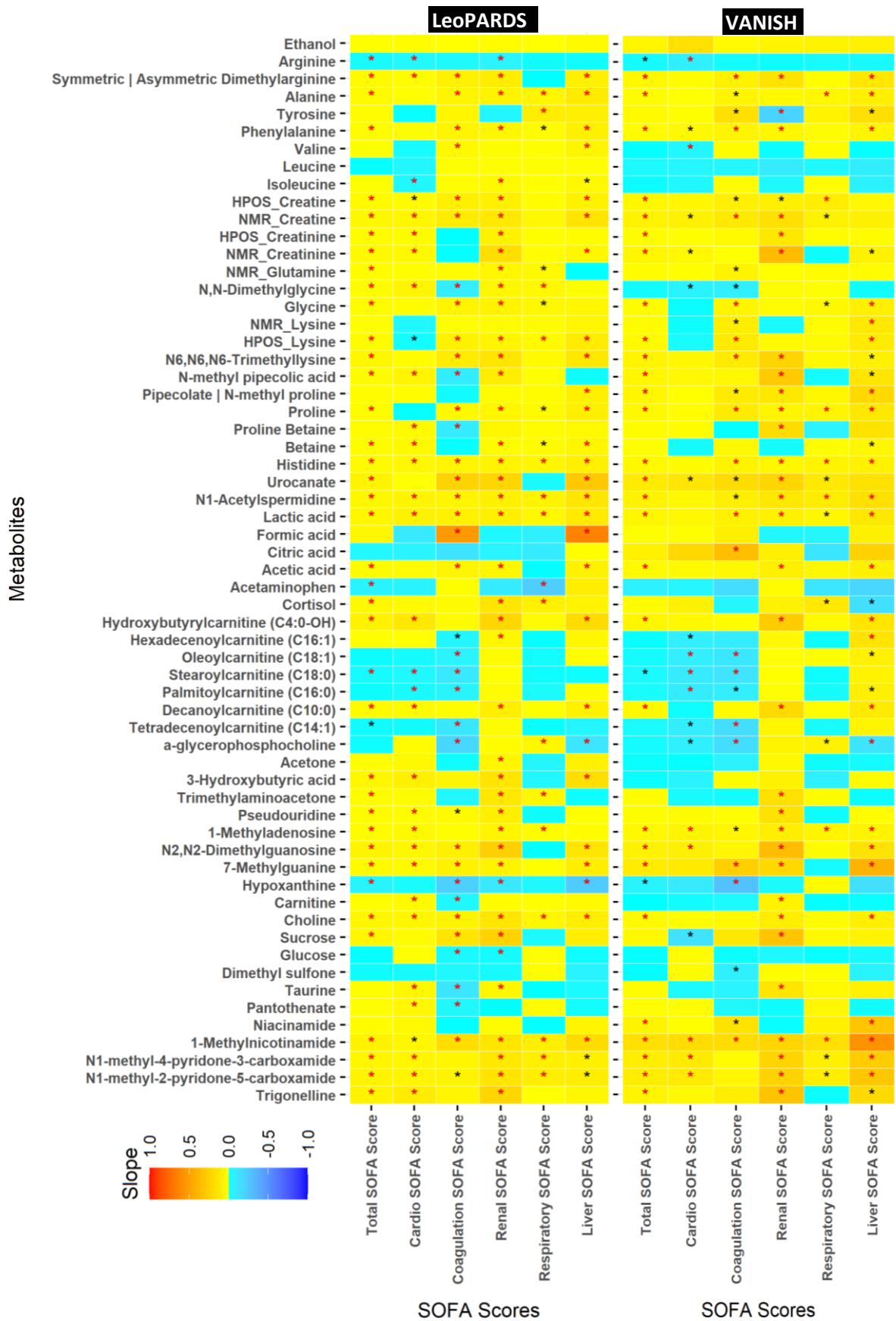


Figure 4.17: The results of LME models based on the SOFA scores for combined SMoIESY-select and HPOS ppR log transformed data with age and sex as confounders for LeoPARDS and VANISH. Identity contrast was performed via contrast coding and the outcomes are shown here. LME model output slope (i.e., change in metabolite levels with SOFA scores) is presented for each SOFA score. The scale for slope ranges from positive to negative values, where positive values indicate higher levels of metabolites when the SOFA score is high whilst negative values indicate lower levels of metabolites when the SOFA score is high and stronger the colour the more the levels of metabolites with higher SOFA scores. Metabolites with slopes which are significantly different from 0 are highlighted in black asterisk whilst those that remained significant after B-H correction are shown in red.

4.5 Discussion

Analytic techniques together with bioinformatic analysis have great potential in helping us to understand sepsis at a biological and molecular level. In this study, metabolomics was used to investigate the metabolic trajectories of septic shock survivors and non-survivors by using NMR and UHPLC-MS methodologies in the two cohorts. We also attempted to validate results from trajectory analysis focusing on outcome using mortality prediction models on the combined metabolomic datasets with the purpose of adding more weight to the similarity of metabolites between the two cohorts. Additionally, this study also looked at the metabolic differences between patients who received specific trial treatments and those who didn't in VANISH and how their metabolic profiles evolve with time and how they differ based on mortality. The findings for this analysis were not validated in LeoPARDS as different treatment was used in the two clinical trials. In this chapter, metabolite changes based on organ failure scores (i.e., SOFA scores) from the two cohorts were also investigated.

Our findings from the trajectory analysis on outcome revealed that most of the small molecule metabolites had the same trend over time in both survivors and non-survivors but there was a lack of significant difference in the overall trajectories of between these two clinical groups in both cohorts. However, the trajectories of some of the metabolites (such as long-chain acylcarnitines (hexadecenoylcarnitine, stearyl carnitine and palmitoylcarnitine), N6,N6,N6-trimethyllysine, purine nucleosides (hypoxanthine and N2,N2-dimethylguanosine, 7-methylguanine), α -glycerophosphocholine and glutamine) were significantly different between survivors and non-survivors, but only in LeoPARDS and these metabolites followed the same trend in VANISH. This reflects higher statistical power in LeoPARDS because of the large sample size in comparison to VANISH.

The metabolic and energetic failure in sepsis patients could lead to increased energy requirements which are fulfilled by increased cellular respiration both in cytoplasm via glycolysis and in mitochondria via TCA cycle (239). Long chain acylcarnitines such as hexadecenoylcarnitine, stearyl carnitine and palmitoylcarnitine decreased over time in patients who went on to die whilst these metabolites increased over time in survivors from both cohorts. These results were also consistent when the EMMs of survivors were compared between day 0 and day 4, and suggest incomplete fatty acid oxidation (i.e., β -oxidation) due to mitochondrial dysfunction (162, 253) in non-survivors with reduced uptake of long chain fatty acids into the mitochondria over time. However, increased levels of these long-chain acyl carnitines in survivors over time might suggest increased uptake of fatty acids into the mitochondria where they can be converted into acetyl-coenzyme A (acetyl-CoA) via β -oxidation which can then enter TCA cycle and generate more energy for survival. Additionally, other short and medium chain acylcarnitines i.e., hydroxybutyrylcarnitine and decanoylcarnitine were significantly elevated at day 0 with respect to day 4 in both survivors and non-survivors which suggests that both groups had enhanced fatty acid oxidation at baseline compared to day4 and at later timepoints there will be lower uptake of fatty acids from cytoplasm into the mitochondria (267) hence lower levels of these carnitines. All these acylcarnitines were significantly higher in non-survivors with respect to survivors on days 0 and 4 indicating increased β -oxidation (162, 253) in patients who went on to die in both cohorts. Higher levels of two acylcarnitines (hydroxybutyrylcarnitine and decanoylcarnitine) were also associated with higher organ failure scores (renal, liver and total SOFA scores) indicating that as the organ dysfunction gets worse or as the patients progress towards organ failure, they will have high levels of these two metabolites. This is supported by evidence that people with liver failure have increased mitochondrial dysfunction and hence enhanced incomplete fatty oxidation (162, 253).

N6,N6,N6-trimethyllysine, a precursor in the biosynthesis of carnitine (268), increased over time in survivors and non-survivors from both cohorts and this increase was steeper in patients who went on to die. This post-translational amino acid was also elevated in patients who went on to die in comparison to those who survive on both days (0 and 4) in both cohorts also consistent with our results from the univariate analysis. These results could

suggest carnitine biosynthesis might be altered in patients who went on to die. This metabolite has not been previously associated with sepsis mortality. Moreover, the levels of N6,N6,N6-trimethyllysine were higher when the organ failure scores (total SOFA, coagulation and renal) were higher indicating patients with increased organ dysfunction might have altered carnitine biosynthesis in septic shock patients. N6,N6,N6-trimethyllysine is also a precursor for the atherogenic metabolite, trimethylamine *N*-oxide (TMAO) and it has also been reported to be a risk predictor of incident cardiovascular disease (269). Increased levels of N6,N6,N6-trimethyllysine in patients with renal failure/dysfunction might indicate increased production of TMAO by the gut microbiota and previously, higher levels of TMAO have been linked with decreased glomerular filtration rate in patients with chronic kidney disease (CKD) (270).

Mitochondrial dysfunction in sepsis patients could also cause a build-up of TCA cycle and glycolysis metabolites including lactate and citric acid (158). These metabolites decreased over time in all patients in both cohorts reflecting reduced glycolysis and TCA cycle activity over time in both groups as some of the energy demands have been already met. Additionally, the levels of lactate were higher in patients who die with respect to those who survive at days 0 and 4 in both cohorts and these results were also supported by other studies (66, 146-149, 157, 158) also in accordance with our previous results from the univariate analysis (chapter 3, section 3.3.3.1). These results suggest that there's an increased stimulation of glycolysis in non-survivors by endogenous epinephrine which leads to an increased generation of pyruvate in the cytoplasm, which is then metabolised in the mitochondria causing increased lactate formation (259) and lactic acidosis (260). Additionally, higher organ failure scores including total SOFA, coagulation, renal and liver were associated with higher lactate levels in patients with sepsis from both cohorts indicating that patients with increased organ dysfunction have higher lactate levels.

Glucose levels are commonly seen to fluctuate in critically ill patients, but we found that trajectories of glucose and sucrose were not significantly different between survivors and non-survivors with no difference between day 0 and day 4 levels. However, we found that glucose was significantly lower in non-survivors with respect to survivors on day 0. Interestingly, sucrose was significantly higher in non-survivors than survivors on day 0. These results suggest that increased glucose is consumed by the cells to generate energy

but not much of the sucrose in the serum is being metabolised to glucose to replace the glucose levels in non-survivors.

The two ketone bodies including acetone and 3-hydroxybutyric acid both decreased over time in survivors and non-survivors in both cohorts. Ketone bodies are formed from the breakdown of free fatty acids and ketogenic amino acids and our results suggest that as the levels of these ketone bodies decrease with time there will be enhanced muscle catabolism with time (242). The breakdown products of protein catabolism such as BCAAs including leucine, isoleucine and valine increased over time in both groups. These results indicate that both survivors and non-survivors have higher protein catabolism at later timepoints, and non-survivors have increased protein breakdown than survivors at baseline and as the disease progresses in non-survivors the levels of the BCAAs get higher than survivors at day 4 in both cohorts. The levels of BCAAs especially valine and isoleucine were also related with organ failure scores.

The two amino acids alanine and glutamine decreased over time in patients who died whilst these metabolites increased over time in patients who survived. Both amino acids were higher in non-survivors than survivors at days 0 and 4 in accordance with other metabolomic studies (66, 147, 149). Our results suggest that sepsis patients who die might have increased muscle catabolism with these amino acids being used as substrates for generating energy to meet the increased energy demand seen in patients with more severe sepsis (239). Additionally, the levels of glutamine were higher in non-survivors at day 0 in comparison to day 4 reflecting that there is increased muscle catabolism at later time to meet with the increased energy demands (239) whilst in non-survivors there was no significant difference in the glutamine levels between day 0 and day 4, but the levels were higher at baseline in both cohorts. The results from the organ failure scores revealed that alanine levels are higher in patients with increased organ dysfunction (based on total SOFA, respiratory and liver) in both cohorts whereas higher levels of glutamine were only associated with higher total SOFA and renal SOFA scores but not across both cohorts.

The aromatic amino acid, phenylalanine is associated with oxidative stress which causes overproduction of hydroxyl radicals converting L-phenylalanine enzymatically in the kidneys to para-tyrosine (234) and the balance between these two amino acids can be disrupted due

to an impaired function of phenylalanine hydroxylase (PAH), which is an enzyme involved in tyrosine synthesis (271). Our results revealed that there was no significant difference between the overall trajectories of phenylalanine for survivors and non-survivors though the levels increased over time in both groups but not across both cohorts. Additionally, the levels of phenylalanine were significantly higher in patients who died with respect to those who survived at both days (0 and 4) as supported by other studies (66, 147, 151, 152, 164-166). Accumulation of this amino acid might also suggest increased muscle proteolysis (235) and this metabolite could be converted into fumarate and enter the TCA cycle (66) to compensate for the increased energy demand in non-surviving patients. Moreover, patients with sepsis had higher phenylalanine levels as the organ dysfunction (based on total SOFA, coagulation, renal and liver in both cohorts) gets worse which might reflect increased muscle breakdown in these patients.

Glycine was found to be significantly higher in survivors at day 4 when compared to baseline whilst in non-survivors there was no significant difference between days 0 and 4. Other studies reported higher concentrations of glycine in patients with sepsis when compared to healthy controls (153, 157) and our results revealed that glycine was significantly higher at day 0 in patients who die than in those who survive which might reflect continuum of the disease. Glycine is a potent antioxidant (236) and our findings of increased levels of glycine might be indicative of increased oxidative stress as well as increased energy demand which might lead to proteolysis to generate amino acid substrates for gluconeogenesis (237) and other processes (238). The levels of glycine also increased with the organ failure scores (including Total SOFA, coagulation and renal) indicating patients who had more dysfunctional coagulation as well as kidney failure tend to have higher levels of this antioxidant.

Histidine, an amino acid which exhibits anti-inflammatory and anti-oxidant roles (272) decreased over time in all patients with a steeper decline in patients who die. The results for the differences between the overall trajectories of the two groups were not significant but the levels of histidine were significantly higher in both survivors and non-survivors on day 0 in comparison to day 4 in both cohorts suggesting increased breakdown of proteins at baseline in both groups (273). Additionally, histidine was higher in patients who go on to die in comparison to those who go on to survive on both days 0 and 4 in both cohorts also in

accordance with findings from Khaliq et al (151). Our results suggest that patients who go on to die might have increased proteolysis in the skeletal muscle (273) to cope with the increased oxidative stress. Additionally, Moreover, the results from the trajectory analysis of CPMG data from VANISH revealed that the post-translational metabolite of histidine, 1-MH also decreased with time in both survivors and non-survivors with a small degree of change in non-survivors. Moreover, higher levels of histidine were also linked with higher organ failure scores including total SOFA, coagulation, renal, respiratory, and liver in patients with septic shock from both cohorts.

Urocanate, which is an intermediate in histidine metabolism increased over time in patients who survived whilst it decreased in patients who survived but not across both cohorts. The levels of urocanate were significantly higher in non-survivors on day 4 in comparison to day 0 in both cohorts whilst urocanate was lower in survivors on day 4 in LeoPARDS with an opposite trend in VANISH. These results reflect that non-survivors had increased histidine metabolism over time in both cohorts to regulate histidine levels whilst patients who survived had decreased histidine metabolism over time but only in LeoPARDS as the levels were not as high as they were at the baseline. Additionally, urocanate was higher in patients who died in comparison to those who survived on days 0 and 4, in both cohorts, but the results were only significant in LeoPARDS. Moreover, higher organ failure scores including total SOFA and renal SOFA were associated with higher urocanate indicating increased histidine metabolism in patients with increased organ dysfunction.

The trajectories of NMR_Creatine, NMR and HPOS_Creatinine showed a less steep decline in non-survivors in comparison to survivors. However, NMR and HPOS_Creatinine were significantly higher at baseline in both survivors and non-survivors in comparison to day 4 in both cohorts whereas NMR_Creatine was only significantly higher in survivors at baseline in comparison to day 4 and this might be a sign of muscular protein catabolic state (274) and renal failure (275, 276). The degree of change for creatinine from day 4 to baseline in patients who survive was higher than the patients who go on to die. Additionally, creatinine (both from NMR and HPOS) was significantly higher in non-survivors in relation to patients who survive at both days (i.e., day 0 and day 4) whilst NMR_Creatine was significant higher in non-survivors than survivors only at day 4 in LeoPARDS. These results indicate both liver and kidney dysfunction as well as enhanced breakdown of proteins (244, 245) in patients

who die also reflected by the results of LME models of SOFA scores which showed that the levels of these two amino acids increase as both renal and liver dysfunction gets worse in sepsis patients from both cohorts.

Trajectory analysis of CPMG data from VANISH also revealed that the degree of change in the levels of N-acetylglycoproteins i.e., GlycA and GlycB was smaller in non-survivors with respect to survivors, although increased over time in both groups. GlycA is a biomarker of systemic inflammation and it is highly prognostic of morbidity and mortality from various range of diseases including chronic inflammatory diseases (such as sepsis, pancreatitis, rheumatoid arthritis), cardiovascular diseases, certain cancers, type II diabetes, renal failure and liver diseases (277-279). Surprisingly, GlycA and GlycB were not found to be discriminant metabolites in my previous work looking at static timepoint levels suggesting that its dynamics are more important than individual levels.

Purines play several roles such as synthesis of nucleotides which are building blocks for deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) modulation of signal transduction and energy metabolism as well as exhibiting crucial roles in the normal functioning of neurotransmission, platelets and muscles (248). Mostly, all the methylated forms of purines, pyrimidines and modified ribonucleoside originate from RNA mainly transfer RNA (tRNA) (280). Alterations in the trajectories of purine metabolites related to oxidative stress such as hypoxanthine and N²,N²-dimethylguanosine were found in surviving and non-surviving patients. These two purine nucleosides increased over time in non-survivors and decreased over time in survivors indicating increased purine metabolism over time in patients who went on to die whilst decreased purine metabolism over time in patients who went on to survive. The levels of N²,N²-dimethylguanosine were higher in non-survivors than survivors on both days 0 and 4 in both cohorts, also in accordance with other studies (145, 146) and these results reflect increased oxidative stress which leads to an enhanced metabolism of tRNA which results in the release of this nucleoside (145) in the cytoplasm. Additionally, N²,N²-dimethylguanosine was higher when the organ dysfunction was higher in patients with sepsis based on SOFA scores (cardiac, renal, liver, and total SOFA) in both cohorts which reflects increased oxidative stress in patients with higher degree of organ dysfunction.

Hypoxanthine, a purine base also associated with ROS which upon oxidation by xanthine oxidase leads to uric acid, an end product of purine metabolism (248) was found to be lower in patients who go on to die in relation to those who survive on days 0 and 4 in both cohorts. Our results suggest that non-survivors might have increased conversion of hypoxanthine into uric acid hence lower levels of hypoxanthine in non-survivors in comparison to survivors. Additionally, our results from LME models with organ failure scores revealed that higher the renal, liver, coagulation and total SOFA score in patients with sepsis the lower the levels of hypoxanthine in both cohorts and these results also relate with our findings from the LME models of mortality.

A modified purine nucleoside, 7-methylguanine is released from the degradation of nucleic acids and it is involved in the competitive inhibition of poly-ADP-ribose polymerases (PARPs) including PARP1 and PARP2 which are DNA repair enzymes (281). 7-methylguanine is also associated with oxidative stress and decreased over time in both survivors and non-survivors and this metabolite showed a steeper decline in patients who survive than those who go on to die but not across both cohorts. The levels of this metabolite were also significantly higher in non-survivors at baseline in comparison to day 4 reflecting increased degradation of nucleic acids due to increased oxidative stress at the start of the septic episode. Moreover, this metabolite was significantly higher in patients who go on to die in comparison to those who survive at both days (0 and 4), but not across both cohorts, also reflecting enhanced degradation of nucleic acids in non-survivors.

Pseudouridine is a modified pyrimidine nucleoside of uridine which is released after RNA metabolism due to increased oxidative stress. This pyrimidine metabolite declines over time in both survivors and non-survivors but with a steeper decline in survivors. Additionally, pyrimidine was also significantly higher at day 4 in patients who die in comparison to those who survive and these results were also in accordance with the previous findings (145). Our results suggest enhanced tRNA metabolism in patients who die and might also hint that non-survivors have increased kidney dysfunction or even renal failure in comparison to survivors (280, 282) which is also reflected in our results where higher renal SOFA score was associated with higher pseudouridine.

Post-translationally modified forms of arginine, SDMA/ADMA (283), which are markers of oxidative stress increased with time in both survivors and non-survivors. The levels of this metabolite were significantly higher in survivors at baseline in comparison to day 4 suggesting increased NO at baseline. Additionally, these metabolites were also higher in patients who die in comparison to those who survive at both days (0 and 4), and these were also in accordance with other studies (154, 155). As SDMA/ADMA are competitive inhibitors of NO synthase (246) therefore increased levels of these markers are probably present to counteract increased NO in the serum of patients who die. SDMA/ADMA are also uremic toxins as well as risk indicators for cardiovascular disease (CVD) (283). Our results revealed the patients with higher organ failure SOFA scores (total SOFA, coagulation, renal and liver) had higher levels of SDMA/ADMA in both cohorts, supporting these previous findings.

Arginine, a metabolite of the urea cycle has crucial roles in the synthesis of creatine, SDMA/ADMA, polyamines and nitric oxide (NO) (155, 247) and this amino acid increased over time in both survivors and non-survivors in both cohorts reflecting upregulation of urea cycle over time or it might also suggest decreased conversion of arginine into SDMA/ADMA as the initial metabolic insult has been dealt. Arginine was higher in patients who survive in comparison to those who go on to die at day 0 in both cohorts also supported by our univariate results (chapter 3, section 3.3.4) and other metabolomic studies (159, 165). These results suggest that there is a downregulation of urea cycle in patients who went on to die or increased production of SDMA/ADMA to counteract increased NO in these patients. Additionally, arginine was lower when the cardio and renal SOFA scores were higher in septic shock patients from both cohorts indicating that patients with increased cardiac and renal dysfunction will have reduced levels of arginine probably because of the reduced arginine synthesis in the kidneys due to renal dysfunction (284). The amino acid lysine is involved in regulating the production of NO (236) and increased over time in both survivors and non-survivors and the increase was steeper in patients who went on to die in both cohorts. These results suggest there is an upregulation of this pathway over time in both clinical groups.

Taurine, an antioxidant sulphur containing amino acid which also exhibits anti-inflammatory, antiatherogenic effects as well as modulation of intracellular calcium concentration and provides protection in lung injury, cardiovascular diseases, and acute

nephrotoxicity (285-289). The overall trajectories of taurine for survivors and non-survivors were not significantly different but taurine decreased over time in both survivors and non-survivors and the decline was steeper in patients who go on to die in both cohorts. Our results suggest that there might be an increased oxidative stress, systemic inflammation, muscle, and organ dysfunction at baseline as recently taurine has been reported to provide protection against systemic inflammation, muscle dysfunction as well as from injuries in the multiple organs including (lungs and kidneys) in septic mice (288, 290-292). Additionally, taurine levels in patients who die were higher than survivors at day 0 but the results were not significant across both cohorts also in accordance with one metabolomic study (164). In another study taurine was reduced in non-survivors but only on days 10 and 14, later than samples were collected in our study, (165) which also fits well with our data where taurine levels were lower in non-survivors than survivors at day 4, but the results were not significant. Recently, the protection mechanism of taurine has been investigated by Ma and co-workers, and they revealed that administering taurine in LPS-challenged neutrophils and monocytes from mice reduced the expression of pro-inflammatory cytokines, TNF- α and IL-1 β in vitro and also scavenged the ROS which resulted in decreased production of neutrophil extracellular traps (288). In addition, it also provided protection by decreasing neutrophil infiltration and synthesis of TNF- α which prevented septic mice from dying as well as it led to improvement in organ injuries (i.e., in the lung, liver, and kidney) (288). Our results from the LME models of organ failure scores revealed that higher the renal SOFA score higher the taurine levels in patients with sepsis from both cohorts which might related to increased oxidative stress whilst higher cardio SOFA score was associated with lower taurine levels but only in LeoPARDS.

Proline, a substrate for the synthesis of superoxide radicals (249) increased over time in patients who go on to die and those who go on to survive in both cohorts and the increase was steeper in survivors in both cohorts. The overall trajectory of proline for the two groups was not significantly different between the two groups but the levels of proline were significantly higher in both groups on day 4 in comparison to day 0. Proline was also significantly higher in patients who went on to die with respect to those who went on to survive at both days but not across both cohorts also supported by the findings of other studies (147, 149, 154). Our findings indicate that non-survivors might have increased

oxidative damage due to aggravated stress during inflammation (249) which might cause damage to kidneys and liver (160). The results from the LME models of organ failure scores revealed that patients with septic shock had higher proline levels with increased organ dysfunction in both cohorts (based on SOFA scores including total SOFA, coagulation, renal and liver). The results of LME models for mortality revealed that proline betaine (i.e., stachydrine) decreased over time in both survivors and non-survivors in both cohorts and the decline was steeper in patients who go on to die in both cohorts. The overall trajectories were not significantly different between the two groups, but the levels of proline were significantly higher in survivors and non-survivors on day 0 in comparison to day 4 in both cohorts. Proline betaine has strong antifibrotic and anti-inflammatory properties as well as it also exhibits cardioprotective, vasoprotective, neuroprotective and anticancer roles (293).

NAD⁺ plays important roles in the regulation of cellular and metabolic signaling pathways by acting as a cofactor (in glycolysis, fatty acid oxidation, and TCA cycle) and a coenzyme in redox reactions as well as playing crucial roles in aging, metabolism, mitochondrial function, immunological pathways, oxidative stress, gene expression, and apoptosis (294). Metabolites related to NAD⁺ metabolism including MNA and its metabolites, (2-Py and 4-Py) increased over time in both survivors and non-survivors in both cohorts. Only MNA was significantly higher in survivors at day 4 in relation to day 0 but not across both cohorts indicating that patients have increased NAD⁺ degradation via the activation of PARPs triggered by the DNA damage under conditions of oxidative stress (294). Additionally, MNA, 2-Py and 4-Py were significantly higher in patients who go on to die than those who go on to survive at both days (0 and 4) in both cohorts and these results suggest that patients who die might have an enhanced NAD⁺ degradation (255) via increased PARPs activation, because of the increased DNA damage under stress which leads to the accumulation of these catabolic products (256, 294). These results were also supported by Seymour and co-workers who reported higher levels of 2-Py in non-survivors (145). All three small molecules (including MNA, 2-Py and 4-Py) were higher in patients with higher organ failure scores (including total SOFA, cardio, renal, respiratory and liver).

N1-acetylspermidine decreased over time in both survivors and non-survivors in both cohorts and the levels of N1-acetylspermidine were higher in patients who go on to die than those who go on to survive on days 0 and 4 in both cohorts. These results suggest that

increased concentrations of this polyamine in non-survivors might activate apoptosis of macrophages in the lungs probably due the increased ROS production via polyamine metabolism (257) and over time there is reduced polyamine metabolism as the initial metabolic insult has passed. Higher levels of N1-acetylspermidine were also associated with higher organ failure scores (including total SOFA, renal, respiratory and liver).

α -glycerophosphocholine, which is an abundant renal medullary organic osmolyte decreased over time in patients who go on to die whilst increasing in patients who go on to survive. Moreover, the levels of α -glycerophosphocholine were also elevated in patients who die in comparison to those who go on to survive indicating patients who die might have higher levels of α -glycerophosphocholine probably to provide protection to renal medullary cells against elevated levels of interstitial sodium chloride and urea (295).

Choline is an essential nutrient (265) with antioxidant and antiapoptotic roles (266) and it increased over time in both survivors and non-survivors. These trajectories were not significantly different between the two groups in both cohorts, but the choline levels were significantly higher in survivors at day 4 in comparison to day 0 but only in LeoPARDS whilst in VANISH the results were not significant, but the trend was the same. The levels of choline were also significantly higher in patients who go on to die in comparison to those who survive on day 0 in both cohorts whilst on day 4, non-survivors also had significantly elevated levels of choline in comparison to survivors but only in LeoPARDS and these are also in accordance with the recent findings from Kumar and co-workers (229). Choline reduces the levels of pro-inflammatory cytokines including TNF- α , IL-1, IL-6, PGE2 and NO and provides protection to the cell membrane from oxidative damage during sepsis (266). Our results showed higher levels of choline over time in both groups suggesting increased oxidative stress and increased choline might cause reduction in the pro-inflammatory cytokines as well as NO in septic patients over time. Septic patients with higher SOFA scores including (liver, renal and total SOFA) had higher levels of choline in both cohorts indicating increased metabolism of glycerophosphocholine to choline (295).

The cortisol levels decreased over time in both survivors and non-survivors. There was no significant difference between the overall trajectories of cortisol for survivors and non-survivors, but the levels of cortisol were significantly higher in both survivors and non-

survivors on day 0 in comparison to day 4. Additionally, cortisol was higher in patients who go on to die with respect to those who survive on both days (0 and 4) in accordance with other metabolomic studies (145, 146). Our results suggest that patients who went on to die might have increased production of cortisol via the activation of the hypothalamopituitary–adrenal axis due to aggravated stress response and it will also enhance the activity of catecholamines (e.g., noradrenaline)(254). Cortisol exerts its effects by indirectly suppressing the expression of inflammatory cytokines, such as IL-1 β , TNF, and IL-6, as well as attenuating the release of ACTH and CRH by the anterior pituitary gland (90, 254). The results from the LME models based on organ failure scores revealed that patients with higher organ dysfunction (based on SOFA scores including total SOFA, renal and respiratory) had higher cortisol levels in septic shock patients from LeOPARDS whilst VANISH also followed the same pattern but the results were not significant.

The results of LME models based on treatment in VANISH revealed that there were not any significant differences based on neither study drug 1 (vasopressin vs noradrenaline) nor study drug2 (hydrocortisone vs placebo) in the trajectory of metabolites except for cortisol. The results from the drug2 showed that only cortisol was found to be significantly higher in patients with hydrocortisone than those on placebo. This suggests conversion of hydrocortisone into cortisol in the liver which can bind to glucocorticoid receptors and will exert many different effects such as causing reduced vasodilation, vascular permeability and leukocyte migration as well as inhibition of phospholipase A2 resulting in reduced production of arachidonic acid (89).

4.6 Conclusion

To conclude, we found that the overall trajectories of small molecules for septic shock survivors and non-survivors were similar reflecting that the two groups tend to move in the same direction despite having baseline differences. Pathways related to energy, amino acid and nucleotide metabolism were disrupted over time. These findings were consistent across the two cohorts, but the mortality prediction models did not perform well probably due to the less commonality in the metabolites incorporated into these models in the two cohorts or due to the metabolic trajectories being similar for the two clinical groups. This study also revealed that there weren't any significant metabolite changes in response to trial drugs except for the elevation of cortisol in patients with septic shock who received

hydrocortisone with respect to those who received placebo. Additionally, this study also revealed that the metabolic profiles of patients with septic shock also vary with organ failure and mostly patients with higher organ dysfunction were associated with higher levels of metabolites. All these findings bring us a step closer in understanding the underlying pathological mechanisms in sepsis patients and it will allow us to monitor the clinical progress of patients over time which is clinically beneficial.

Chapter 5 – Baseline Metabolic Differences in Lipids and Lipoproteins

5.1 Introduction

It is well known that fatty acid, lipid, and lipoprotein metabolism is disturbed in sepsis. Alterations in lipid and lipoprotein profiles in patients with sepsis have been previously investigated using metabolomics and these studies have allowed us to gain a better understanding of sepsis associated mortality (144). Metabolomic studies have shown that patients who go on to die have reduced levels of several lipid classes such as glycerophospholipids (including PCs, LPCs, LPG (22:0), PIs (including PI (18:0/22:5), and PI (18:0/22:6)), phosphatidylethanolamines (PEs), lysophosphatidylethanolamines (LPEs) (including LPE (18:0), LPE(18:2), LPE(20:3), LPE(20:4)), sphingolipids (including SMs such as SM-OH (C14:1, C16:1, C22:2, C23:0) and SM(C16:0, C18:0, C18:1, C20:2, C22:3, C24:0, C26:0) (151)) as well as several lipoproteins including high density lipoprotein (HDL), low density lipoprotein (LDL) and apolipoprotein-A1 (Apo-A1), triglycerides (TGs), cholesterol and HDL cholesterol whilst acylcarnitines (C3, C4, C5, C5-OH, C5:1, C6, C10, C12), glycerophosphocholine, fatty acids (FAs) (including 10-hepatodecenoic acid), glycerophospholipids (phosphatidylglycerols (PGs) (including PG 22:2 (13Z, 16Z)/0:0) and lysophosphatidylinositol (LPI) were accumulated in non-survivors than survivors (145, 148, 150, 163). However, one study showed conflicting results in comparison to other studies and showed that LPC (14:0) was elevated in patients who went on to die than those who didn't (147) whilst acylcarnitines (C16, C18) and FAs including (palmitic acid, stearic acid (147) were reduced in non-survivors with respect to survivors (147). These inconsistencies might be due to the limitations of the studies as the sample sizes were relatively small except for Wang et al study (158) (N = 188; 134 survivors & 54 non-survivors) and of those, only three studies (145, 146, 148) validated their findings. (144)

As previous metabolomic studies conducted on lipids and lipoproteins were relatively small and lacked validation our focus was to perform in depth analysis using larger cohorts to understand the serum lipid and lipoprotein metabolome fingerprints of patients with septic shock and see how they differ based on survival. The present study uses metabolites measured on two different platforms, NMR and UHPLC-MS to investigate the metabolic differences in lipids and lipoproteins between survivors and non-survivors of septic shock in serum samples taken from the VANISH (92) and LeoPARDS trials (88) with the purpose of understanding the molecular biology of sepsis to help understand the trajectories of lipids

and lipoproteins in the later analysis. In this study, metabolic phenotypes related to patient survival at day 29 will be explored for both cohorts (VANISH and LeoPARDS). This will be taken a step further where the findings from one cohort will be validated in the other cohort and vice versa. To date this type of validation has not been previously done in other metabolic comparisons of survivors and non-survivors.

5.2 Aims

The main purpose of this study was to use metabolomic techniques to determine metabolic differences in sepsis patients based on mortality and validate the findings.

The aims of this chapter are as follows:

- 1) To determine if there are baseline differences in the metabolic profiles of survivors and non-survivors at ICU admission and at later sampling times.
- 2) To validate whether the metabolic differences between the two clinical groups are consistent across the two independent cohorts of sepsis patients studied in this project.
- 3) To evaluate whether the models generated using metabolic signatures of septic shock survivors and non-survivors from one cohort can predict mortality in another cohort.

5.3 Statistical analysis methods

The methods for all the analyses are listed in chapter 2, section 2.3 (sub-section 2.3.3).

5.4 Results

5.4.1 Mortality analysis of targeted data

5.4.1.1 NMR targeted data (BI LISA)

Univariate analysis of 110 lipoprotein subfractions from BI LISA was performed for all timepoints on a total of 475 serum samples from VANISH and 1405 samples from LeoPARDS, figure 5.1, and figure 5.2. Samples were excluded from both cohorts as previously mentioned in chapter 2, section 2.2.1.3. An additional 22 samples from VANISH (18 samples had no sample timings recorded and 4 samples belonged to a patient whose samples were

collected after several days of receiving the study drug) and 10 samples with no sample timings available from LeoPARDS were excluded from this analysis.

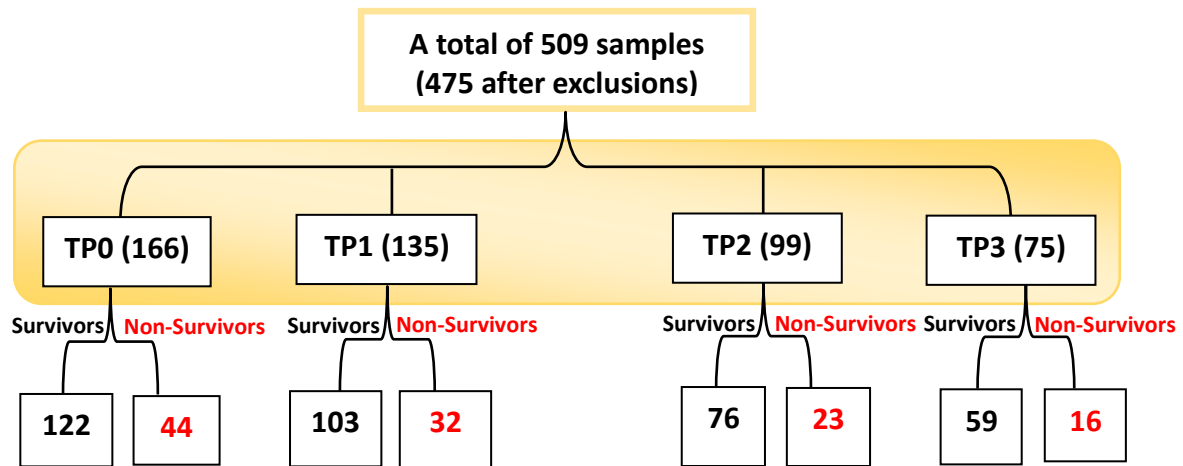


Figure 5.1: Flow chart for the samples included in the univariate analysis of BI LISA from VANISH.

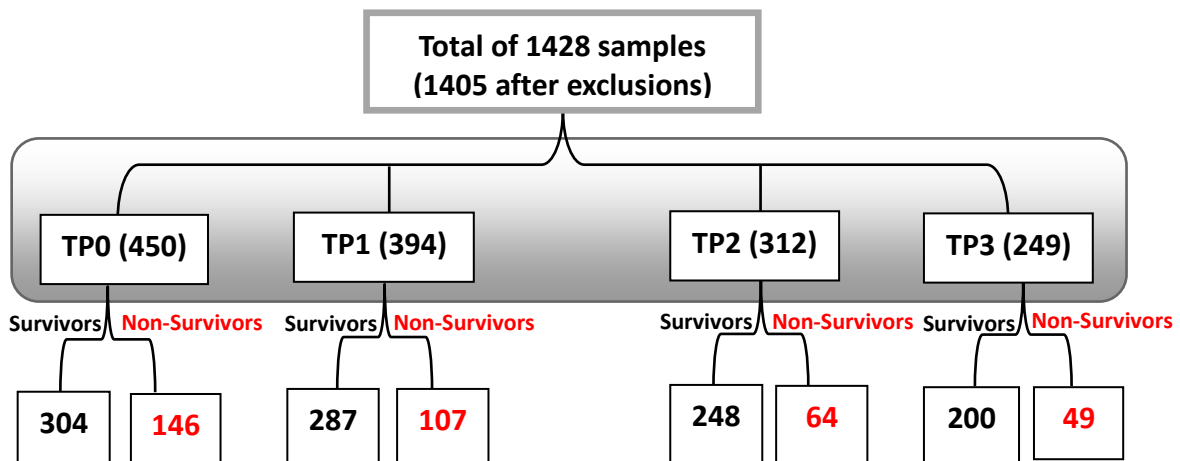


Figure 5.2: Flow chart for the samples included in the univariate analysis of BI LISA from LeoPARDS.

A summary of univariate results for all timepoints in both cohorts is presented in tables 5.1, 5.2, 5.3 and 5.4, differentiating survivors from non-survivors at all three timepoints (TP0-TP3). Overall, HDL apolipoprotein-A2 (Apo-A2) subtraction 2 and 3, HDL phospholipid subfraction 3 and HDL triglyceride subfraction 4 were significantly lower in non-survivors with respect to survivors after 24 h of admission in both cohorts ($p < 0.05$). However, only HDL Apo-A2 subfraction 2 and 3 remained significant after B-H correction and that only in VANISH ($p_{Adj} < 0.05$) (table 5.2).

Additionally, other lipoproteins such as total plasma Apo-A2, HDL Apo-A2, subfractions 4, very-low density lipoprotein (VLDL) apolipoprotein-B100 (Apo-B100), VLDL cholesterol and its subfractions 3 & 4, VLDL free cholesterol and its subfraction 4, VLDL phospholipid subfractions 3 and 4, HDL phospholipid subfraction 4, VLDL triglyceride subfraction 4 and VLDL particle number were significantly lower in non-survivors with respect to survivors at TP1 in VANISH and most of these lipoproteins except for few also had the same pattern in LeoPARDS, but the results were not significant after B-H correction.

At TP2, only HDL Apo-A2 and its subfraction 2 were significantly reduced in patients who went on to die in comparison to those who survive in LeoPARDS ($p_{Adj} < 0.05$) and the pattern for this metabolite was also the same in the alternate cohort (table 5.3).

Table 5.1: Welch two sample *t*-test results of NMR BI LISA data for the VANISH and LeoPARDS cohorts at TP0. Adjusted p-values were calculated with B-H correction. Adjusted p-values (p_{Adj}) and un-adjusted p-values (p) <0.05 are highlighted in light green; Mean intensity values are highlighted for survivors and non-survivors (Yellow represents higher mean value; peach represents lower mean value). Metabolites significant in the two cohorts are highlighted in different colours (green = In both cohorts; red = Only in VANISH; blue = Only in LeoPARDS). Abbreviations: A1 = Apo-A1; A2 = Apo-A2; HD = High density lipoprotein (HDL); HDA1 = HDL Apo A1; H2A2 = HDL Apo-A2 subfraction 2; TPA1 = Total Plasma A1, TG = Triglyceride; H4TG = HDL Triglyceride subfraction 4; PL = Phospholipid; V2PL = VLDL Phospholipid subfraction 3; HDPL = HDLPL; V2PL = VLDL Phospholipid subfraction 3; H3PL = HDL Phospholipid subfraction 3.

Metabolite	VANISH _{TP0} (N = 166, n = 122 & 44)				LeoPARDS _{TP0} (N = 450, n = 304 & 146)			
	Mean concentration (mg/dL)		p	p_{Adj}	Mean concentration (mg/dL)		p	p_{Adj}
	Survivors	Non-survivors			Survivors	Non-survivors		
H2A2	3.25	2.70	0.015	0.728	2.10	2.03	0.757	0.877
HDA1	95.43	75.35	0.036	0.728	78.41	81.64	0.608	0.812
TPA1	93.03	74.76	0.021	0.728	77.87	82.00	0.400	0.686
H2A1	12.68	11.55	0.323	0.728	10.18	8.69	6.42E-03	0.353
H3PL	9.65	9.00	0.418	0.728	7.75	6.43	7.72E-04	0.085
H4TG	2.85	2.80	0.873	0.963	3.12	2.75	0.023	0.593
HDPL	49.43	44.03	0.192	0.728	38.64	33.85	0.017	0.593
V2PL	3.75	3.34	0.441	0.746	2.70	2.20	0.036	0.593

Table 5.2: Welch two sample *t*-test results of NMR BI LISA data for the VANISH and LeoPARDS cohorts at TP1. Adjusted p-values were calculated with B-H correction. Adjusted p-values (p_{Adj}) and un-adjusted p-values (p) <0.05 are highlighted in light green; Mean intensity values are highlighted for survivors and non-survivors (Yellow represents higher mean value; peach represents lower mean value). Metabolites significant in the two cohorts are highlighted in different colours (green = In both cohorts; red = Only in VANISH; blue = Only in LeoPARDS). Abbreviations: CH = Cholesterol; FC = Free Cholesterol; PL = Phospholipid; TG = Triglycerides; A1 = Apo-A1; A2 = Apo-A2; AB = Apo-B100; HD = High density lipoprotein (HDL); VL = Very low density lipoprotein (VLDL); ID = Intermediate density lipoprotein (IDL); LD = Low density lipoprotein; TPCH = Total plasma Cholesterol; TPA1 = Total Plasma A1; TPA2 = Total plasma A2; IDAB = IDLAB; VLCH = VLDL Cholesterol; HDCH = HDL Cholesterol; IDAB = IDL Apo-B100; IDCH = IDL Cholesterol; IDFC = IDL Free Cholesterol; IDPN = IDL Particle Number; LDTG = LDL Triglyceride; H1-H4A2 = HDL subfractions 1-4 of Apo-A2; H4A1 = HDL Apo A1 subfraction 4; H1-4TG = HDL subfractions 1-4 of TG; H4CH = HDL cholesterol subfraction 4; H2-H4PL = HDL Phospholipid subfractions 2-4; VLPN = VLDL Particle Number; VLFC = VLDL FC; V3-V5FC = VLDL Free Cholesterol subfractions 3-5; V3-V5TG = VLDL Triglyceride subfractions 3-5; V2-V5PL = VLDL Phospholipid subfractions 2-5; VLAB= VLDL Apo-B100; V3-V5CH = VLDL Cholesterol subfractions 3-5; L1FC & L4-L6FC = LDL Free Cholesterol subfractions 1 & 4-6; L1CH & L4-L5CH = LDL Cholesterol subfractions 1 & 4-5; L1 & L5PL = LDL Phospholipid subfractions 1 & 5; LDTG = LDL Triglyceride; L1 – L3TG = LDL Triglyceride subfractions 1 -3; L1-L4PN = LDL PN subfractions 1 & 4. Particle numbers are reported in nmol/L and are highlighted in asterisk.

Metabolite	VANISH _{TP1} (N = 135, n = 103 & 32)				LeoPARDS _{TP1} (N = 394, n = 287 & 107)			
	Mean concentration (mg/dL) or (nmol/L)*		p	p_{Adj}	Mean concentration (mg/dL) or (nmol/L)*		p	p_{Adj}
	Survivors	Non-survivors			Survivors	Non-survivors		
H2A2	3.35	2.54	1.49E-03	0.023	2.08	1.64	4.93E-03	0.069
H3A2	5.11	3.74	1.14E-03	0.023	3.76	3.07	0.013	0.078
H3PL	10.46	8.37	0.022	0.092	8.24	6.93	6.39E-03	0.069
H4A1	31.09	23.30	0.031	0.110	30.37	39.85	0.012	0.078
H4CH	7.29	4.35	9.67E-03	0.056	7.29	9.30	0.040	0.145
H4TG	3.78	2.82	1.97E-03	0.026	3.53	3.06	0.030	0.135
HDA2	21.17	14.98	2.54E-04	0.014	16.14	16.80	0.599	0.725
H4A2	8.86	5.15	1.33E-03	0.023	8.15	9.49	0.154	0.308
HDCH	26.32	19.59	0.039	0.126	22.35	25.49	0.346	0.541
IDAB	10.42	8.34	0.032	0.110	7.85	6.67	0.060	0.158
IDCH	22.92	17.69	0.025	0.093	17.05	16.00	0.536	0.719
IDFC	6.47	4.96	0.038	0.126	4.75	4.24	0.356	0.543
IDPL	6.50	4.05	0.016	0.074	3.25	3.16	0.900	0.943
H4PL	13.04	9.34	6.11E-03	0.040	12.38	12.77	0.670	0.760

IDPN*	189.49	151.67	0.032	0.110	142.76	121.35	0.060	0.158
TPA2	19.09	12.79	1.28E-04	0.014	13.16	13.85	0.534	0.719
TPCH	109.09	86.04	0.011	0.058	80.26	87.85	0.215	0.408
V2PL	4.16	2.77	0.024	0.093	2.40	2.40	0.999	0.999
V3CH	6.17	3.85	6.15E-04	0.023	3.44	3.44	0.993	0.999
V3FC	3.43	2.48	0.024	0.093	2.41	2.53	0.650	0.755
V3PL	6.96	4.67	5.14E-03	0.040	4.06	3.77	0.580	0.722
V3TG	21.66	14.99	0.012	0.062	13.82	14.12	0.854	0.913
V4CH	10.52	6.61	9.98E-04	0.023	6.98	5.71	0.066	0.158
V4FC	5.62	3.87	4.03E-03	0.037	3.84	3.46	0.329	0.531
V4PL	10.47	7.04	2.68E-03	0.029	7.16	6.13	0.151	0.307
V4TG	22.93	15.33	3.03E-03	0.030	15.92	14.64	0.455	0.641
V5CH	2.81	1.92	0.011	0.058	2.44	2.23	0.360	0.543
V5FC	1.35	0.86	0.046	0.144	1.92	2.09	0.545	0.722
V5PL	2.99	2.08	0.015	0.074	2.76	2.63	0.587	0.722
V5TG	5.59	4.35	0.018	0.080	5.44	5.34	0.739	0.821
VLAB	18.55	14.14	5.93E-03	0.040	15.10	14.20	0.440	0.629
VLCH	32.24	22.22	2.09E-03	0.026	24.11	24.16	0.983	0.999
VLFC	14.80	9.93	6.24E-03	0.040	13.16	14.44	0.333	0.531
VLPL	29.43	18.81	9.49E-03	0.056	25.23	26.07	0.763	0.836
VLPN*	337.35	257.10	5.92E-03	0.040	274.53	258.16	0.440	0.629
H1A2	2.46	1.90	0.103	0.290	1.42	1.07	0.019	0.099
H2A1	12.32	10.36	0.139	0.368	9.58	7.74	1.53E-03	0.069
H2PL	10.66	10.30	0.787	0.929	8.01	6.44	4.47E-03	0.069
HDTG	17.24	15.85	0.430	0.719	14.36	11.73	1.34E-03	0.069
H1TG	6.80	6.93	0.910	0.948	5.71	4.61	0.010	0.075
H2TG	3.54	3.44	0.861	0.929	2.77	2.34	0.039	0.145
H3TG	3.37	3.13	0.600	0.815	2.95	2.48	0.025	0.121

H3FC	0.91	0.55	0.152	0.370	0.54	1.01	0.014	0.079
H4FC	1.73	1.01	0.075	0.223	1.54	2.53	6.49E-03	0.069
HDFC	6.52	7.99	0.543	0.777	6.50	9.73	9.32E-03	0.075
L1AB	15.93	14.95	0.634	0.831	14.61	11.63	6.05E-03	0.069
L1CH	20.66	18.86	0.572	0.796	18.00	13.71	7.56E-03	0.069
L1FC	6.30	5.89	0.667	0.864	5.19	4.11	0.021	0.105
L1PL	14.44	13.49	0.620	0.831	12.79	9.85	3.39E-03	0.069
L1PN*	289.56	271.85	0.634	0.831	265.68	211.54	6.06E-03	0.069
L1TG	16.19	15.65	0.734	0.906	15.32	12.73	9.49E-03	0.075
L2TG	5.36	5.55	0.741	0.906	4.63	3.68	4.01E-03	0.069
L3TG	3.98	3.75	0.544	0.777	3.71	3.13	7.11E-03	0.069
L4AB	5.09	5.38	0.875	0.929	4.12	6.29	0.042	0.145
L4CH	4.54	4.82	0.914	0.948	3.12	6.28	0.031	0.135
L4FC	1.85	2.46	0.539	0.777	1.67	2.71	0.033	0.135
L4PN*	92.48	97.79	0.875	0.929	74.95	114.45	0.042	0.145
L5CH	4.99	2.42	0.197	0.441	3.24	7.30	0.032	0.135
L5FC	1.86	1.44	0.587	0.807	1.63	2.96	0.046	0.152
L5PL	3.09	1.73	0.158	0.370	1.79	3.51	0.042	0.145
L6FC	2.44	2.72	0.844	0.929	2.21	3.68	0.047	0.152
LDFC	18.19	20.12	0.503	0.777	15.37	18.93	0.013	0.078
LDTG	38.29	39.66	0.718	0.898	33.82	28.99	0.018	0.098

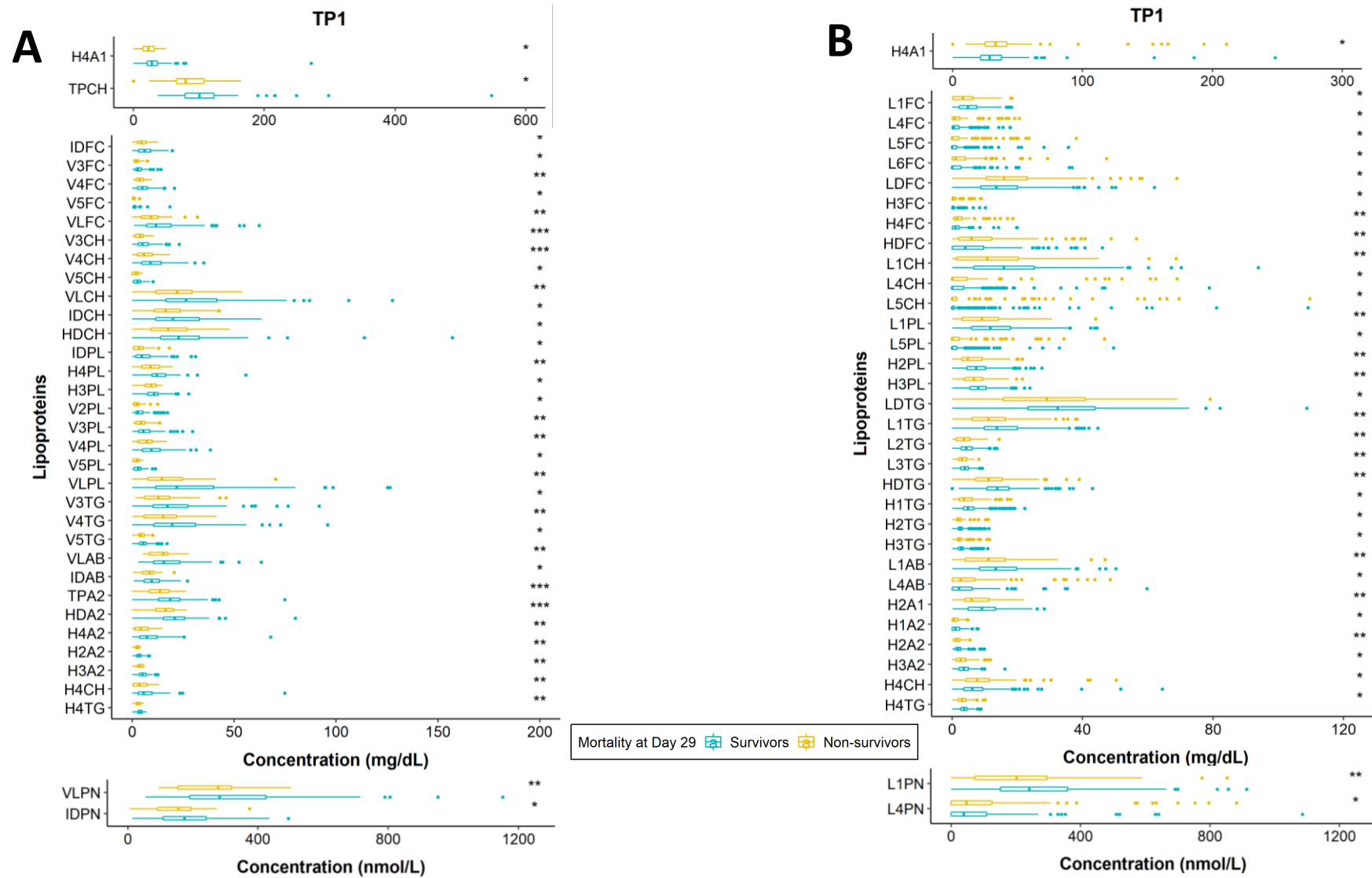


Figure 5.3: Box plots for lipoproteins which were significantly different between survivors and non-survivors at TP1 in VANISH (A) and LeOPARDS (B) BI LISA datasets.

Table 5.3: Welch two sample *t*-test results of NMR BI LISA data for the VANISH and LeoPARDS cohorts at TP2. Adjusted p-values were calculated with B-H correction. Adjusted p-values (p_{Adj}) and un-adjusted p-values (p) <0.05 are highlighted in light green; Mean intensity values are highlighted for survivors and non-survivors (Yellow represents higher mean value; peach represents lower mean value). Metabolites significant in the two cohorts are highlighted in different colours (green = In both cohorts; red = Only in VANISH; blue = Only in LeoPARDS). Abbreviations: CH = Cholesterol; FC = Free Cholesterol; PL = Phospholipid; TG = Triglycerides; A1 = Apo-A1; A2 = Apo-A2; AB = Apo-B100; HD = High density lipoprotein (HDL); VL = Very low density lipoprotein (VLDL); ID = Intermediate density lipoprotein (IDL); LD = Low density lipoprotein; TPC = Total plasma Cholesterol; TPA2 = Total plasma A2; IDAB = IDLAB; IDPN = IDL Particle Number; IDPL = IDL Phospholipids; IDTG = IDL Triglyceride; HDCH = HDL Cholesterol, HDFC = HDL Free Cholesterol; HDPL = HDL Phospholipid; H2A1 = HDL Apo A1 subfraction 2; H2A2: HDL Apo-A2; H1-H3A2 = HDL Apo-A2 subfractions 1-3; H3CH = HDL cholesterol subfraction 3; H4TG = HDL Triglyceride subfraction 4; H3PL = HDL Phospholipid subfraction 3; IDAB = IDL Apo-B100; L1CH = LDL Cholesterol subfraction 1; L1FC = LDL Free Cholesterol subfraction 1; L1PL = LDL Phospholipid subfraction 1; L1PN = LDL PN subfraction 1. Particle numbers are reported in nmol/L and are highlighted in asterisk.

Lipoprotein	VANISH _{TP2} (N = 92, n =76 & 23)				LeoPARDS _{TP2} (N = 312, n = 248 & 64)			
	Mean concentration (mg/dL) or (nmol/L)*		p	p_{Adj}	Mean concentration (mg/dL) or (nmol/L)*		p	p_{Adj}
	Survivors	Non-survivors			Survivors	Non-survivors		
H2A2	3.36	2.61	0.020	0.558	2.40	1.80	1.62E-04	0.018
HDA2	23.77	18.80	0.026	0.558	20.51	17.88	0.032	0.217
HDCH	31.13	21.94	0.024	0.558	22.64	20.63	0.408	0.633
H1A2	2.35	1.59	0.035	0.558	1.30	1.07	0.197	0.451
H3CH	5.84	4.33	6.64E-03	0.558	5.13	4.68	0.164	0.420
HDPL	49.91	40.76	0.050	0.604	43.19	38.54	0.098	0.326
TPA2	20.83	16.08	0.022	0.558	16.76	14.78	0.080	0.314
TPCH	108.89	89.05	0.034	0.558	99.51	94.42	0.350	0.629
HDFC	5.05	3.84	0.409	0.852	4.51	6.79	0.018	0.193
IDAB	11.27	10.02	0.318	0.850	10.58	8.60	0.025	0.212
IDPL	5.80	4.50	0.238	0.846	4.93	3.22	0.011	0.193
IDPN	204.86	182.15	0.318	0.850	192.39	156.29	0.025	0.212
IDTG	13.80	12.54	0.657	0.856	16.32	12.26	0.042	0.241
L1AB	20.18	18.00	0.328	0.850	20.76	17.05	0.012	0.193
L1CH	26.61	22.46	0.221	0.846	26.35	21.01	0.012	0.193

L1FC	7.73	6.62	0.245	0.846	7.32	6.20	0.050	0.273
L1PL	18.55	15.99	0.231	0.846	18.65	15.11	0.012	0.193
L1PN	366.85	327.29	0.328	0.850	377.45	309.98	0.012	0.193
L1TG	18.71	17.50	0.547	0.852	20.58	17.56	0.031	0.217
L2TG	6.58	6.10	0.524	0.852	6.93	5.76	0.016	0.193
L3TG	4.74	4.46	0.516	0.852	5.07	4.47	0.030	0.217
V2PL	3.26	3.11	0.798	0.904	3.01	2.38	0.041	0.241
H3PL	11.93	10.06	0.076	0.666	11.38	9.95	0.019	0.193
H4TG	3.63	3.44	0.632	0.856	4.04	3.48	0.017	0.193
H3A2	5.78	4.63	0.058	0.604	5.15	4.15	4.51E-03	0.193
H2A1	13.20	10.85	0.079	0.666	12.03	10.43	0.036	0.231

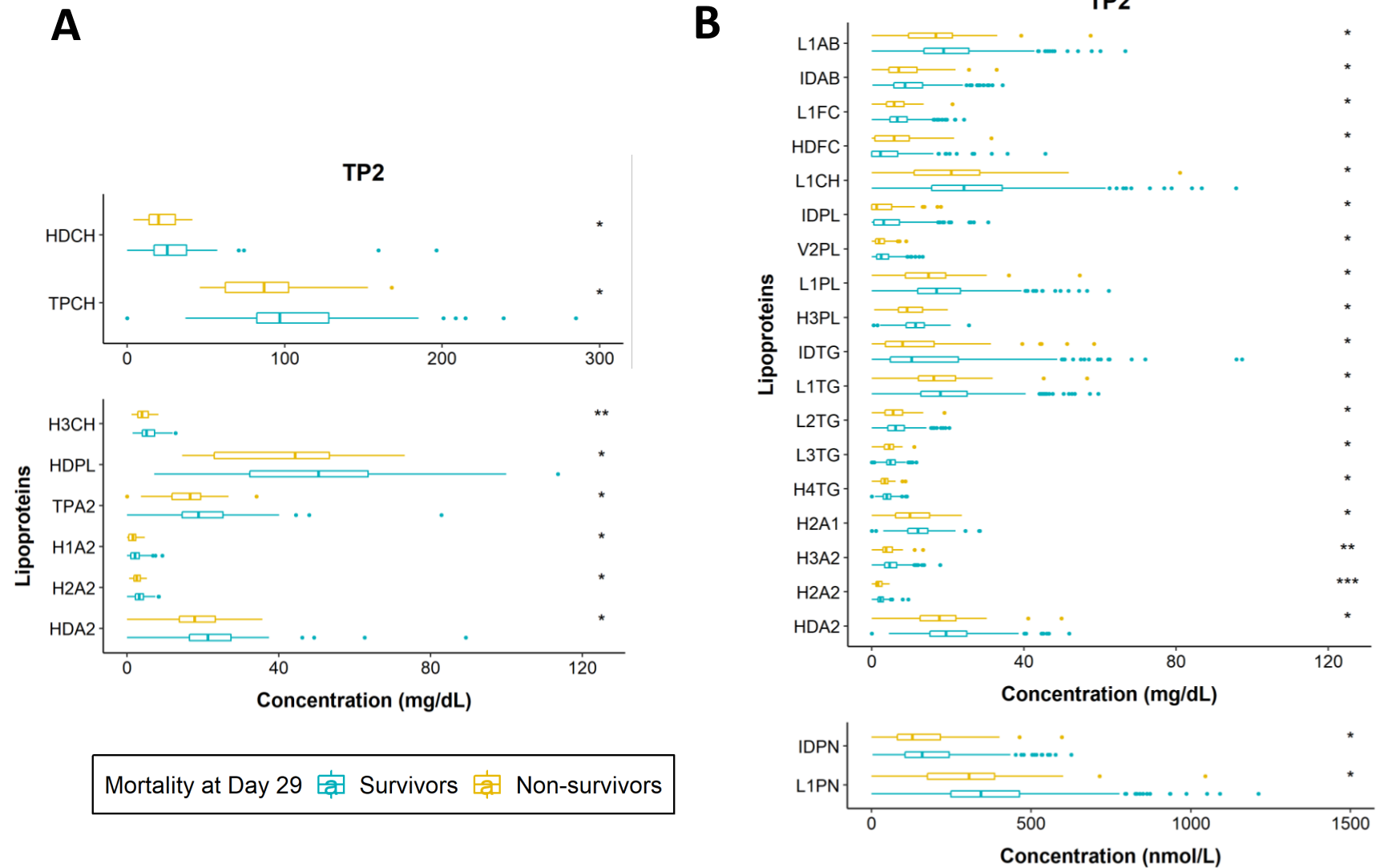


Figure 5.4: Box plots for lipoproteins which were significantly different between survivors and non-survivors at TP2 in VANISH (A) and LeoPARDS (B) BI LISA datasets.

Table 5.4: Welch two sample *t*-test results of NMR BI LISA data for the VANISH and LeoPARDS cohorts at TP2. Adjusted p-values were calculated with B-H correction. Adjusted p-values (p_{Adj}) and un-adjusted p-values (p) <0.05 are highlighted in light green; Mean intensity values are highlighted for survivors and non-survivors (Yellow represents higher mean value; peach represents lower mean value). Metabolites significant in the two cohorts are highlighted in different colours (green = In both cohorts; red = Only in VANISH; blue = Only in LeoPARDS). Abbreviations: CH = Cholesterol; FC = Free Cholesterol; PL = Phospholipid; TG = Triglycerides; A2 = Apo-A2; AB = Apo-B100; HD = High density lipoprotein (HDL); VL = Very low density lipoprotein (VLDL); ID = Intermediate density lipoprotein (IDL); H2FC = HDL Free Cholesterol subfraction 2; H1-H2A2 = HDL Apo-A2 subfractions 1-2; V2-V3CH = VLDL cholesterol subfraction 2-3; V1FC = VLDL Free Cholesterol subfraction 2; V3-V3TG = VLDL subfractions 2-3; VLPL = VLDL Phospholipid ; V3PL = VLDL Phospholipid subfractions 3.

Lipoprotein	VANISH _{TP3} (N = 75, n = 59 & 16)				LeoPARDS _{TP3} (N = 249, n = 200 & 49)			
	Mean concentration (mg/dL)		p	p_{Adj}	Mean concentration (mg/dL)		p	p_{Adj}
	Survivors	Non-survivors			Survivors	Non-survivors		
H2FC	1.35	0.95	0.047	0.970	0.97	0.87	0.475	0.736
H1A2	2.00	1.62	0.414	0.970	1.72	1.21	9.43E-03	0.185
H2A2	3.28	3.45	0.636	0.970	2.50	2.00	1.50E-03	0.082
V1FC	1.32	1.20	0.835	0.973	2.51	1.48	0.029	0.327
V2CH	3.11	2.19	0.103	0.970	3.81	2.64	5.75E-03	0.185
V2PL	3.57	3.05	0.469	0.970	3.76	2.42	1.19E-03	0.082
V2TG	15.88	14.17	0.617	0.970	17.16	12.36	9.69E-03	0.185
V3CH	5.10	4.41	0.566	0.970	4.59	3.12	0.010	0.185
V3PL	5.82	5.94	0.927	0.994	5.10	3.69	0.019	0.261
V3TG	17.86	17.80	0.988	0.996	17.17	13.03	0.030	0.327
VLPL	22.37	21.33	0.816	0.973	25.59	19.34	0.014	0.220

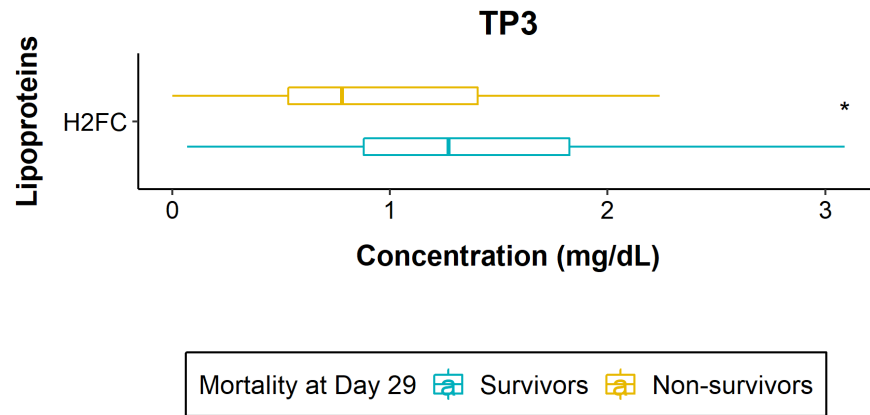
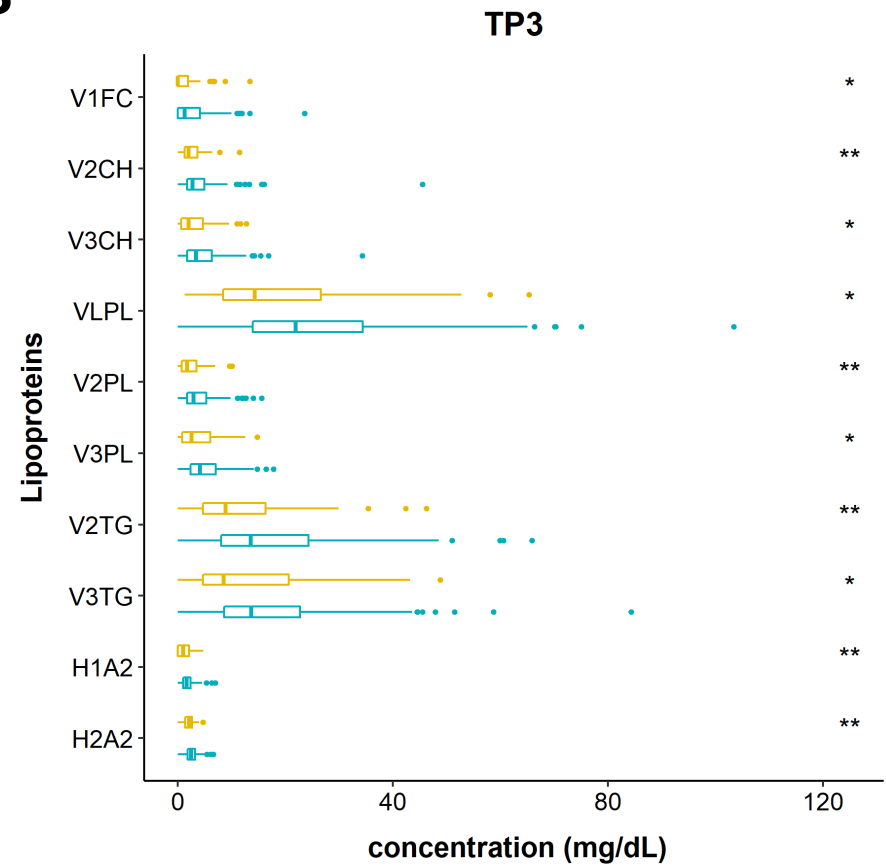
A**B**

Figure 5.5: Box plots for lipoproteins which were significantly different between survivors and non-survivors at TP3 in VANISH (A) and LeoPARDS (B) BI LISA datasets.

5.4.1.2 UHPLC-MS targeted data

5.4.1.2.1 LPOS ppR

Univariate analysis of 246 lipids from LPOS ppR data was performed for all timepoints on a total of 488 serum samples from VANISH and 1437 samples from LeoPARDS, figure 5.6 and figure 5.7. In total 23 samples were excluded from VANISH and of those, 19 samples had no sample timing recorded and 4 samples belonged to a patient whose samples were collected after several days of receiving the study drug. Same samples were excluded from LeoPARDS as mentioned previously in chapter 2.2.2.3. However, 10 additional samples which had no sample timings recorded were also excluded from LeoPARDS prior to analysis.

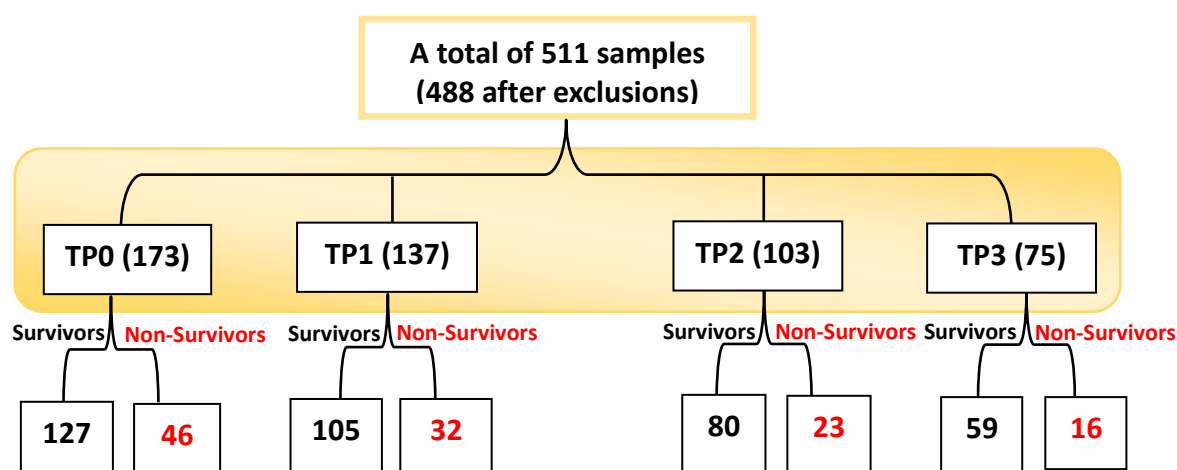


Figure 5.6: Flow chart for the samples included in the univariate analysis of LPOS ppR data from VANISH.

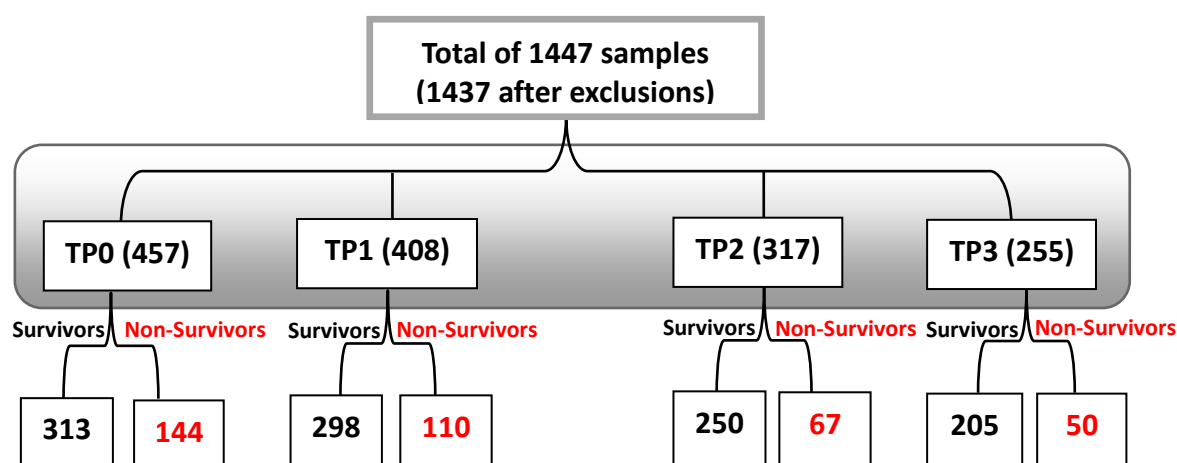


Figure 5.7: Flow chart for the samples included in the univariate analysis of LPOS ppR data from LeoPARDS.

A summary of univariate results for all timepoints in both cohorts is presented in tables 5.5, 5.6, 5.7 and 5.8, differentiating survivors from non-survivors at each timepoint (tables 5.5-5.8).

The results from the univariate analysis revealed that 96 lipids were significantly ($p < 0.05$) different between survivors and non-survivors in LeoPARDS at TP0 and of those 49 lipids remained significant after correcting for multiple comparisons ($p_{\text{Adj}} < 0.05$). We found that six fatty acyls including medium- and long- chain acyl carnitines (such as CAR(8:0), CAR(10:0), CAR(12:0-OH), CAR(12:1-OH), CAR(20:0) and CAR(20:1)) were significantly higher in patients who went on to die than those who survived at baseline in both cohorts ($p_{\text{Adj}} < 0.05$). Glycerophospholipids (including 11 PCs, 7 LPCs and PE(O-18:1/22:6) and/or PE(P-18:0/22:6)), sphingolipids (including 12 SMs and Cer(d18:1/16:0)) and 4 TGs (TG(46:0), TG(49:1), TG(51:4) and TG(58:9)) were significantly lower in patients who went on to die than those who didn't in LeoPARDS at TP0 ($p_{\text{Adj}} \leq 0.05$) and all these lipid classes had the same pattern in VANISH, but none of them remained significant after correction. Of these lipid classes only glycerophospholipids (including 2 PCs PC(14:0/20:4), PC(16:0/20:5), 4 LPCs (LPC(0:0/16:0), LPC(0:0/18:0), LPC(16:0/0:0), and LPC(18:0/0:0) and PE(O-18:1/22:6) and/or PE(P-18:0/22:6)) and Cer(d18:1/16:0) were significantly different between survivors and non-survivors at baseline in both cohorts based on un-adjusted p-values ($p < 0.05$) (table 5.5).

Lipid classes including fatty acyls, sphingolipids, glycerolipids and glycerophospholipids, followed the same pattern at TP1 as TP0 in both cohorts. In brief, five acylcarnitines including (CAR(12:1-OH)CAR(16:2), CAR(18:1-DC), CAR(20:0) and CAR(20:1)) were significantly higher in non-survivors than survivors at TP1 in both cohorts whilst Glycerophospholipids (including 4 PCs, 4 LPCs and PE(O-18:1/22:6) and/or PE(P-18:0/22:6)) and sphingolipids (two SMs (SM(d18:1/18:0), SM(d18:2/16:0)_1)) and four Cers), glycerolipids (including 7 TGs) were significantly lower in patients who went on to die before day 29 than those who didn't in both cohorts at TP1 ($p < 0.05$). All these lipid species remained significant after correcting for multiple comparisons, but only in LeoPARDS ($p_{\text{Adj}} < 0.05$). Additionally, cholesterol and cholesteryl esters (CEs) including CE(20:4), CE(20:5) were also significantly lower in patients who went on to die than those who didn't in LeoPARDS

($p_{\text{Adj}} < 0.05$) and the trend for this was also the same in VANISH, but the results were not significant.

At later timepoints there were not much significant differences between survivors and non-survivors. Medium chain acylcarnitine CAR(12:1-OH) was higher in non-survivors with respect to survivors at TP2 in both cohorts ($p < 0.05$) and this acylcarnitine did not remain significant after correcting for multiple comparisons in both cohorts. Glycerophospholipids (including eight PCs, 38 LPCs and 6 LPEs) sphingolipids (including 14 SMs, three Cers and lactosylceramide (LacCer)(d18:1/16:0)), glycerolipids including 22 TGs and 5 DGs) and steroid esters (2 CEs including CE(20:4), CE(20:5)) were significantly lower in patients who went on to die in comparison to those who didn't in LeoPARDS at TP2 ($p_{\text{Adj}} < 0.05$). All these lipids except for 7 (three TGs, three LPCs and LacCer (d18:1/16:0)) followed the same pattern in VANISH as LeoPARDS, but the results for all these lipids were not significant.

The univariate analysis revealed that 128 lipids were significantly different between survivors and non-survivors in LeoPARDS ($p_{\text{Adj}} < 0.05$) at TP3 (table 5.8). Of those 3 acylcarnitines (CAR(12:1-OH), CAR(18:1-DC), CAR(8:0)) were significantly higher in patients who went on to die than those who didn't in LeoPARDS ($p_{\text{Adj}} < 0.05$) whilst glycerophospholipids (including 15 PCs, 38 LPCs, 5 LPEs and PE(O-18:1/22:6) and/or PE(P-18:0/22:6)), sphingolipids (including 16 SMs, 3 Cers), glycerolipids (including 6 DGs and 35 TGs) and steroid ester (CE(20:5)) were significantly lower in non-survivors in comparison to those who didn't in LeoPARDS ($p_{\text{Adj}} < 0.05$). The pattern for these lipid classes except for 26 TGs, 2 PCs (PC(15:0/18:2), and PC(O-24:1/20:3) and/or PC(P-24:0/20:3)), LPC(20:5/0:0), and DG(32:1) was the same in VANISH as LeoPARDS. Of those, only 13 lipids including sphingolipids (two SMs (SM(d18:1/23:0), SM(d18:1/16:0)), glycerophospholipids (including 9 LPCs, PE(O-18:1/22:6) and/or PE(P-18:0/22:6) and LPE(18:2/0:0) were significantly lower in patients who went on to die than those who didn't in both cohorts at TP3 ($p < 0.05$) but these lipids only remained significant after correction in LeoPARDS, but not in VANISH.

Table 5.5: Welch two sample *t*-test results of LPOS ppR data for the VANISH and LeoPARDS cohorts at TP0. Adjusted p-values were calculated with B-H correction. Adjusted p-values (p_{Adj}) and un-adjusted p-values (p) <0.05 are highlighted in light green; Mean intensity values are highlighted for survivors and non-survivors (Yellow represents higher mean value; peach represents lower mean value). Metabolites significant in the two cohorts are highlighted in different colours (green = In both cohorts; red = Only in VANISH; blue = Only in LeoPARDS). Abbreviations: PC = Phosphatidylcholine; LPC = Lysophosphatidylcholine; PE = Phosphatidylethanolamine; SM = Sphingomyelin; Cer = Ceramide; CE = Cholesteryl esters; DG = Diacylglycerol; TG = Triglycerides; CAR = Carnitine. Note: Carbon atoms and double bonds in all lipid species are represented as (C atoms:double bond); d indicates 2 hydroxyl groups in the sphenoid base of ceramides and sphigomyelins; DC denotes for dicarboxylic acid; Prefixes "OH", "O" and "P" represents different substituents/linkages in the lipid species: "OH" denotes for hydroxyl group, "O" represents an alkyl ether linkage whilst "P" refers to the plasmalogen link. Lipid species with abbreviations _1 and _2 indicate same m/z value but different retention times for those lipids.

Lipids	VANISH _{TP0} (N = 173, n = 127 & 46)				LeoPARDS _{TP0} (N = 457, n = 313 & 144)			
	Log Mean integrated area (a.u)		p	p_{Adj}	Log Mean integrated area (a.u)		p	p_{Adj}
	Survivors	Non-survivors			Survivors	Non-survivors		
Arachidyl carnitine CAR(20:0)	8.59	9.29	3.35E-05	2.74E-03	7.70	8.15	3.09E-07	7.59E-05
Eicoseneoylcarnitine CAR(20:1)	9.91	10.50	2.67E-05	2.74E-03	8.93	9.31	1.48E-05	1.13E-03
Hydroxydodecenoylcarnitine CAR(12:1-OH)	11.31	11.92	1.24E-05	2.74E-03	10.47	10.86	2.29E-05	1.13E-03
Hexadecadienoylcarnitine CAR(16:2)	9.48	10.01	2.97E-03	0.081	8.51	8.89	6.80E-05	2.41E-03
Decanoylcarnitine CAR(10:0)	13.41	13.93	1.63E-04	1.00E-02	12.71	12.93	1.01E-03	0.016
Eicosadienoylcarnitine CAR(20:2)	9.07	9.40	2.22E-03	0.068	8.17	8.38	1.13E-03	0.016
Octanoylcarnitine CAR(8:0)	12.82	13.29	3.49E-04	0.017	12.08	12.30	1.42E-03	0.018
Palmitoylcarnitine CAR(16:0)	12.52	12.93	2.05E-03	0.068	11.69	11.90	3.17E-03	0.024
Tetradecadienoylcarnitine CAR(14:2)	11.17	11.53	0.042	0.302	10.02	10.35	3.75E-03	0.026
Hydroxydodecanoylcarnitine CAR(12:0-OH)	10.81	11.32	5.50E-04	0.023	9.94	10.15	3.87E-03	0.026
Hydroxypalmitoleoylcarnitine CAR(16:1-OH)	10.32	10.64	5.13E-03	0.101	9.29	9.47	8.17E-03	0.044
Hydroxytetradecanoylcarnitine CAR(14:0-OH)	10.40	10.77	4.59E-03	0.101	9.36	9.53	0.029	0.091

Arachidonoylcarnitine CAR(20:4)	9.49	9.76	0.027	0.246	8.05	8.21	0.035	0.099
PC(16:0/20:5)	17.11	16.85	0.044	0.302	15.56	15.24	7.25E-06	8.92E-04
PC(14:0/20:4)	13.49	13.23	0.047	0.302	11.33	11.14	1.66E-03	0.020
LPC(16:0/0:0)	16.77	16.50	0.038	0.302	15.11	14.86	1.82E-03	0.021
LPC(0:0/16:0)	14.99	14.66	0.038	0.302	13.01	12.73	1.97E-03	0.021
LPC(18:0/0:0)	15.82	15.55	0.046	0.302	13.89	13.65	3.73E-03	0.026
LPC(0:0/18:0)	13.81	13.49	0.046	0.302	11.68	11.43	4.38E-03	0.028
LPC(20:5/0:0)	12.54	11.98	7.91E-03	0.122	10.18	9.88	0.012	0.056
LPC(0:0/20:5)	10.36	9.83	8.48E-03	0.123	7.87	7.55	0.015	0.065
PE(O-18:1/22:6) and/or PE(P- 18:0/22:6)	12.89	12.65	0.021	0.227	11.51	11.38	9.10E-03	0.048
Cer(d18:1/16:0)	13.14	13.32	0.042	0.302	11.65	11.80	0.011	0.054
LPC(18:3/0:0)_1	11.64	11.22	0.015	0.183	9.41	9.22	0.068	0.160
LPC(20:3/0:0)_1	13.06	12.71	0.033	0.279	10.84	10.66	0.084	0.185
LPC(0:0/20:4)	12.64	12.25	0.018	0.212	10.41	10.25	0.086	0.185
LPC(0:0/22:5)_1	9.82	9.46	5.32E-03	0.101	7.62	7.48	0.110	0.218
LPC(20:4/0:0)	14.55	14.16	0.015	0.183	12.41	12.27	0.131	0.244
LPC(0:0/22:6)	11.50	11.09	6.63E-03	0.116	9.21	9.07	0.161	0.278
LPC(22:5/0:0)_1	12.15	11.71	4.50E-03	0.101	9.78	9.65	0.167	0.281
LPC(22:6/0:0)	13.67	13.23	7.88E-03	0.122	11.36	11.23	0.173	0.285
LPC(0:0/18:2)	13.54	13.19	0.032	0.279	11.42	11.32	0.318	0.443
LPC(18:2/0:0)	15.48	15.14	0.027	0.246	13.49	13.40	0.342	0.470
LPC(0:0/18:3)	9.43	9.04	0.015	0.183	7.19	7.10	0.393	0.515
LPC(20:3/0:0)_2	10.65	10.32	0.042	0.302	8.34	8.29	0.614	0.726
LPC(22:5/0:0)_2	10.31	9.97	0.023	0.233	8.38	8.34	0.693	0.783
Cer(t18:0/24:1)	11.30	11.04	0.026	0.246	8.90	8.86	0.415	0.537
PC(O-16:1/22:6) and/or PC(P- 16:0/22:6)	14.06	13.80	0.019	0.214	12.45	12.41	0.433	0.552

PC(14:0/22:6)	12.70	12.43	0.060	0.333	10.62	10.33	2.13E-05	1.13E-03
PC(16:0/20:4)_1	15.24	15.13	0.382	0.734	13.12	12.83	6.86E-05	2.41E-03
PC(18:0/20:3)	17.03	16.90	0.276	0.601	15.43	15.19	1.73E-04	5.30E-03
SM(d18:2/18:0)	16.06	15.86	0.077	0.391	14.74	14.56	1.94E-04	5.30E-03
PC(18:0/22:6)	16.50	16.30	0.059	0.333	15.13	14.94	2.66E-04	5.98E-03
SM(d18:1/18:0)	16.72	16.52	0.057	0.333	15.35	15.18	2.67E-04	5.98E-03
PC(16:0/22:6)	18.09	17.90	0.065	0.349	16.58	16.40	3.42E-04	6.76E-03
PC(18:0/20:4)	17.78	17.64	0.091	0.423	16.60	16.47	3.57E-04	6.76E-03
PC(15:0/18:2)	14.41	14.31	0.392	0.736	13.07	12.87	4.90E-04	8.61E-03
TG(46:0)	12.33	12.14	0.340	0.696	9.87	9.61	9.35E-04	0.015
PC(16:0/20:4)_3	18.07	17.94	0.124	0.462	16.93	16.82	1.33E-03	0.018
Carboxyheptadecenoylcarnitine CAR(18:1-DC)	12.72	12.98	0.151	0.485	11.80	12.16	1.85E-03	0.021
LPC(15:0/0:0)	12.02	11.72	0.115	0.462	9.90	9.60	2.38E-03	0.023
SM(d35:1); SM(d17:1/18:0) SM(d18:1/17:0)	14.18	14.02	0.150	0.485	12.69	12.54	2.53E-03	0.023
LPC(17:0/0:0)	12.55	12.22	0.059	0.333	10.25	9.96	2.69E-03	0.023
SM(d18:1/24:0)	16.11	15.95	0.154	0.485	14.23	14.08	2.70E-03	0.023
PC(18:0/22:5)	14.61	14.47	0.253	0.587	12.81	12.63	2.71E-03	0.023
SM(d18:1/23:0)	15.85	15.67	0.122	0.462	13.77	13.61	2.76E-03	0.023
SM(d18:1/20:1)	15.21	15.02	0.130	0.470	13.73	13.57	2.91E-03	0.024
SM(d18:1/22:0)	16.45	16.30	0.140	0.484	14.86	14.73	3.02E-03	0.024
LPC(0:0/15:0)	10.05	9.78	0.126	0.462	8.04	7.81	3.38E-03	0.025
SM(d18:2/24:0)	15.83	15.68	0.165	0.487	13.82	13.68	3.51E-03	0.025
SM(d30:1); SM(d16:1/14:0) SM(d18:1/12:0)	12.98	12.86	0.346	0.698	10.66	10.50	5.09E-03	0.032
SM(d18:2/22:0)	16.06	15.90	0.167	0.487	14.37	14.23	5.86E-03	0.036
SM(d18:1/20:0)	15.83	15.66	0.108	0.460	14.12	13.97	6.02E-03	0.036
SM(d18:2/23:0)	15.25	15.06	0.166	0.487	13.10	12.94	6.60E-03	0.039

LPC(0:0/14:0)	10.19	9.88	0.098	0.432	7.95	7.68	7.00E-03	0.040
TG(49:1)	14.28	14.26	0.914	0.957	11.69	11.46	7.22E-03	0.040
TG(51:4)	13.18	13.00	0.264	0.591	10.36	10.11	7.24E-03	0.040
TG(58:9)_1	12.30	12.22	0.483	0.757	10.06	9.88	9.54E-03	0.049
TG(48:0)	12.53	12.44	0.439	0.741	10.04	9.86	9.81E-03	0.049
SM(d18:2/14:0)	13.77	13.61	0.172	0.487	11.39	11.25	0.011	0.053
LPC(14:0/0:0)	12.50	12.19	0.098	0.432	7.87	7.61	0.012	0.056
TG(48:1)	15.72	15.66	0.683	0.858	13.22	12.98	0.012	0.057
Carboxyheptadecanoylcarnitine CAR(18:0-DC)	11.56	11.81	0.093	0.423	10.76	10.98	0.014	0.062
PC(16:0/18:2)	19.07	19.05	0.646	0.823	18.08	18.01	0.015	0.065
TG(50:3)	16.65	16.56	0.451	0.741	14.13	13.92	0.016	0.065
TG(50:1)	16.98	16.96	0.811	0.915	14.75	14.58	0.016	0.065
TG(60:9)	9.99	9.91	0.404	0.741	8.15	8.00	0.016	0.067
TG(56:6)_1	15.60	15.44	0.227	0.574	12.99	12.79	0.017	0.068
CE(20:5)	8.47	8.26	0.140	0.484	9.21	9.00	0.018	0.073
PC(O-24:0/20:4)	12.26	12.13	0.296	0.628	9.92	9.79	0.020	0.076
Luteine	11.01	10.91	0.544	0.778	9.58	9.39	0.020	0.076
SM(d17:1/16:0)	15.73	15.67	0.509	0.770	13.83	13.71	0.022	0.081
TG(52:4)_1	17.34	17.25	0.369	0.714	15.15	14.98	0.022	0.081
DG(16:0/16:0)	11.58	11.35	0.113	0.462	10.22	10.07	0.023	0.081
PC(16:0/18:1)_2	19.13	19.11	0.705	0.863	18.18	18.11	0.023	0.081
Cer(d41:1); Cer(d18:1/23:0) Cer(d17:1/24:0)	14.44	14.28	0.206	0.552	11.99	11.86	0.023	0.081
TG(52:3)	18.12	18.07	0.533	0.777	15.91	15.77	0.024	0.083
TG(53:4)	14.04	13.92	0.407	0.741	11.38	11.18	0.024	0.083
TG(51:3)	14.71	14.60	0.435	0.741	11.91	11.71	0.025	0.083
TG(50:4)	15.07	14.87	0.229	0.574	12.38	12.17	0.025	0.083
TG(53:1)	13.26	13.21	0.761	0.888	9.58	9.40	0.025	0.083

TG(58:6)_2	12.11	12.09	0.808	0.915	10.07	9.92	0.025	0.083
PC(14:0/18:2)	15.19	15.06	0.287	0.613	13.10	12.96	0.029	0.091
TG(49:2)	13.99	13.93	0.687	0.858	11.14	10.94	0.029	0.091
Cer(d18:1/24:0)	15.29	15.16	0.239	0.583	12.89	12.78	0.029	0.091
PC(O-24:0/18:2)	10.97	10.83	0.233	0.579	8.48	8.34	0.031	0.095
TG(51:2)	15.44	15.42	0.905	0.951	12.75	12.56	0.031	0.095
SM(d32:1); SM(d16:1/16:0) SM(d18:1/14:0)	16.35	16.30	0.516	0.770	14.46	14.36	0.032	0.096
TG(50:2)	17.26	17.24	0.881	0.939	14.98	14.83	0.034	0.099
TG(46:1)	13.89	13.78	0.632	0.823	11.27	11.04	0.034	0.099
Sphingosine-1-phosphate	10.05	9.95	0.324	0.671	8.50	8.34	0.034	0.099
TG(52:1)	16.06	16.02	0.750	0.888	13.60	13.43	0.035	0.099
TG(53:5)	12.42	12.24	0.221	0.569	9.92	9.72	0.036	0.099
TG(48:2)	15.52	15.46	0.727	0.880	12.87	12.67	0.044	0.120
TG(53:3)	15.00	14.95	0.723	0.880	12.33	12.17	0.046	0.125
PC(16:0/18:3)	14.83	14.79	0.703	0.863	13.08	12.94	0.046	0.125
DG(16:0/18:1)	13.35	13.13	0.108	0.460	11.83	11.68	0.047	0.125
PC(18:0/18:2)	18.68	18.67	0.932	0.964	17.55	17.48	0.049	0.126
LPC(0:0/20:3)	11.15	10.86	0.062	0.338	8.76	8.57	0.049	0.126
DG(32:1)	11.18	10.94	0.135	0.483	9.73	9.58	0.049	0.126
TG(49:3)	12.63	12.52	0.517	0.770	9.78	9.61	0.049	0.126

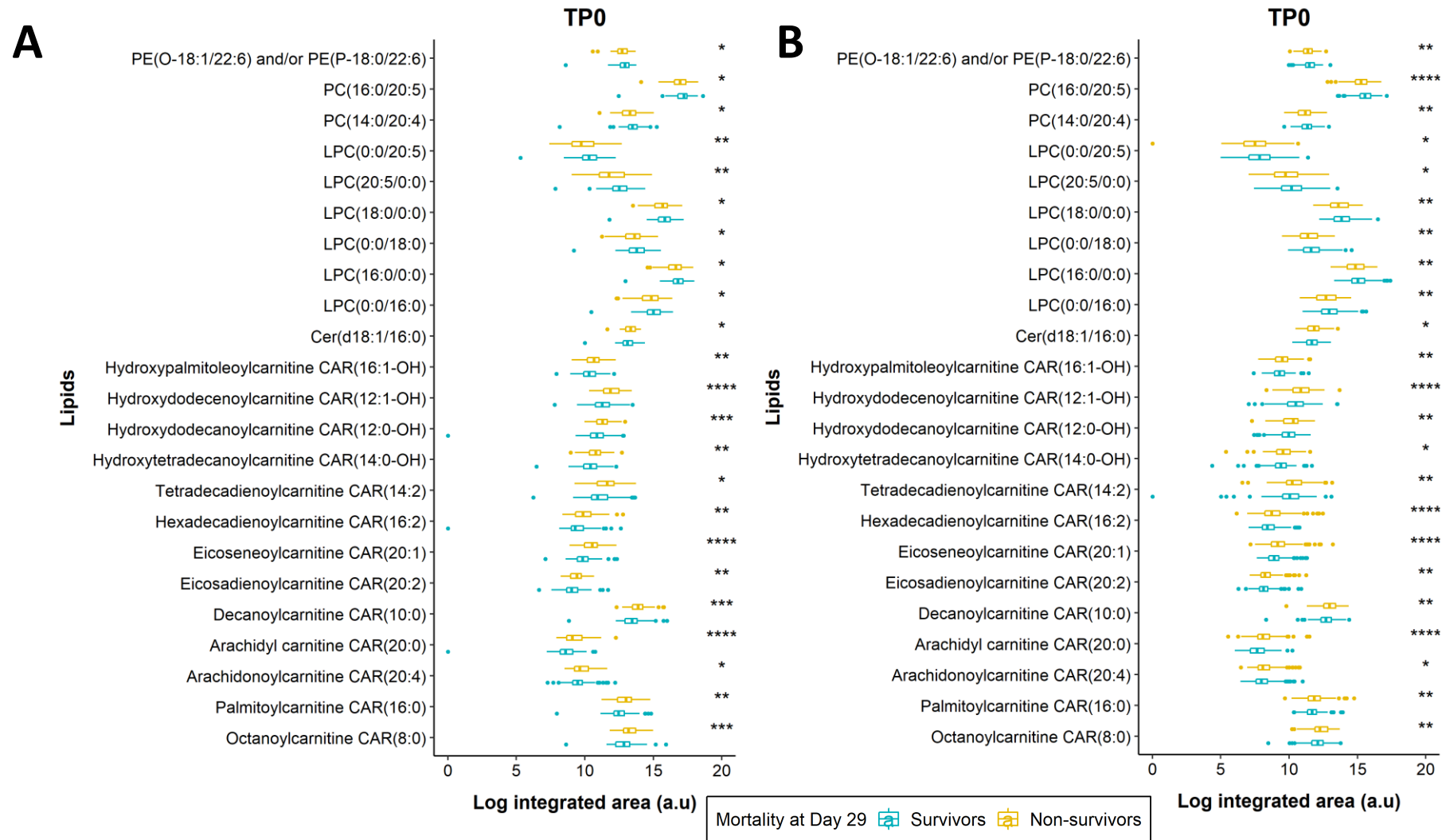


Figure 5.8: Box plots for common lipids which were significantly different between survivors and non-survivors at TP0 in VANISH (A) and LeoPARDS (B) LPOS ppr datasets.

Table 5.6: Welch two sample *t*-test results of LPOS ppR data for the VANISH and LeoPARDS cohorts at TP1. Adjusted p-values were calculated with B-H correction. Adjusted p-values (p_{Adj}) and un-adjusted p-values (p) <0.05 are highlighted in light green; Mean intensity values are highlighted for survivors and non-survivors (Yellow represents higher mean value; peach represents lower mean value). Metabolites significant in the two cohorts are highlighted in different colours (green = In both cohorts; red = Only in VANISH; blue = Only in LeoPARDS). Abbreviations: PC = Phosphatidylcholine; LPC = Lysophosphatidylcholine; PE = Phosphatidylethanolamine; LPE = Lysophosphatidylethanolamine; SM = Sphingomyelin; Cer = Ceramide; LacCer = Lactosylceramide; CE = Cholesteryl esters; DG = Diacylglycerol; TG = Triglycerides; CAR = Carnitine. Note: Carbon atoms and double bonds in all lipid species are represented as (C atoms:double bond); d indicates two hydroxyl groups in the sphingoid base whilst t indicates three hydroxyl groups; DC denotes for dicarboxylic acid; Prefixes “OH”, “O” and “P” represents different substituents/linkages in the lipid species: “OH” denotes for hydroxyl group, “O” represents an alkyl ether linkage whilst “P” refers to the plasmalogen link. Lipid species with abbreviations _1 and _2 indicate same m/z value but different retention times for those lipids.

Lipids	VANISH _{TP1} (N =137, n = 105 & 32)				LeoPARDS _{TP1} (N = 408, n = 298 & 110)			
	Log Mean integrated area (a.u)		p	p_{Adj}	Log Mean integrated area (a.u)		p	p_{Adj}
	Survivors	Non-survivors			Survivors	Non-survivors		
Octanoylcarnitine CAR(8:0)	12.75	13.11	0.033	0.277	12.00	12.16	0.047	0.080
Decanoylcarnitine CAR(10:0)	13.39	13.80	0.014	0.277	12.67	12.82	0.048	0.081
Hydroxydodecenoylcarnitine CAR(12:1-OH)	11.23	11.76	1.44E-03	0.277	10.38	10.70	2.21E-03	9.39E-03
Hydroxypalmitoleoylcarnitine CAR(16:1-OH)	10.28	10.53	0.040	0.289	9.20	9.35	0.036	0.065
Carboxyheptadecenoylcarnitine CAR(18:1-DC)	12.83	13.30	0.029	0.277	11.94	12.27	0.019	0.040
Eicoseneoylcarnitine CAR(20:1)	9.88	10.21	0.012	0.277	8.94	9.16	4.00E-03	0.014
Arachidyl carnitine CAR(20:0)	8.74	9.10	3.15E-03	0.277	7.82	8.15	1.24E-04	1.79E-03
PC(16:0/20:5)	17.23	16.90	0.012	0.277	15.70	15.27	3.49E-08	8.58E-06
PC(14:0/22:6)	12.69	12.41	0.044	0.289	10.68	10.34	1.77E-05	7.26E-04
PC(18:0/22:6)	16.58	16.33	0.013	0.277	15.16	14.87	2.14E-05	7.53E-04
PC(16:0/22:6)	18.14	17.88	0.012	0.277	16.58	16.32	5.48E-05	1.05E-03
LPC(16:0/0:0)	16.91	16.64	0.029	0.277	15.16	14.79	1.92E-04	2.25E-03
LPC(0:0/16:0)	15.16	14.82	0.024	0.277	13.06	12.66	2.43E-04	2.46E-03
LPC(18:0/0:0)	16.04	15.74	0.017	0.277	14.00	13.64	2.50E-04	2.46E-03

LPC(0:0/18:0)	14.08	13.72	0.015	0.277	11.80	11.43	3.04E-04	2.67E-03
PE(O-18:1/22:6) and/or PE(P-18:0/22:6)	12.95	12.72	0.011	0.277	11.61	11.46	9.90E-03	0.028
SM(d18:1/18:0)	16.70	16.47	0.030	0.277	15.32	15.02	9.25E-07	7.58E-05
SM(d18:2/16:0)_1	16.91	16.77	0.049	0.289	15.13	15.00	0.022	0.043
DG(16:0/18:1)	13.53	13.19	0.038	0.289	11.87	11.64	0.011	0.029
DG(16:0/16:0)	11.73	11.31	0.023	0.277	10.23	10.03	0.019	0.040
Cer(d18:1/24:0)	15.43	15.20	0.017	0.277	13.04	12.77	1.58E-05	7.26E-04
Cer(d41:1); Cer(d18:1/23:0) Cer(d17:1/24:0)	14.59	14.27	8.92E-03	0.277	12.09	11.80	5.55E-05	1.05E-03
Cer(d18:2/24:0)	13.95	13.65	0.026	0.277	11.41	11.14	1.08E-04	1.66E-03
Cer(d18:1/22:0)	14.71	14.49	0.046	0.289	12.59	12.35	1.70E-04	2.21E-03
Cer(t18:0/24:1)	11.50	11.23	0.033	0.277	9.06	8.93	0.049	0.081
TG(48:0)	12.51	12.17	0.030	0.277	9.94	9.67	6.79E-04	4.54E-03
TG(52:3)	18.21	18.04	0.040	0.289	15.94	15.70	9.02E-04	5.13E-03
TG(52:4)_1	17.45	17.22	0.038	0.289	15.16	14.90	2.07E-03	9.08E-03
TG(50:3)	16.72	16.44	0.043	0.289	14.14	13.85	2.43E-03	9.78E-03
TG(52:5)_1	15.87	15.54	0.037	0.289	13.41	13.10	2.66E-03	0.011
TG(50:4)	15.14	14.75	0.031	0.277	12.48	12.19	3.82E-03	0.014
TG(56:6)_1	15.71	15.42	0.046	0.289	13.02	12.68	7.02E-04	4.54E-03
Hydroxydodecanoylcarnitine CAR(12:0-OH)	10.72	11.06	0.021	0.277	9.77	9.92	0.103	0.149
Carboxyheptadecanoylcarnitine CAR(18:0-DC)	11.76	12.16	0.013	0.277	10.95	11.11	0.125	0.173
Eicosadienoylcarnitine CAR(20:2)	8.94	9.21	0.029	0.277	8.14	8.17	0.538	0.605
PE(O-20:1/20:4) and/or PE(P-20:0/20:4)	10.62	10.33	0.023	0.277	9.12	9.03	0.156	0.202
PE(O-18:1/20:4) and/or PE(P-18:0/20:4)	13.13	12.89	0.022	0.277	11.46	11.44	0.762	0.812

LPE(0:0/18:0)	9.83	9.63	0.030	0.277	8.25	8.15	0.058	0.093
DG(34:2)	12.15	11.83	0.020	0.277	10.90	10.76	0.114	0.160
DG(32:1)	11.26	10.90	0.042	0.289	9.77	9.67	0.218	0.272
SM(d18:2/23:0)	15.25	15.04	0.080	0.308	13.04	12.84	4.38E-03	0.015
PC(18:0/20:3)	17.12	16.90	0.063	0.292	15.49	15.08	5.61E-07	6.90E-05
PC(15:0/18:2)	14.48	14.30	0.121	0.366	13.15	12.83	3.32E-06	2.04E-04
PC(18:0/20:4)	17.82	17.69	0.079	0.307	16.60	16.41	3.16E-05	9.72E-04
PC(16:0/20:4)_3	18.09	17.95	0.094	0.338	16.91	16.72	4.14E-05	9.93E-04
SM(d18:1/20:0)	15.80	15.63	0.070	0.296	14.11	13.84	4.20E-05	9.93E-04
SM(d18:1/22:0)	16.45	16.32	0.076	0.305	14.86	14.64	4.44E-05	9.93E-04
PC(16:0/20:4)_1	15.26	15.14	0.228	0.467	13.20	12.89	6.46E-05	1.13E-03
SM(d18:2/18:0)	16.02	15.82	0.053	0.289	14.65	14.43	9.29E-05	1.52E-03
LPC(17:0/0:0)	12.74	12.44	0.070	0.296	10.32	9.88	1.66E-04	2.21E-03
PC(16:0/18:2)	19.13	19.07	0.152	0.399	18.15	18.01	1.83E-04	2.25E-03
TG(51:4)	13.29	12.97	0.071	0.296	10.44	10.04	2.21E-04	2.46E-03
SM(d35:1); SM(d17:1/18:0) SM(d18:1/17:0)	14.07	13.98	0.281	0.519	12.60	12.38	2.50E-04	2.46E-03
LPC(0:0/20:5)	10.68	10.35	0.158	0.403	8.27	7.67	2.65E-04	2.51E-03
SM(d18:1/23:0)	15.84	15.67	0.061	0.291	13.73	13.49	2.85E-04	2.59E-03
LPC(15:0/0:0)	12.13	11.89	0.165	0.403	9.94	9.50	3.48E-04	2.95E-03
TG(51:3)	14.81	14.56	0.122	0.366	11.96	11.60	3.72E-04	3.05E-03
Cer(d18:1/20:0)	13.38	13.14	0.109	0.354	11.23	10.96	4.40E-04	3.50E-03
TG(50:1)	17.03	16.82	0.088	0.328	14.71	14.41	5.68E-04	4.26E-03
Cer(d18:1/18:0)	13.09	12.82	0.109	0.354	11.30	11.03	5.72E-04	4.26E-03
TG(53:4)	14.23	14.04	0.152	0.399	11.50	11.16	6.13E-04	4.44E-03
SM(d18:1/24:0)	16.05	15.92	0.115	0.362	14.18	13.99	6.33E-04	4.45E-03
SM(d19:1/18:0)	12.49	12.34	0.363	0.593	11.08	10.78	6.96E-04	4.54E-03
TG(49:1)	14.27	14.12	0.373	0.593	11.62	11.28	7.29E-04	4.60E-03

SM(d18:1/20:1)	15.14	14.95	0.109	0.354	13.64	13.42	7.77E-04	4.78E-03
PC(14:0/20:4)	13.48	13.28	0.072	0.296	11.43	11.19	8.54E-04	5.12E-03
TG(46:0)	12.18	11.80	0.094	0.338	9.73	9.43	9.17E-04	5.13E-03
LPC(0:0/20:3)	11.34	11.24	0.568	0.732	8.98	8.60	9.18E-04	5.13E-03
TG(51:2)	15.56	15.43	0.318	0.553	12.79	12.46	9.50E-04	5.19E-03
TG(53:3)	15.22	15.07	0.269	0.518	12.45	12.14	1.06E-03	5.69E-03
TG(52:2)	18.25	18.13	0.145	0.399	16.02	15.80	1.20E-03	6.23E-03
LPC(20:5/0:0)	12.85	12.54	0.195	0.433	10.51	10.03	1.21E-03	6.23E-03
TG(53:1)	13.51	13.40	0.510	0.677	9.63	9.34	1.26E-03	6.32E-03
TG(58:9)_1	12.62	12.50	0.369	0.593	10.24	10.01	1.46E-03	7.21E-03
TG(60:9)	10.17	10.20	0.770	0.879	8.22	7.99	1.54E-03	7.45E-03
SM(d18:2/22:0)	16.04	15.89	0.116	0.362	14.32	14.12	1.61E-03	7.63E-03
TG(53:2)	15.18	15.08	0.512	0.677	12.29	11.98	1.73E-03	8.01E-03
TG(52:1)	16.18	15.98	0.171	0.403	13.59	13.29	1.81E-03	8.23E-03
LPC(20:3/0:0)_1	13.26	13.17	0.615	0.762	11.07	10.69	1.85E-03	8.25E-03
PC(O-24:0/20:4)	12.32	12.19	0.365	0.593	9.90	9.68	2.20E-03	9.39E-03
SM(d17:1/16:0)	15.79	15.72	0.391	0.609	13.85	13.66	2.26E-03	9.41E-03
TG(49:2)	13.97	13.77	0.240	0.483	11.12	10.80	2.40E-03	9.78E-03
Cer(d18:2/22:0)	12.98	12.71	0.057	0.289	10.76	10.55	2.71E-03	0.011
PC(16:0/18:3)	14.96	14.91	0.632	0.767	13.39	13.14	2.85E-03	0.011
PE(16:0/20:4)	13.98	13.82	0.213	0.451	12.67	12.45	3.32E-03	0.013
LPC(14:0/0:0)	12.60	12.34	0.157	0.403	7.98	7.58	3.42E-03	0.013
SM(d18:2/24:0)	15.82	15.65	0.072	0.296	13.78	13.61	3.43E-03	0.013
PC(18:0/18:2)	18.84	18.78	0.270	0.518	17.69	17.57	3.53E-03	0.013
TG(53:5)	12.59	12.36	0.131	0.372	10.02	9.71	4.12E-03	0.014
TG(54:5)_2	16.13	15.96	0.194	0.433	13.42	13.16	4.22E-03	0.014
Cer(d18:1/24:1)	15.45	15.35	0.283	0.519	13.42	13.25	4.28E-03	0.014
Cholesterol	15.07	15.01	0.224	0.463	14.38	14.24	4.69E-03	0.015

TG(50:2)	17.32	17.12	0.096	0.338	14.98	14.74	5.11E-03	0.017
LPC(0:0/14:0)	10.30	10.01	0.111	0.354	8.05	7.70	5.94E-03	0.019
PC(18:0/22:5)	14.77	14.55	0.128	0.367	12.88	12.67	6.39E-03	0.020
DG(18:2/18:1)	13.27	13.01	0.054	0.289	12.04	11.81	7.52E-03	0.023
TG(48:1)	15.70	15.47	0.163	0.403	13.16	12.86	7.79E-03	0.024
SM(d18:1/16:0)	18.35	18.28	0.087	0.328	16.96	16.86	7.82E-03	0.024
CE(20:5)	8.32	8.18	0.356	0.593	9.09	8.83	8.09E-03	0.024
PE(18:0/20:4)	15.03	14.89	0.159	0.403	13.64	13.46	8.46E-03	0.025
LPC(17:1/0:0)	11.22	11.12	0.500	0.672	9.07	8.83	8.80E-03	0.026
SM(d18:1/24:1)	17.62	17.57	0.446	0.635	15.96	15.85	9.33E-03	0.027
LPC(0:0/15:0)	10.17	9.94	0.172	0.403	8.06	7.82	9.43E-03	0.027
LPC(0:0/20:2)	9.13	9.01	0.268	0.518	7.38	7.07	1.00E-02	0.028
TG(55:2)	12.05	12.04	0.945	0.979	9.47	9.24	0.010	0.028
LPC(0:0/18:3)	9.79	9.62	0.319	0.553	7.63	7.18	0.011	0.029
LPC(18:3/0:0)_1	12.03	11.88	0.411	0.613	9.79	9.39	0.011	0.029
TG(54:1)	13.76	13.59	0.333	0.568	11.05	10.81	0.011	0.029
PC(18:1/20:4)	16.63	16.51	0.195	0.433	15.11	14.95	0.011	0.029
LPC(0:0/22:6)	11.77	11.55	0.203	0.439	9.46	9.16	0.011	0.029
PC(16:0/18:1)_2	19.23	19.17	0.166	0.403	18.21	18.12	0.011	0.029
TG(54:2)	16.89	16.79	0.449	0.635	14.26	14.03	0.013	0.031
SM(d18:2/24:1)	17.11	17.03	0.281	0.519	15.44	15.31	0.013	0.031
LPC(20:2/0:0)	11.55	11.36	0.198	0.433	9.11	8.87	0.013	0.031
LPC(0:0/20:4)	12.86	12.69	0.366	0.593	10.58	10.29	0.013	0.031
PC(O-26:1/20:4) and/or PC(P-26:0/20:4)	11.68	11.57	0.470	0.649	9.34	9.17	0.013	0.031
SM(d32:1); SM(d16:1/16:0) SM(d18:1/14:0)	16.41	16.29	0.078	0.307	14.48	14.34	0.013	0.032
SM(d18:0/16:0)	15.64	15.52	0.050	0.289	13.89	13.76	0.014	0.034
TG(55:4)	12.73	12.65	0.528	0.690	10.16	9.96	0.014	0.034

LPC(20:4/0:0)	14.72	14.58	0.414	0.614	12.58	12.29	0.015	0.035
PC(18:0/22:4)	14.73	14.56	0.121	0.366	12.84	12.67	0.015	0.036
LPC(22:5/0:0)_1	12.35	12.11	0.161	0.403	9.95	9.69	0.016	0.036
TG(58:6)_2	12.32	12.22	0.300	0.539	10.09	9.92	0.016	0.036
TG(49:3)	12.65	12.39	0.151	0.399	9.81	9.57	0.016	0.036
SM(d17:1/24:1)	15.41	15.35	0.451	0.635	13.26	13.11	0.017	0.037
SM(d18:2/14:0)	13.78	13.60	0.096	0.338	11.40	11.25	0.017	0.037
TG(58:7)_2	11.00	11.05	0.640	0.767	9.56	9.41	0.017	0.037
LPC(0:0/18:2)	13.93	13.77	0.364	0.593	11.82	11.55	0.017	0.037
Zeaxanthin	10.97	10.91	0.720	0.832	8.94	8.72	0.017	0.037
LPC(22:6/0:0)	13.93	13.75	0.312	0.548	11.62	11.33	0.018	0.038
TG(54:4)	17.62	17.52	0.277	0.519	15.26	15.08	0.018	0.038
Anhydrolutein I	8.35	8.41	0.703	0.820	9.14	8.94	0.018	0.038
PC(16:0/17:0)	13.54	13.56	0.877	0.942	12.10	11.95	0.020	0.041
LPC(18:2/0:0)	15.80	15.68	0.406	0.613	13.89	13.62	0.020	0.041
PC(14:0/18:2)	15.20	14.99	0.053	0.289	13.17	13.00	0.020	0.041
SM(d30:1); SM(d16:1/14:0) SM(d18:1/12:0)	12.98	12.81	0.178	0.411	10.65	10.48	0.020	0.041
TG(58:8)	12.84	12.86	0.862	0.936	10.62	10.47	0.022	0.043
PC(O-24:0/18:2)	10.88	10.76	0.277	0.519	8.42	8.26	0.024	0.046
PC(33:1); PC(15:0/18:1) PC(16:0/17:1)	15.41	15.42	0.924	0.971	14.15	14.01	0.024	0.046
Hexadecadienoylcarnitine CAR(16:2)	9.50	9.73	0.136	0.380	8.50	8.68	0.025	0.049
CE(20:4)	8.88	8.83	0.549	0.711	10.65	10.38	0.026	0.049
Luteine	10.98	10.92	0.703	0.820	9.54	9.34	0.032	0.060
Cer(d42:3); Cer(d18:2/24:1) Cer(d18:1/24:2)	14.08	13.91	0.192	0.433	11.88	11.74	0.032	0.060
SM(d16:1/22:0)	14.96	14.82	0.215	0.451	7.84	7.49	0.033	0.062

LPC(18:3/0:0)_2	11.08	11.08	1.000	1.000	8.87	8.57	0.033	0.062
TG(56:5)_2	13.58	13.58	0.975	0.987	11.26	11.14	0.034	0.062
LPE(16:0/0:0)	11.47	11.25	0.055	0.289	10.06	9.89	0.034	0.062
TG(48:2)	15.53	15.25	0.128	0.367	12.85	12.61	0.036	0.065
TG(54:3)	17.74	17.69	0.594	0.754	15.37	15.22	0.037	0.067
LacCer(d18:1/16:0)	13.16	13.21	0.411	0.613	11.72	11.59	0.040	0.070
TG(56:4)	12.02	12.04	0.900	0.950	9.75	9.59	0.040	0.070
PC(O-16:1/18:2) and/or PC(P-16:0/18:2)	15.06	14.90	0.106	0.354	13.57	13.45	0.040	0.071
DG(36:2); DG(18:1/18:1) DG(18:0/18:2)	13.15	12.99	0.206	0.440	12.18	12.01	0.042	0.072
LPE(20:4/0:0)	11.88	11.75	0.505	0.676	10.23	10.00	0.042	0.072
PC(18:0/18:1)	17.97	17.92	0.500	0.672	16.58	16.48	0.046	0.079
DG(18:0/16:0)	11.51	11.35	0.061	0.291	9.69	9.58	0.047	0.079
LPC(16:1/0:0)	13.92	13.71	0.251	0.499	11.66	11.44	0.050	0.082

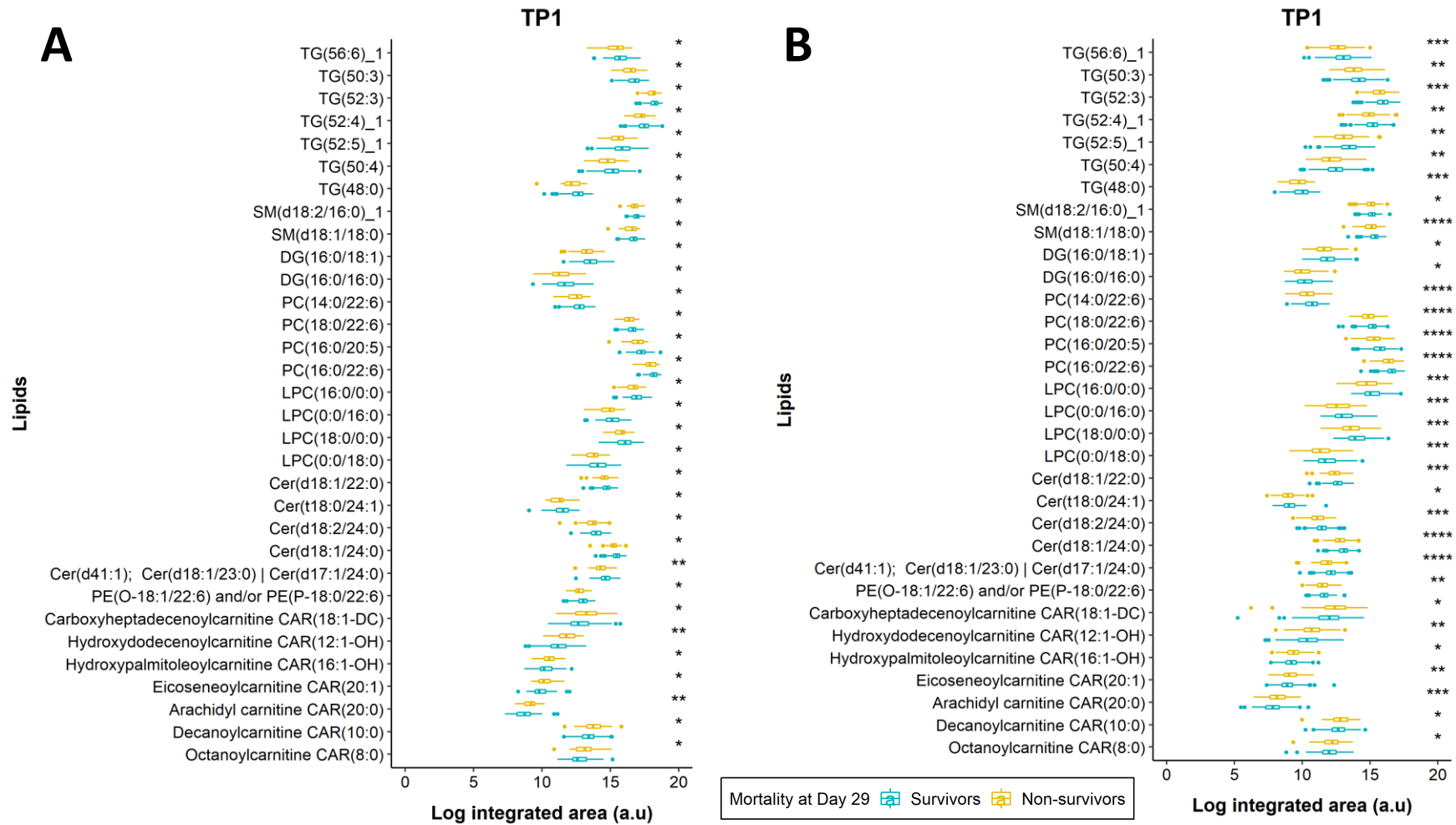


Figure 5.9: Box plots for common lipids which were significantly different between survivors and non-survivors at TP1 in VANISH (A) and LeoPARDS (B) LPOS ppR datasets.

Table 5.7: Welch two sample *t*-test results of LPOS ppR data for the VANISH and LeoPARDS cohorts at TP2. Adjusted p-values were calculated with B-H correction. Adjusted p-values (p_{Adj}) and un-adjusted p-values (p) <0.05 are highlighted in light green; Mean intensity values are highlighted for survivors and non-survivors (Yellow represents higher mean value; peach represents lower mean value). Metabolites significant in the two cohorts are highlighted in different colours (green = In both cohorts; red = Only in VANISH; blue = Only in LeoPARDS). Abbreviations: PC = Phosphatidylcholine; LPC = Lysophosphatidylcholine; PE = Phosphatidylethanolamine; LPE = Lysophosphatidylethanolamine; SM = Sphingomyelin; Cer = Ceramide; LacCer = Lactosylceramide; DG = Diacylglycerol; TG = Triglycerides; CAR = Carnitine. Note: Carbon atoms and double bonds in all lipid species are represented as (C atoms:double bond); d indicates two hydroxyl groups in the sphingoid base; DC denotes for dicarboxylic acid; Prefixes “OH”, “O” and “P” represents different substituents/linkages in the lipid species: “OH” denotes for hydroxyl group, “O” represents an alkyl ether linkage whilst “P” refers to the plasmalogen link. Lipid species with abbreviations _1 and _2 indicate same m/z value but different retention times for those lipids.

Lipids	VANISH _{TP2} (N = 103, n = 80 & 23)				LeoPARDS _{TP2} (N = 317, n = 250 & 67)			
	Log Mean integrated area (a.u)		p	p_{Adj}	Log Mean integrated area (a.u)		p	p_{Adj}
	Survivors	Non-survivors			Survivors	Non-survivors		
SM(d18:2/18:0)	16.07	15.88	0.021	0.719	14.77	14.55	6.75E-04	7.61E-03
Hydroxydodecenoylcarnitine CAR(12:1-OH)	11.24	11.76	0.011	0.719	10.20	10.51	0.041	0.081
Hydroxytetradecanoylcarnitine CAR(14:0-OH)	10.50	10.91	0.023	0.719	9.37	9.13	0.055	0.102
Arachidyl carnitine CAR(20:0)	9.07	9.49	0.042	0.999	8.27	8.02	0.134	0.201
Decanoylcarnitine CAR(10:0)	13.51	13.98	0.015	0.719	12.47	12.31	0.458	0.551
Eicoseneoylcarnitine CAR(20:1)	10.12	10.63	0.023	0.719	9.19	9.11	0.499	0.582
Hydroxydodecanoylcarnitine CAR(12:0-OH)	10.72	11.19	9.84E-03	0.719	9.57	9.52	0.706	0.765
Octanoylcarnitine CAR(8:0)	12.81	13.24	0.022	0.719	11.73	11.65	0.710	0.766
Hydroxypalmitoleoylcarnitine CAR(16:1-OH)	10.36	10.78	8.25E-03	0.719	9.29	9.26	0.755	0.807
Carboxyheptadecanoylcarnitine CAR(18:0-DC)	11.84	12.30	0.048	0.999	10.85	10.86	0.954	0.966
LPC(15:0/0:0)	12.48	12.14	0.118	0.999	10.70	10.04	1.69E-05	1.64E-03
LPC(17:0/0:0)	13.09	12.80	0.141	0.999	11.03	10.39	1.92E-05	1.64E-03
LPC(0:0/16:0)	15.39	15.10	0.117	0.999	13.71	13.13	2.39E-05	1.64E-03

LPC(16:0/0:0)	17.11	16.88	0.124	0.999	15.73	15.21	2.67E-05	1.64E-03
LPC(17:1/0:0)	11.62	11.46	0.393	0.999	9.82	9.30	4.70E-05	2.31E-03
LPC(0:0/15:0)	10.52	10.25	0.177	0.999	8.67	8.18	6.65E-05	2.73E-03
SM(d19:1/18:0)	12.51	12.41	0.578	0.999	11.33	10.85	8.80E-05	3.09E-03
TG(53:2)	15.32	15.39	0.632	0.999	12.57	12.14	1.11E-04	3.33E-03
TG(51:2)	15.61	15.66	0.713	0.999	13.04	12.63	1.36E-04	3.33E-03
LPC(18:0/0:0)	16.27	16.04	0.112	0.999	14.58	14.12	1.41E-04	3.33E-03
LPC(0:0/18:0)	14.36	14.08	0.114	0.999	12.41	11.92	1.53E-04	3.33E-03
TG(53:3)	15.36	15.36	0.955	0.999	12.75	12.33	1.63E-04	3.33E-03
SM(d18:1/18:0)	16.72	16.51	0.062	0.999	15.45	15.19	2.05E-04	3.65E-03
TG(51:3)	14.88	14.85	0.901	0.999	12.20	11.76	2.08E-04	3.65E-03
SM(d18:1/20:0)	15.88	15.77	0.288	0.999	14.42	14.15	3.25E-04	5.32E-03
LPC(0:0/20:3)	11.66	11.59	0.710	0.999	9.69	9.19	4.72E-04	7.26E-03
TG(51:4)	13.35	13.27	0.727	0.999	10.66	10.22	6.10E-04	7.61E-03
LPC(20:3/0:0)_1	13.60	13.56	0.833	0.999	11.81	11.31	6.20E-04	7.61E-03
LPC(0:0/20:5)	11.04	10.73	0.289	0.999	9.01	8.41	6.70E-04	7.61E-03
LPC(0:0/18:2)	14.33	14.14	0.379	0.999	12.52	12.04	7.08E-04	7.61E-03
LPE(18:2/0:0)	12.14	11.95	0.417	0.999	10.80	10.34	7.49E-04	7.61E-03
LPE(16:0/0:0)	11.59	11.46	0.386	0.999	10.38	10.08	7.65E-04	7.61E-03
LPC(14:0/0:0)	12.94	12.70	0.287	0.999	8.80	8.24	8.05E-04	7.61E-03
LPC(20:5/0:0)	13.22	12.91	0.289	0.999	11.28	10.71	8.22E-04	7.61E-03
TG(53:4)	14.39	14.34	0.734	0.999	11.76	11.35	8.29E-04	7.61E-03
LPC(18:2/0:0)	16.17	15.99	0.328	0.999	14.58	14.12	8.57E-04	7.61E-03
SM(d35:1); SM(d17:1/18:0) SM(d18:1/17:0)	14.11	13.99	0.126	0.999	12.70	12.49	8.66E-04	7.61E-03
PC(18:0/20:3)	17.18	17.08	0.372	0.999	15.70	15.42	9.97E-04	8.46E-03
TG(49:1)	14.18	14.25	0.677	0.999	11.74	11.34	1.15E-03	8.94E-03
TG(52:3)	18.25	18.18	0.465	0.999	16.10	15.81	1.16E-03	8.94E-03

PC(16:0/17:0)	13.47	13.56	0.414	0.999	12.17	11.94	1.20E-03	8.94E-03
LPC(P-16:0/0:0)	12.70	12.60	0.688	0.999	11.07	10.53	1.26E-03	8.94E-03
SM(d18:1/22:0)	16.54	16.46	0.208	0.999	15.13	14.95	1.31E-03	8.94E-03
LPC(20:4/0:0)	14.86	14.73	0.520	0.999	13.13	12.70	1.33E-03	8.94E-03
LPC(O-16:0/0:0)	12.88	12.85	0.850	0.999	11.10	10.66	1.34E-03	8.94E-03
TG(52:2)	18.29	18.27	0.838	0.999	16.18	15.94	1.39E-03	8.94E-03
LPC(O-18:1/0:0)_1	12.86	12.86	0.998	0.999	10.92	10.49	1.41E-03	8.94E-03
LPE(20:4/0:0)	12.21	12.09	0.614	0.999	10.77	10.38	1.42E-03	8.94E-03
LPE(0:0/18:2)	9.82	9.65	0.398	0.999	10.79	10.38	1.61E-03	9.93E-03
CE(20:5)	8.43	8.26	0.353	0.999	9.48	9.07	1.70E-03	0.010
LPC(16:1/0:0)	14.26	14.08	0.474	0.999	12.48	12.03	1.77E-03	0.010
LPC(0:0/14:0)	10.65	10.37	0.215	0.999	8.83	8.35	1.80E-03	0.010
TG(56:6)_1	15.71	15.64	0.647	0.999	13.16	12.81	2.08E-03	0.012
LPC(0:0/20:4)	13.08	12.92	0.493	0.999	11.15	10.74	2.16E-03	0.012
TG(49:2)	13.93	13.98	0.780	0.999	11.29	10.94	2.25E-03	0.012
LPC(20:2/0:0)	11.96	11.82	0.421	0.999	9.79	9.41	2.56E-03	0.013
TG(52:4)_1	17.47	17.38	0.509	0.999	15.33	14.99	2.63E-03	0.013
DG(18:2/18:1)	13.31	13.29	0.897	0.999	12.20	11.85	2.71E-03	0.014
SM(d18:1/20:1)	15.19	15.07	0.167	0.999	13.81	13.60	2.98E-03	0.014
LPC(19:0/0:0)	10.51	10.30	0.241	0.999	8.43	8.03	2.99E-03	0.014
LPC(22:5/0:0)_1	12.54	12.39	0.512	0.999	10.39	10.00	3.05E-03	0.014
LPC(18:1/0:0)_1	16.32	16.24	0.673	0.999	14.77	14.39	3.12E-03	0.014
PC(16:0/20:5)	17.22	17.00	0.117	0.999	15.82	15.56	3.29E-03	0.015
PC(16:0/22:6)	18.08	17.94	0.136	0.999	16.54	16.32	3.40E-03	0.015
LPC(0:0/16:1)	12.18	11.97	0.365	0.999	10.16	9.75	3.78E-03	0.016
LPC(0:0/18:1)	14.36	14.27	0.643	0.999	12.63	12.24	3.79E-03	0.016
TG(53:5)	12.74	12.65	0.668	0.999	10.28	9.88	4.00E-03	0.017
Cer(d18:1/24:0)	15.49	15.41	0.349	0.999	13.16	12.95	4.08E-03	0.017

PC(15:0/18:2)	14.46	14.35	0.416	0.999	13.19	12.95	4.17E-03	0.017
LPC(20:1/0:0)	12.41	12.37	0.793	0.999	10.25	9.90	4.20E-03	0.017
SM(d18:1/23:0)	15.91	15.78	0.100	0.999	13.97	13.76	4.22E-03	0.017
LPC(0:0/20:2)	9.41	9.32	0.494	0.999	7.87	7.56	4.29E-03	0.017
TG(54:5)_2	16.17	16.16	0.906	0.999	13.65	13.37	4.57E-03	0.017
LPE(0:0/18:0)	9.92	9.85	0.613	0.999	8.38	8.22	4.60E-03	0.017
LPC(0:0/22:6)	11.97	11.87	0.615	0.999	9.84	9.47	4.63E-03	0.017
DG(36:2); DG(18:1/18:1) DG(18:0/18:2)	13.15	13.23	0.556	0.999	12.37	12.10	4.87E-03	0.018
LPC(0:0/22:5)_1	10.01	9.92	0.571	0.999	8.10	7.76	5.01E-03	0.018
SM(d18:1/24:1)	17.73	17.71	0.764	0.999	16.21	16.09	5.28E-03	0.019
TG(50:3)	16.72	16.69	0.852	0.999	14.33	14.03	5.76E-03	0.020
TG(53:1)	13.64	13.70	0.715	0.999	9.77	9.44	5.91E-03	0.020
LPC(22:6/0:0)	14.14	13.97	0.440	0.999	12.02	11.64	6.21E-03	0.021
DG(16:0/18:1)	13.46	13.50	0.822	0.999	11.99	11.71	6.56E-03	0.022
LPC(20:3/0:0)_2	11.18	11.18	0.999	0.999	9.34	8.97	6.68E-03	0.022
LPC(0:0/18:3)	10.15	9.99	0.477	0.999	8.18	7.79	6.73E-03	0.022
SM(d17:1/16:0)	15.86	15.79	0.384	0.999	13.96	13.76	7.18E-03	0.023
SM(d17:1/24:1)	15.49	15.47	0.737	0.999	13.49	13.31	7.55E-03	0.024
DG(34:2)	12.07	12.07	0.969	0.999	10.98	10.71	7.98E-03	0.025
TG(49:3)	12.63	12.67	0.808	0.999	9.97	9.66	8.05E-03	0.025
PC(14:0/22:6)	12.70	12.58	0.416	0.999	10.89	10.62	8.10E-03	0.025
TG(50:2)	17.30	17.30	0.956	0.999	15.11	14.87	8.53E-03	0.026
Cer(d41:1); Cer(d18:1/23:0) Cer(d17:1/24:0)	14.60	14.45	0.231	0.999	12.10	11.89	8.67E-03	0.026
Cer(d18:2/24:0)	13.96	13.92	0.722	0.999	11.40	11.22	9.49E-03	0.028
SM(d18:1/16:0)	18.40	18.35	0.250	0.999	17.09	16.99	9.52E-03	0.028
TG(52:5)_1	15.94	15.81	0.518	0.999	13.72	13.41	0.011	0.031
LPE(18:0/0:0)	12.06	11.99	0.588	0.999	10.73	10.54	0.011	0.031

LPC(18:3/0:0)_2	11.84	11.63	0.470	0.999	9.89	9.44	0.011	0.032
PC(16:0/20:4)_1	15.47	15.46	0.938	0.999	13.63	13.38	0.012	0.032
LacCer(d18:1/16:0)	13.25	13.28	0.732	0.999	11.90	11.71	0.012	0.034
CE(20:4)	8.87	8.86	0.952	0.999	10.95	10.64	0.013	0.034
TG(55:2)	12.15	12.33	0.295	0.999	9.63	9.35	0.013	0.035
SM(d18:1/24:0)	16.15	16.05	0.275	0.999	14.47	14.32	0.013	0.035
SM(d18:0/16:0)	15.75	15.67	0.264	0.999	14.18	14.02	0.013	0.035
TG(50:4)	15.17	15.09	0.716	0.999	12.75	12.48	0.014	0.037
LPC(20:0/0:0)	11.09	10.91	0.356	0.999	9.08	8.76	0.014	0.037
DG(18:0/18:1)	11.89	11.96	0.672	0.999	10.33	10.08	0.015	0.037
PC(16:0/18:2)	19.18	19.14	0.448	0.999	18.17	18.06	0.018	0.045
LPC(P-18:0/0:0)	10.86	10.73	0.515	0.999	9.05	8.76	0.018	0.046
TG(48:2)	15.47	15.53	0.748	0.999	13.03	12.74	0.019	0.046
LPC(O-18:0/0:0)	11.72	11.74	0.932	0.999	9.88	9.61	0.020	0.050
SM(d18:2/24:1)	17.22	17.18	0.560	0.999	15.65	15.54	0.021	0.051
TG(50:1)	16.97	16.94	0.821	0.999	14.78	14.54	0.022	0.052
LPC(18:3/0:0)_1	12.40	12.26	0.563	0.999	10.44	9.94	0.022	0.052
TG(48:4)_1	11.96	12.07	0.659	0.999	9.72	9.39	0.025	0.059
DG(16:0/16:0)	11.55	11.57	0.945	0.999	10.24	10.03	0.026	0.060
PE(O-18:1/22:6) and/or PE(P-18:0/22:6)	13.05	12.89	0.104	0.999	11.79	11.63	0.026	0.060
TG(48:1)	15.62	15.65	0.849	0.999	13.27	12.98	0.026	0.060
PC(18:0/22:6)	16.54	16.36	0.078	0.999	15.07	14.90	0.027	0.060
Cer(d18:1/22:0)	14.74	14.66	0.499	0.999	12.65	12.49	0.027	0.060
TG(54:4)	17.74	17.74	0.973	0.999	15.48	15.26	0.028	0.061
TG(58:9)_1	12.59	12.55	0.779	0.999	10.29	10.10	0.028	0.061
DG(32:1)	11.12	11.17	0.805	0.999	9.85	9.65	0.029	0.063
LPC(O-24:1/0:0)	11.67	11.54	0.573	0.999	9.72	9.44	0.029	0.064

TG(48:3)	14.20	14.24	0.859	0.999	11.66	11.39	0.031	0.066
Cer(d18:1/24:1)	15.50	15.55	0.660	0.999	13.47	13.32	0.031	0.066
SM(d18:2/22:0)	16.10	16.03	0.294	0.999	14.50	14.36	0.031	0.066
TG(54:2)	16.97	17.04	0.595	0.999	14.45	14.21	0.032	0.068
Cer(d18:1/18:0)	12.97	12.85	0.512	0.999	11.23	11.02	0.034	0.070
Cer(d18:1/20:0)	13.38	13.33	0.782	0.999	11.31	11.12	0.034	0.071
Cer(t18:0/24:1)	11.68	11.44	0.132	0.999	9.31	9.12	0.037	0.075
Tetracosanoylcarnitine CAR(24:0)	11.10	11.22	0.390	0.999	10.15	9.92	0.038	0.077
PE(16:0/18:2)	13.80	13.82	0.897	0.999	12.55	12.35	0.039	0.078
DG(36:4)_1; DG(18:2/18:2) DG(18:1/18:3)	11.30	11.20	0.681	0.999	10.20	9.93	0.040	0.081
TG(56:5)_2	13.63	13.66	0.719	0.999	11.35	11.22	0.041	0.081
PE(16:0/20:4)	13.96	13.98	0.909	0.999	12.74	12.57	0.045	0.088
TG(46:1)	13.73	13.81	0.728	0.999	11.30	11.03	0.049	0.095

Table 5.8: Welch two sample *t*-test results of LPOS ppR data for the VANISH and LeoPARDS cohorts at TP3. Adjusted p-values were calculated with B-H correction. Adjusted p-values (p_{Adj}) and un-adjusted p-values (p) <0.05 are highlighted in light green; Mean intensity values are highlighted for survivors and non-survivors (Yellow represents higher mean value; peach represents lower mean value). Metabolites significant in the two cohorts are highlighted in different colours (green = In both cohorts; red = Only in VANISH; blue = Only in LeoPARDS). Abbreviations: PC = Phosphatidylcholine; LPC = Lysophosphatidylcholine; PE = Phosphatidylethanolamine; LPE = Lysophosphatidylethanolamine; SM = Sphingomyelin; Cer = Ceramide; LacCer = Lactosylceramide; CE = Cholesteryl esters; DG = Diacylglycerol; TG = Triglycerides; CAR = Carnitine. Note: Carbon atoms and double bonds in all lipid species are represented as (C atoms:double bond); d indicates two hydroxyl groups in the sphingoid base; DC denotes for dicarboxylic acid; Prefixes “OH”, “O” and “P” represents different substituents/linkages in the lipid species: “OH” denotes for hydroxyl group, “O” represents an alkyl ether linkage whilst “P” refers to the plasmalogen link. Lipid species with abbreviations _1 and _2 indicate same m/z value but different retention times for those lipids.

Lipids	VANISH _{TP3} (N = 75, n = 59 & 16)				LeoPARDS _{TP3} (N = 255, n = 205 & 50)			
	Log Mean integrated area (a.u)		p	p_{Adj}	Log Mean integrated area (a.u)		p	p_{Adj}
	Survivors	Non-survivors			Survivors	Non-survivors		
SM(d18:1/23:0)	15.94	15.74	0.023	0.529	14.08	13.82	1.23E-03	6.57E-03
SM(d18:1/16:0)	18.41	18.29	0.036	0.529	17.11	17.00	0.026	0.049
PE(O-18:1/22:6) and/or PE(P-18:0/22:6)	12.98	12.71	0.013	0.529	11.76	11.58	0.022	0.044
LPE(0:0/18:2)	10.11	9.64	0.046	0.529	10.93	10.57	0.029	0.054
LPE(18:2/0:0)	12.44	11.91	0.040	0.529	10.95	10.53	0.013	0.030
LPC(0:0/20:3)	11.97	11.47	0.038	0.529	9.96	9.45	1.28E-03	6.64E-03
LPC(0:0/20:4)	13.34	12.80	0.040	0.529	11.39	10.93	1.35E-03	6.70E-03
LPC(20:4/0:0)	15.07	14.64	0.041	0.529	13.33	12.87	1.77E-03	7.42E-03
LPC(0:0/22:6)	12.12	11.49	0.013	0.529	9.98	9.54	4.28E-03	0.015
LPC(0:0/18:2)	14.63	14.04	0.010	0.529	12.73	12.26	4.31E-03	0.015
LPC(20:2/0:0)	12.29	11.81	0.027	0.529	10.07	9.69	5.05E-03	0.016
LPC(22:6/0:0)	14.31	13.70	0.017	0.529	12.16	11.72	5.13E-03	0.016
LPC(18:2/0:0)	16.44	15.94	0.011	0.529	14.79	14.34	5.26E-03	0.017
LPC(22:5/0:0)_1	12.71	12.19	0.040	0.529	10.57	10.20	9.04E-03	0.023
LPC(22:5/0:0)_2	10.94	10.50	0.036	0.529	9.16	8.96	0.219	0.279

SM(d18:0/16:0)	15.81	15.59	8.70E-03	0.529	14.21	14.10	0.167	0.225
TG(49:3)	12.54	12.74	0.197	0.634	10.09	9.51	1.18E-05	1.61E-03
LPC(15:0/0:0)	12.72	12.53	0.436	0.733	11.09	10.40	3.25E-05	1.61E-03
LPC(16:0/0:0)	17.24	16.96	0.097	0.529	15.99	15.46	4.03E-05	1.61E-03
LPC(0:0/18:0)	14.57	14.22	0.078	0.529	12.71	12.11	4.20E-05	1.61E-03
LPC(17:0/0:0)	13.33	13.08	0.308	0.684	11.39	10.73	4.53E-05	1.61E-03
LPC(0:0/16:0)	15.56	15.22	0.095	0.529	14.01	13.41	4.91E-05	1.61E-03
LPC(18:0/0:0)	16.45	16.18	0.098	0.529	14.85	14.30	4.99E-05	1.61E-03
LPC(0:0/15:0)	10.74	10.54	0.413	0.731	9.01	8.47	5.80E-05	1.61E-03
TG(49:2)	13.86	14.11	0.166	0.619	11.36	10.85	5.89E-05	1.61E-03
TG(46:0)	11.86	12.05	0.390	0.707	9.79	9.34	8.14E-05	2.00E-03
LPC(0:0/14:0)	10.93	10.64	0.321	0.687	9.27	8.63	1.14E-04	2.55E-03
TG(48:4)_1	11.86	12.05	0.365	0.707	9.85	9.15	1.36E-04	2.64E-03
TG(51:4)	13.27	13.34	0.700	0.915	10.80	10.19	1.55E-04	2.64E-03
LPC(14:0/0:0)	13.24	12.95	0.310	0.684	9.24	8.61	1.68E-04	2.64E-03
TG(51:3)	14.82	14.95	0.385	0.707	12.28	11.75	1.69E-04	2.64E-03
TG(50:4)	15.09	15.07	0.910	0.995	12.92	12.40	1.72E-04	2.64E-03
TG(49:1)	14.11	14.44	0.055	0.529	11.81	11.32	1.93E-04	2.71E-03
LPC(O-24:1/0:0)	11.94	11.64	0.366	0.707	10.20	9.67	1.98E-04	2.71E-03
TG(52:5)_1	15.88	15.78	0.613	0.852	13.91	13.33	2.16E-04	2.79E-03
TG(48:3)	14.07	14.23	0.438	0.733	11.76	11.23	2.35E-04	2.88E-03
LPC(17:1/0:0)	11.87	11.63	0.264	0.684	10.08	9.62	2.93E-04	3.29E-03
TG(46:1)	13.65	14.03	0.123	0.581	11.35	10.81	2.94E-04	3.29E-03
TG(48:2)	15.40	15.59	0.304	0.684	13.09	12.59	3.13E-04	3.30E-03
TG(50:3)	16.64	16.69	0.777	0.960	14.43	13.96	3.43E-04	3.30E-03
LPC(P-16:0/0:0)	13.08	12.67	0.172	0.620	11.55	10.92	3.44E-04	3.30E-03
PC(18:0/22:6)	16.39	16.17	0.097	0.529	15.09	14.75	3.49E-04	3.30E-03
LPC(O-16:0/0:0)	13.15	12.87	0.201	0.634	11.47	10.98	3.96E-04	3.61E-03

LPC(19:0/0:0)	10.78	10.47	0.217	0.652	8.76	8.29	4.29E-04	3.77E-03
TG(51:2)	15.57	15.80	0.102	0.529	13.09	12.67	4.51E-04	3.82E-03
LPC(20:5/0:0)	13.56	12.89	0.052	0.529	11.52	10.84	5.00E-04	4.10E-03
LPC(O-18:0/0:0)	12.02	11.74	0.208	0.648	10.22	9.79	6.20E-04	4.75E-03
PC(14:0/22:6)	12.63	12.56	0.675	0.902	11.04	10.62	6.92E-04	4.75E-03
TG(53:3)	15.35	15.49	0.354	0.707	12.79	12.37	6.97E-04	4.75E-03
LPC(0:0/20:5)	11.35	10.69	0.058	0.529	9.27	8.58	6.98E-04	4.75E-03
TG(44:1)	13.04	13.64	0.138	0.601	10.76	10.17	7.06E-04	4.75E-03
PC(18:0/20:3)	17.26	17.16	0.485	0.764	15.81	15.46	7.15E-04	4.75E-03
TG(53:4)	14.36	14.43	0.685	0.911	11.85	11.37	7.21E-04	4.75E-03
PC(16:0/22:6)	17.96	17.74	0.056	0.529	16.57	16.24	7.34E-04	4.75E-03
SM(d18:1/22:0)	16.63	16.47	0.084	0.529	15.23	14.99	8.75E-04	5.52E-03
LPC(P-18:0/0:0)	11.19	10.81	0.158	0.616	9.45	8.99	9.23E-04	5.68E-03
LPC(O-24:0/0:0)	12.39	12.15	0.370	0.707	10.52	10.09	1.02E-03	6.03E-03
TG(53:5)	12.68	12.71	0.880	0.992	10.42	9.90	1.04E-03	6.03E-03
LPE(16:0/0:0)	11.74	11.59	0.371	0.707	10.56	10.22	1.05E-03	6.03E-03
TG(52:4)_1	17.42	17.39	0.818	0.969	15.42	14.99	1.14E-03	6.35E-03
TG(48:1)	15.55	15.77	0.240	0.666	13.31	12.87	1.23E-03	6.57E-03
SM(d18:1/20:0)	15.95	15.76	0.153	0.616	14.53	14.22	1.29E-03	6.64E-03
PC(16:0/20:4)_1	15.56	15.58	0.891	0.992	13.87	13.48	1.38E-03	6.70E-03
TG(46:2)	14.26	14.65	0.246	0.666	11.60	10.99	1.39E-03	6.70E-03
PC(16:0/20:5)	17.17	16.99	0.231	0.654	15.82	15.46	1.52E-03	7.20E-03
CE(20:5)	8.52	8.14	0.069	0.529	9.68	9.29	1.63E-03	7.32E-03
TG(52:3)	18.22	18.20	0.804	0.960	16.15	15.82	1.63E-03	7.32E-03
TG(50:2)	17.26	17.35	0.508	0.777	15.15	14.82	1.64E-03	7.32E-03
TG(48:0)	12.33	12.41	0.644	0.875	10.10	9.74	1.73E-03	7.42E-03
LPC(20:3/0:0)_1	13.97	13.47	0.047	0.529	12.09	11.57	1.77E-03	7.42E-03
LPC(16:1/0:0)	14.53	14.07	0.129	0.600	12.76	12.31	1.78E-03	7.42E-03

Hydroxydodecenoylcarnitine CAR(12:1-OH)	11.14	11.52	0.106	0.534	10.04	10.56	1.87E-03	7.54E-03
SM(d18:1/24:0)	16.26	16.12	0.139	0.601	14.64	14.40	1.87E-03	7.54E-03
SM(d18:1/18:0)	16.69	16.43	0.066	0.529	15.46	15.19	2.20E-03	8.73E-03
LPC(20:0/0:0)	11.43	11.11	0.228	0.652	9.46	9.01	2.40E-03	9.36E-03
LPE(18:0/0:0)	12.22	12.09	0.383	0.707	10.87	10.60	2.59E-03	9.95E-03
LPC(0:0/20:2)	9.64	9.32	0.063	0.529	8.10	7.77	2.72E-03	0.010
LPC(0:0/16:1)	12.46	11.95	0.071	0.529	10.43	10.01	2.77E-03	0.010
SM(d18:1/20:1)	15.16	14.97	0.190	0.634	13.91	13.65	3.06E-03	0.011
SM(d18:2/22:0)	16.12	15.98	0.201	0.634	14.61	14.37	3.13E-03	0.011
TG(56:6)_1	15.64	15.64	0.979	0.996	13.17	12.76	3.19E-03	0.011
TG(50:1)	16.93	17.02	0.528	0.797	14.81	14.49	3.80E-03	0.013
TG(53:2)	15.34	15.58	0.175	0.625	12.55	12.21	4.07E-03	0.014
SM(d18:2/24:0)	15.90	15.82	0.373	0.707	14.06	13.85	4.47E-03	0.015
LPC(O-18:1/0:0)_1	13.17	12.84	0.163	0.616	11.28	10.90	4.95E-03	0.016
TG(54:5)_2	16.18	16.25	0.718	0.925	13.72	13.39	5.35E-03	0.017
Octanoylcarnitine CAR(8:0)	12.61	12.91	0.134	0.601	11.68	11.99	5.53E-03	0.017
PC(O-16:1/22:6) and/or PC(P-16:0/22:6)	14.03	13.91	0.345	0.707	12.77	12.57	5.73E-03	0.017
DG(32:1)	11.08	11.13	0.847	0.974	9.89	9.57	5.86E-03	0.018
LPE(20:4/0:0)	12.44	11.97	0.066	0.529	10.87	10.46	6.04E-03	0.018
DG(18:2/18:1)	13.26	13.22	0.796	0.960	12.28	11.92	6.35E-03	0.019
DG(34:2)	12.01	11.95	0.693	0.915	11.05	10.73	6.90E-03	0.020
TG(52:1)	16.14	16.32	0.329	0.692	13.62	13.27	7.47E-03	0.021
TG(52:2)	18.27	18.31	0.573	0.820	16.19	15.97	7.60E-03	0.021
TG(53:1)	13.67	13.91	0.191	0.634	9.69	9.35	7.65E-03	0.021
SM(d35:1); SM(d17:1/18:0) SM(d18:1/17:0)	14.07	14.00	0.495	0.771	12.75	12.54	7.66E-03	0.021
PC(O-22:0/20:3)	10.54	10.53	0.946	0.996	8.59	8.34	7.93E-03	0.022

SM(d18:2/18:0)	16.04	15.79	0.052	0.529	14.77	14.57	8.30E-03	0.022
TG(46:3)	11.86	12.25	0.311	0.684	9.14	8.77	8.38E-03	0.022
PC(O-18:0/20:3)	12.85	12.82	0.843	0.973	11.38	11.13	8.40E-03	0.022
SM(d18:2/23:0)	15.22	15.00	0.094	0.529	13.20	12.99	8.72E-03	0.023
PC(15:0/18:2)	14.38	14.45	0.558	0.817	13.24	12.96	8.78E-03	0.023
Cer(d18:2/24:0)	13.88	13.85	0.867	0.983	11.36	11.13	8.84E-03	0.023
Carboxyheptadecenoylcarnitine CAR(18:1-DC)	12.74	13.03	0.375	0.707	11.11	11.77	9.53E-03	0.024
LPC(0:0/18:3)	10.41	9.93	0.076	0.529	8.45	8.02	9.79E-03	0.024
LPC(0:0/22:5)_1	10.09	9.71	0.062	0.529	8.26	7.93	0.010	0.025
TG(58:9)_1	12.51	12.39	0.435	0.733	10.19	9.91	0.011	0.026
DG(16:0/18:1)	13.47	13.42	0.803	0.960	12.03	11.73	0.012	0.028
SM(d19:1/18:0)	12.48	12.27	0.357	0.707	11.33	10.95	0.013	0.030
LPC(18:3/0:0)_1	12.71	12.21	0.068	0.529	10.74	10.25	0.013	0.030
LPE(0:0/18:0)	10.07	9.98	0.507	0.777	8.49	8.32	0.013	0.031
LPC(18:3/0:0)_2	12.23	11.84	0.281	0.684	10.17	9.66	0.014	0.032
LPC(18:1/0:0)_1	16.59	16.28	0.091	0.529	15.00	14.66	0.014	0.032
DG(36:4)_1; DG(18:2/18:2) DG(18:1/18:3)	11.24	11.18	0.825	0.971	10.32	9.95	0.014	0.033
SM(d17:1/24:1)	15.53	15.49	0.699	0.915	13.61	13.40	0.015	0.033
PC(O-24:1/20:3) and/or PC(P- 24:0/20:3)	11.58	11.58	0.978	0.996	9.66	9.36	0.016	0.034
LPC(0:0/18:1)	14.68	14.31	0.068	0.529	12.88	12.53	0.016	0.034
LPC(24:0/0:0)	11.80	11.53	0.299	0.684	10.25	9.91	0.016	0.034
DG(16:0/16:0)	11.55	11.52	0.902	0.992	10.26	9.97	0.016	0.034
PC(O-18:1/22:6) and/or PC(P- 18:0/22:6)	14.76	14.61	0.292	0.684	13.18	12.97	0.016	0.035
PC(14:0/20:4)	13.50	13.50	0.993	0.996	11.77	11.51	0.016	0.035
TG(42:1)	10.84	11.47	0.119	0.574	8.84	8.65	0.017	0.035

Cer(d18:1/24:0)	15.50	15.41	0.480	0.764	13.14	12.94	0.017	0.035
SM(d18:2/24:1)	17.25	17.15	0.269	0.684	15.72	15.58	0.018	0.038
LPC(20:3/0:0)_2	11.53	11.12	0.076	0.529	9.54	9.18	0.019	0.038
SM(d17:1/16:0)	15.84	15.77	0.391	0.707	13.98	13.77	0.021	0.043
LPC(20:1/0:0)	12.73	12.39	0.103	0.529	10.53	10.24	0.023	0.045
PC(O-24:0/18:2)	11.01	10.80	0.133	0.601	8.75	8.56	0.024	0.047
Cer(d41:1); Cer(d18:1/23:0) Cer(d17:1/24:0)	14.52	14.33	0.198	0.634	12.03	11.83	0.024	0.047
TG(44:3)	10.82	11.27	0.346	0.707	8.64	8.38	0.025	0.049
PC(18:0/22:5)	14.65	14.53	0.449	0.747	12.92	12.70	0.026	0.049
PC(16:0/17:0)	13.49	13.64	0.245	0.666	12.20	12.01	0.028	0.053
TG(44:2)	12.86	13.52	0.189	0.634	10.51	9.44	0.032	0.059
PC(O-24:2/20:4) and/or PC(P- 24:1/20:4)	13.49	13.39	0.608	0.849	11.54	11.28	0.032	0.059
PC(O-22:0/18:2)	11.48	11.39	0.536	0.804	9.44	9.26	0.032	0.060
PC(16:0/18:2)	19.15	19.05	0.171	0.620	18.16	18.03	0.035	0.064
Decanoylcarnitine CAR(10:0)	13.34	13.57	0.274	0.684	12.41	12.64	0.036	0.065
PC(O-18:0/20:4)	15.54	15.51	0.768	0.959	13.97	13.78	0.036	0.066
SM(d18:1/24:1)	17.80	17.74	0.379	0.707	16.29	16.18	0.037	0.067
DG(18:0/18:1)	11.94	12.05	0.625	0.859	10.27	10.02	0.040	0.071
TG(54:1)	13.81	14.12	0.221	0.652	11.09	10.82	0.043	0.075
Palmitoylcarnitine CAR(16:0)	12.75	12.62	0.561	0.817	11.84	11.63	0.047	0.083
DG(18:0/16:0)	11.43	11.47	0.759	0.958	9.64	9.50	0.047	0.083
TG(54:5)_1	17.04	17.05	0.988	0.996	14.85	14.57	0.048	0.084

5.4.1.2.2 LNEG ppR

Univariate analysis of 63 lipids obtained by feature extraction of LNEG dataset via peakPanther (LNEG ppR) was performed for all timepoints on a total of 488 serum samples from VANISH and 1443 samples from LeoPARDS, figure 5.10 and figure 5.11. In total 23 samples were excluded from VANISH and of those, 19 samples had no sample timing recorded and 4 samples belonged to a patient whose samples were collected after several days of receiving the study drug. Samples were excluded from LeoPARDS as discussed previously in chapter 2 (section 2.2.2.3). 10 additional samples were excluded from LeoPARDS as these samples had no sample timings recorded.

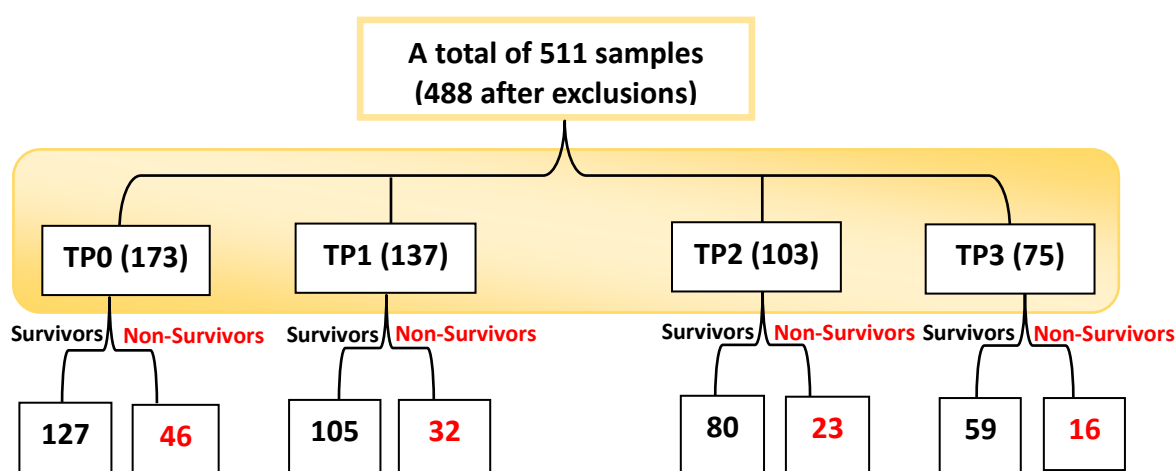


Figure 5.10: Flow chart for the samples included in the univariate analysis of LNEG ppR data from VANISH.

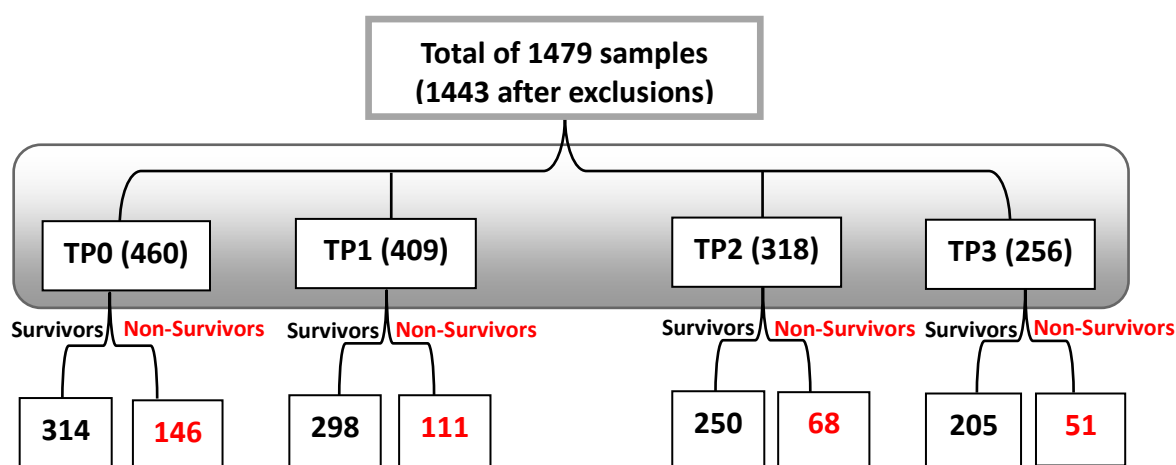


Figure 5.11: Flow chart for the samples included in the univariate analysis of LNEG ppR data from LeoPARDS.

A summary of univariate results for all timepoints in both cohorts is presented in tables 5.9, 5.10, 5.11 and 5.12, differentiating survivors from non-survivors at each timepoint (tables 5.9-5.12).

The results from the univariate analysis of mortality revealed that 18 lipids were significantly ($p < 0.05$) different between survivors and non-survivors in LeoPARDS at TP0 and of those 5 lipids remained significant after correcting for multiple comparisons ($p_{\text{Adj}} < 0.05$). In brief, eight fatty acids (FAs) (FA(20:4), FA(20:2), FA(20:3), FA(20:5), FA(22:4), FA(22:5)_1, FA(22:5)_2 and FA(24:4)) were significantly lower in patients who went on to die than those who didn't in LeoPARDS at TP0 and of these only two FAs (FA(20:3), FA(20:5)) remained significant after correcting for multiple comparisons. The pattern of all FAs was the same in the alternate cohort and only FA(20:5) was significant in VANISH ($p < 0.05$) but not after correction. Additionally, glycerophospholipids such as LPGs (LPG(18:1/0:0), LPG(18:2/0:0)), lysophosphatidylinositols (LPIs) (LPI(16:1/0:0), LPI(18:0/0:0), LPI(18:1/0:0)_1), phosphatidic acid (PA)(36:2);PA(18:0/18:2) | PA(18:1/18:1)) and lysophosphatidic acid (LPA) (22:6/0:0)) were significantly higher in non-survivors than survivors in LeoPARDS. However, only two glycerophospholipids (PA(36:2);PA(18:0/18:2) | PA(18:1/18:1)) and LPI(18:0/0:0) remained significant after correction in LeoPARDS. All these glycerophospholipids except for LPA (22:6/0:0)) had the same trend in VANISH as LeoPARDS. Additionally, other glycerophospholipids such as two PEs (including PE(18:0/22:6), PE(O-16:1/22:6) and/or PE(P-16:0/22:6)), and sphingolipid such as sulfohexosylceramide (SulfoHexCer) (d18:1/24:0(OH)) were significantly lower in patients who die before day 29 than those who survive in LeoPARDS ($p < 0.05$) but these results did not remain significant after correction. The pattern of these glycerophospholipids was also the same in the alternate cohort.

In LeoPARDS, 19 lipids were significantly different between patients who went on to die than those who didn't at TP1 ($p < 0.05$) and of those eight lipids (including FA(20:3), FA(20:4), LPI(18:1/0:0)_1, LPI(16:1/0:0), LPG(18:2/0:0), PE(18:0/22:6), PE(O-16:1/22:6) and/or PE(P-16:0/22:6), PA(36:2);PA(18:0/18:2) | PA(18:1/18:1)) remained significant after correcting for multiple comparisons. In brief, five FAs (including FA(20:2), FA(20:3), FA(20:4), FA(20:5), FA(22:5)_1), two LPEs (LPE(0:0/22:6), LPE(22:6/0:0)), two PEs (including PE(18:0/22:5), PE(18:0/22:6), PE(O-16:1/22:6) and/or PE(P-16:0/22:6)) and Cer(d34:0);Cer(d18:0/16:0) |

Cer(d16:0/18:0) were significantly lower in non-survivors than survivors in LeoPARDS at TP1 ($p < 0.05$) whilst bilirubin and glycerophospholipids such as LPG(18:2/0:0), 3 LPIs (LPI(16:1/0:0), LPI(18:1/0:0)_1, LPI(20:3/0:0)_2) and two PAs (PA(16:0/18:1), PA(36:2);PA(18:0/18:2) | PA(18:1/18:1)) were significantly higher in patients who went on to die than those who didn't in LeoPARDS ($p < 0.05$). These lipids also had the same pattern in VANISH and 4 lipids (LPI(18:1/0:0)_1, LPI(20:3/0:0)_2, PE(18:0/22:5), PE(O-16:1/22:6) and/or PE(P-16:0/22:6)) were common between the two cohorts based on the raw p-values.

Alterations in the lipids were also observed at later timepoints. In LeoPARDS, four glycerophospholipids (PE(18:0/22:6) and three LPEs (LPE(22:6/0:0), LPE(0:0/20:4), LPE(0:0/22:6)) were significantly lower in non-survivors with respect to survivors whilst glycerophospholipids (PA(36:2);PA(18:0/18:2) | PA(18:1/18:1) and LPI(18:1/0:0)_1)) were significantly higher in patients who went on to die than those who survived ($p_{Adj} < 0.05$). All these lipids except for PE(18:0/22:6) had the same pattern in the alternate cohort.

The results of univariate analysis also showed that glycerophospholipids such as three LPEs (including LPE(22:6/0:0), LPE(0:0/22:6), LPE(0:0/20:4)), two PEs (including PE(O-16:1/22:6) and/or PE(P-16:0/22:6), PE(18:0/22:6)) and LPI(22:5/0:0)) were significantly lower in non-survivors than survivors in LeoPARDS ($p < 0.05$) at TP3 whilst PA(36:2);PA(18:0/18:2) | PA(18:1/18:1) and two LPIs (including LPI(18:1/0:0)_1, LPI(16:1/0:0)) were significantly higher in patient who went on to die than those who didn't in LeoPARDS ($p < 0.05$) at TP3. All these lipids did not remain significant after correction in LeoPARDS and the trend for these lipids except for LPI(16:1/0:0) was the same in VANISH.

Table 5.9: Welch two sample *t*-test results of LNEG ppR data for the VANISH and LeoPARDS cohorts at TP0. Adjusted p-values were calculated with B-H correction. Adjusted p-values (p_{Adj}) and un-adjusted p-values (p) <0.05 are highlighted in light green; Mean intensity values are highlighted for survivors and non-survivors (Yellow represents higher mean value; peach represents lower mean value). Metabolites significant in the two cohorts are highlighted in different colours (green = In both cohorts; red = Only in VANISH; blue = Only in LeoPARDS). Abbreviations: LPI = Lysophosphatidylinositol; LPG = Lysophosphatidylglycerol; LPA = Lysophosphatidic acid; LPS = Lysophosphatidylserine; PE = Phosphatidylethanolamine; PA = Phosphatidic acid; SulfoHexCer = Sulfohexosylceramide; FA = Fatty acid. Note: Carbon atoms and double bonds in all lipid species are represented as (C atoms:double bond); d indicates two hydroxyl groups in the sphingoid base; Prefixes “OH”, “O” and “P” represents different substituents/linkages in the lipid species: “OH” denotes for hydroxyl group, “O” represents an alkyl ether linkage whilst “P” refers to the plasmalogen link. Lipid species with abbreviations _1 and _2 indicate same m/z value but different retention times for those lipids.

Lipids	VANISH _{TP0} (N = 173, n = 127 & 46)				LeoPARDS _{TP0} (N = 460, n = 314 & 146)			
	Log mean integrated area (a.u)		p	p_{Adj}	Log mean integrated area (a.u)		p	p_{Adj}
	Survivors	Non-survivors			Survivors	Non-survivors		
LPI(18:0/0:0)	11.49	11.80	4.56E-03	0.287	11.52	11.74	0.015	0.103
LPI(18:1/0:0)_1	10.72	10.99	0.022	0.415	9.86	10.10	7.54E-05	2.37E-03
PE(O-16:1/22:6) and/or PE(P-16:0/22:6)	13.18	12.88	0.046	0.479	12.33	12.20	0.025	0.126
Eicosapentaenoic acid FA(20:5)	10.28	10.05	0.026	0.415	9.32	9.11	3.18E-03	0.033
LPS(18:0/0:0)	8.69	9.09	0.041	0.479	7.41	7.51	0.375	0.656
Hexacosanoic acid FA(26:0)	9.11	9.01	0.024	0.415	9.02	9.02	0.900	0.920
Docosapentaenoic acid FA(22:5)_1	11.07	10.87	0.132	0.538	10.45	10.27	7.37E-03	0.058
Docosapentaenoic acid FA(22:5)_2	9.26	9.20	0.680	0.839	8.59	8.44	0.030	0.126
Docosatetraenoic acid FA(22:4)	10.67	10.56	0.400	0.800	10.41	10.24	0.029	0.126
Eicosadienoic acid FA(20:2)	10.75	10.60	0.179	0.538	10.13	9.99	0.024	0.126
Eicosatetraenoic acid FA(20:4)	12.23	12.11	0.295	0.715	11.56	11.43	0.022	0.126
Tetracosatetraenoic acid FA(24:4)	8.18	7.98	0.101	0.538	7.56	7.34	6.66E-03	0.058

Eicosatrienoic acid FA(20:3)	10.86	10.63	0.057	0.512	10.20	10.00	1.07E-03	0.023
LPA(22:6/0:0)	7.80	7.71	0.462	0.800	6.88	7.09	0.034	0.134
LPG(18:1/0:0)	9.31	9.54	0.116	0.538	8.84	9.07	2.94E-03	0.033
LPG(18:2/0:0)	8.43	8.60	0.144	0.538	7.89	8.22	3.80E-06	2.39E-04
LPI(16:1/0:0)	8.77	8.99	0.241	0.606	7.99	8.19	0.042	0.155
PA(36:2);PA(18:0/18:2) PA(18:1/18:1)	9.39	9.47	0.508	0.800	8.95	9.11	2.16E-03	0.033
PE(18:0/22:6)	14.52	14.32	0.178	0.538	13.36	13.23	0.027	0.126
SulfoHexCer(d18:1/24:0(OH))	11.36	11.28	0.616	0.830	10.34	10.19	0.047	0.164

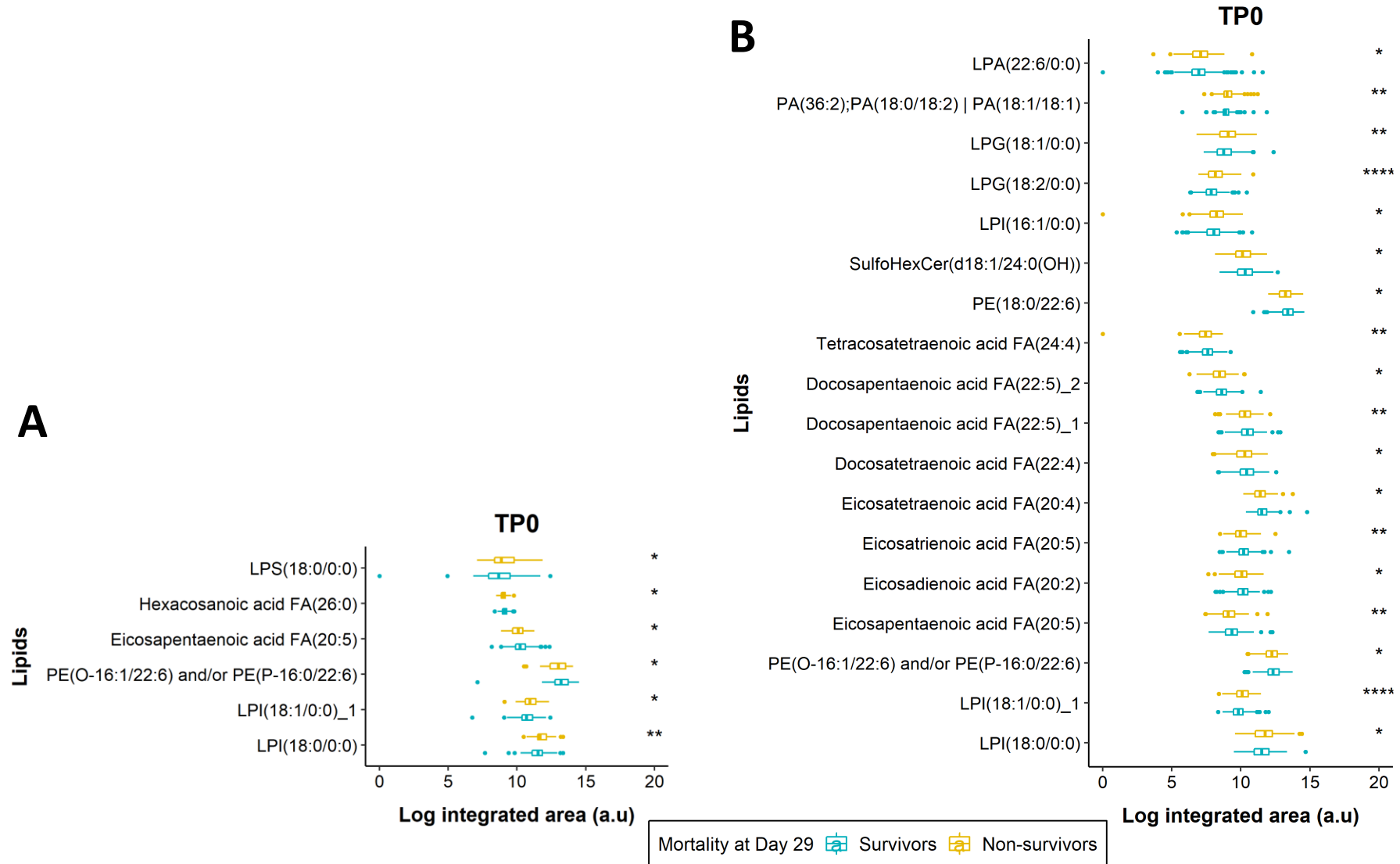


Figure 5.12: Box plots for lipids which were significantly different between survivors and non-survivors at TP0 in VANISH (A) and LeoPARDS (B) LNEG ppR datasets.

Table 5.10: Welch two sample *t*-test results of LNEG ppR data for the VANISH and LeoPARDS cohorts at TP1. Adjusted p-values were calculated with B-H correction. Adjusted p-values (p_{Adj}) and un-adjusted p-values (p) <0.05 are highlighted in light green; Mean intensity values are highlighted for survivors and non-survivors (Yellow represents higher mean value; peach represents lower mean value). Metabolites significant in the two cohorts are highlighted in different colours (green = In both cohorts; red = Only in VANISH; blue = Only in LeoPARDS). Abbreviations: LPI = Lysophosphatidylinositol; LPG = Lysophosphatidylglycerol; LPE = Lysophosphatidylethanolamine; PE = Phosphatidylethanolamine; PA = Phosphatidic acid; Cer = Ceramide; SulfoHexCer = Sulfohexosylceramide; FA = Fatty acid. Note: Carbon atoms and double bonds in all lipid species are represented as (C atoms:double bond); d indicates two hydroxyl groups in the sphingoid base; Prefixes “OH”, “O” and “P” represents different substituents/linkages in the lipid species: “OH” denotes for hydroxyl group, “O” represents an alkyl ether linkage whilst “P” refers to the plasmalogen link. Lipid species with abbreviations _1 and _2 indicate same m/z value but different retention times for those lipids.

Metabolite	VANISH _{TP1} (N = 137, n = 105 & 32)				LeoPARDS _{TP1} (N = 409, n = 298 & 111)			
	Log mean integrated area (a.u)		p	p_{Adj}	Log mean integrated area (a.u)		p	p_{Adj}
	Survivors	Non-survivors			Survivors	Non-survivors		
LPI(18:1/0:0)_1	10.84	11.18	2.13E-03	0.134	9.77	10.10	4.91E-06	3.09E-04
LPI(20:3/0:0)_2	8.48	8.83	0.012	0.244	7.33	7.54	7.52E-03	0.050
PE(18:0/22:5)	12.21	11.89	0.032	0.338	10.82	10.58	8.80E-03	0.050
PE(O-16:1/22:6) and/or PE(P-16:0/22:6)	13.30	13.00	0.022	0.338	12.44	12.21	2.30E-03	0.024
Capric acid FA(10:0)	8.88	9.41	0.049	0.384	9.05	9.29	0.161	0.338
PE(O-16:1/20:4) and/or PE(P-16:0/20:4)	13.79	13.61	0.043	0.384	12.63	12.60	0.635	0.783
PE(O-16:1/22:5) and/or PE(P-16:0/22:5)	12.80	12.61	0.032	0.338	11.56	11.46	0.096	0.218
SulfoHexCer(d18:2/16:0(OH))	10.17	9.80	0.010	0.244	9.35	9.31	0.637	0.783
Bilirubin	11.84	12.04	0.375	0.762	9.80	10.09	8.59E-03	0.050
Cer(d34:0);Cer(d18:0/16:0) Cer(d16:0/18:0)	7.27	7.17	0.558	0.767	8.74	8.54	0.019	0.084
Docosapentaenoic acid FA(22:5)_1	11.02	10.93	0.520	0.767	10.32	10.17	0.049	0.165
Eicosadienoic acid FA(20:2)	10.62	10.57	0.662	0.802	9.95	9.80	0.047	0.165
Eicosapentaenoic acid FA(20:5)	10.33	10.17	0.196	0.706	9.31	9.11	0.024	0.101

Eicosatetraenoic acid FA(20:4)	12.22	12.04	0.080	0.421	11.42	11.25	2.64E-03	0.024
Eicosatrienoic acid FA(20:3)	10.80	10.60	0.080	0.421	10.03	9.79	2.47E-04	5.19E-03
LPE(0:0/22:6)	8.87	8.63	0.202	0.706	7.49	7.10	0.010	0.054
LPE(22:6/0:0)	11.00	10.74	0.214	0.708	9.85	9.52	0.011	0.054
LPG(18:2/0:0)	8.70	8.89	0.075	0.421	8.02	8.23	5.24E-03	0.041
LPI(16:1/0:0)	8.82	8.98	0.348	0.762	7.85	8.12	2.46E-03	0.024
PA(16:0/18:1)	9.77	9.88	0.391	0.764	8.93	9.06	0.048	0.165
PA(36:2);PA(18:0/18:2) PA(18:1/18:1)	9.33	9.42	0.461	0.767	8.96	9.11	7.82E-04	0.012
PE(18:0/22:6)	14.70	14.42	0.066	0.421	13.45	13.16	3.62E-05	1.14E-03

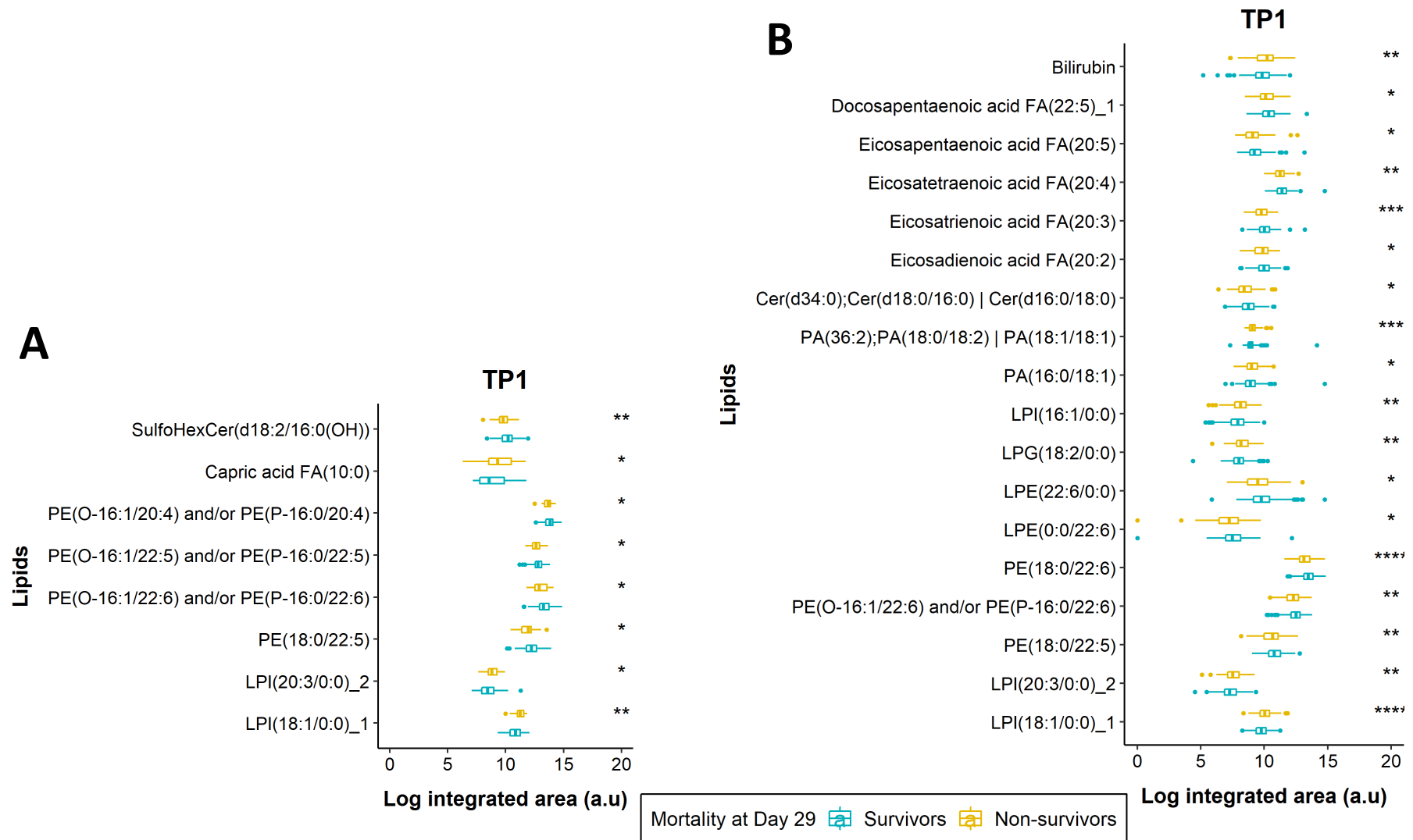


Figure 5.13: Box plots for lipids which were significantly different between survivors and non-survivors at TP1 in VANISH (A) and LeOPARDS (B) LNEG ppR datasets.

Table 5.11: Welch two sample *t*-test results of LNEG ppR data for the VANISH and LeoPARDS cohorts at TP2. Adjusted p-values were calculated with B-H correction. Adjusted p-values (p_{Adj}) and un-adjusted p-values (p) <0.05 are highlighted in light green; Mean intensity values are highlighted for survivors and non-survivors (Yellow represents higher mean value; peach represents lower mean value). Metabolites significant in the two cohorts are highlighted in different colours (green = In both cohorts; red = Only in VANISH; blue = Only in LeoPARDS). Abbreviations: LPI = Lysophosphatidylinositol; LPE = Lysophosphatidylethanolamine; PE = Phosphatidylethanolamine; PA = Phosphatidic acid; SulfoHexCer = Sulfohexosylceramide; FA = Fatty acid. Note: Carbon atoms and double bonds in all lipid species are represented as (C atoms:double bond); d indicates two hydroxyl groups in the sphingoid base; Prefixes “OH”, “O” and “P” represents different substituents/linkages in the lipid species: “OH” denotes for hydroxyl group, “O” represents an alkyl ether linkage whilst “P” refers to the plasmalogen link. Lipid species with abbreviations _1 and _2 indicate same m/z value but different retention times for those lipids.

Metabolite	VANISH _{TP2} (N = 103 , n = 80 & 23)				LeoPARDS _{TP2} (N = 318, n =250 & 68)			
	Log mean integrated area (a.u)		p	p_{Adj}	Log mean integrated area (a.u)		p	p_{Adj}
	Survivors	Non-survivors			Survivors	Non-survivors		
LPI(18:1/0:0)_1	10.87	11.26	1.79E-03	0.113	9.94	10.20	1.99E-03	0.031
LPI(18:0/0:0)	11.86	12.15	0.024	0.428	11.98	11.96	0.898	0.984
LPI(20:3/0:0)_2	8.53	8.93	0.013	0.408	7.59	7.67	0.388	0.788
PA(16:0/18:1)	9.78	10.21	0.035	0.428	8.96	9.08	0.062	0.279
Tetracosenoic acid FA(24:1)	10.65	10.97	0.031	0.428	10.05	10.05	0.976	0.984
Bilirubin	11.81	12.25	0.116	0.489	9.88	10.17	0.019	0.132
LPE(0:0/20:4)	9.11	9.11	0.985	0.993	8.24	7.77	1.48E-03	0.031
LPE(0:0/22:6)	9.09	9.06	0.926	0.993	7.89	7.31	4.39E-03	0.046
LPE(22:6/0:0)	11.25	11.15	0.705	0.972	10.39	9.87	6.67E-04	0.021
LPI(16:1/0:0)	8.74	9.04	0.089	0.467	7.95	8.21	0.013	0.102
PA(36:2);PA(18:0/18:2) PA(18:1/18:1)	9.34	9.73	0.050	0.428	8.94	9.16	1.63E-05	1.02E-03
PE(18:0/22:5)	12.02	12.03	0.945	0.993	10.80	10.58	0.024	0.148
PE(18:0/22:6)	14.52	14.56	0.787	0.972	13.41	13.17	4.30E-03	0.046
PE(O-16:1/22:6) and/or PE(P- 16:0/22:6)	13.32	13.17	0.364	0.917	12.58	12.38	0.013	0.102
SulfoHexCer(d18:2/16:0(OH))	10.13	9.89	0.117	0.489	9.43	9.22	0.046	0.264

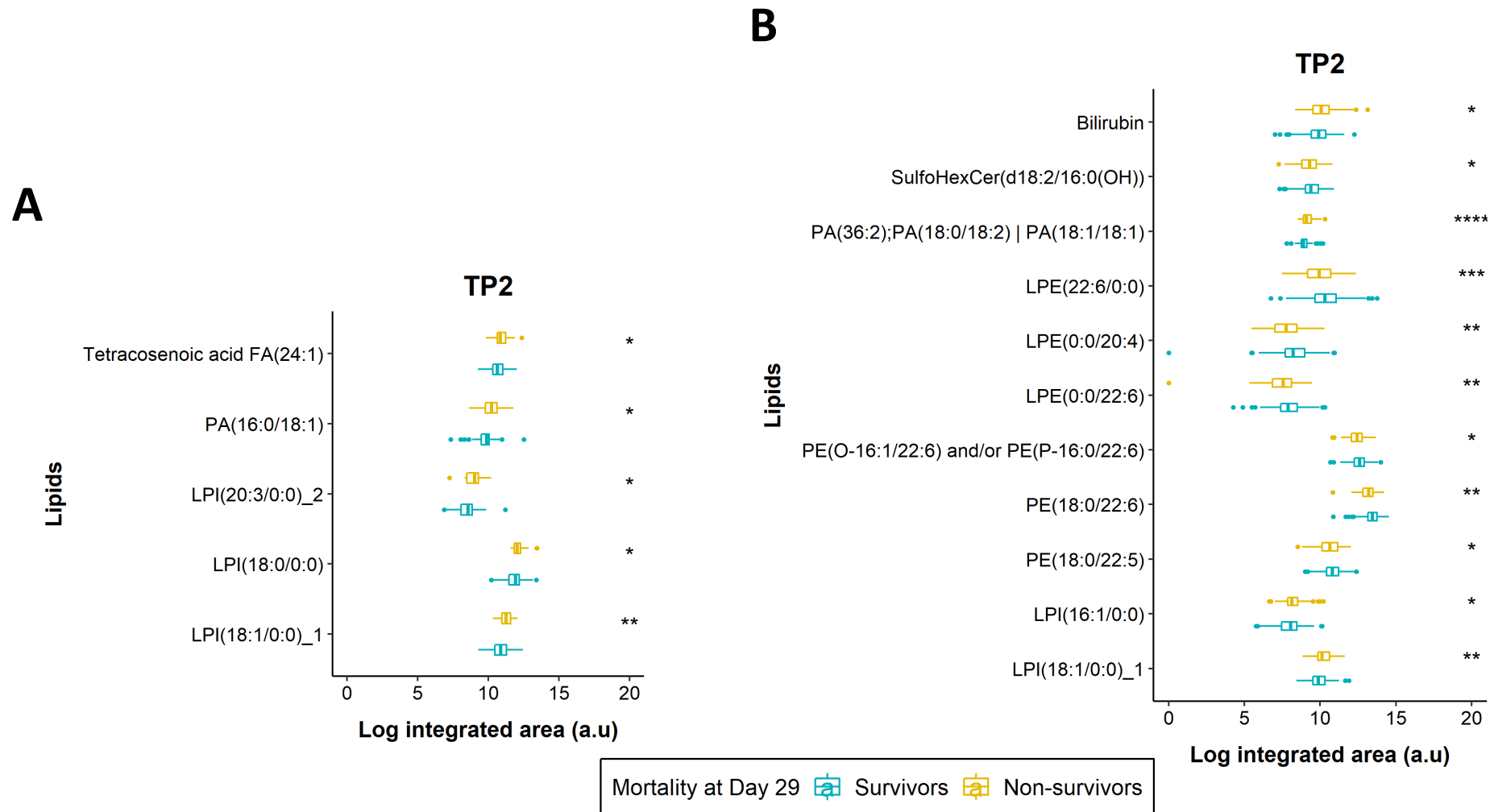


Figure 5.14: Box plots for lipids which were significantly different between survivors and non-survivors at TP2 in VANISH (A) and LeoPARDS (B) LNEG ppR datasets.

Table 5.12: Welch two sample *t*-test results of LNEG ppR data for the VANISH and LeoPARDS cohorts at TP3. Adjusted p-values were calculated with B-H correction. Adjusted p-values (p_{Adj}) and un-adjusted p-values (p) <0.05 are highlighted in light green; Mean intensity values are highlighted for survivors and non-survivors (Yellow represents higher mean value; peach represents lower mean value). Metabolites significant in the two cohorts are highlighted in different colours (green = In both cohorts; red = Only in VANISH; blue = Only in LeoPARDS). Abbreviations: LPI = Lysophosphatidylinositol; LPE = Lysophosphatidylethanolamine; PE = Phosphatidylethanolamine; PA = Phosphatidic acid; SulfoHexCer = Sulfohexosylceramide; FA = Fatty acid. Note: Carbon atoms and double bonds in all lipid species are represented as (C atoms:double bond); Prefixes “OH”, “O” and “P” represents different substituents/linkages in the lipid species: “OH” denotes for hydroxyl group, “O” represents an alkyl ether linkage whilst “P” refers to the plasmalogen link. Lipid species with abbreviations _1 and _2 indicate same m/z value but different retention times for those lipids.

Lipids	VANISH _{TP3} (N = 75, n = 59 & 16)				LeoPARDS _{TP3} (N = 256, n = 205 & 51)			
	Log mean integrated area (a.u)		p	p_{Adj}	Log mean integrated area (a.u)		p	p_{Adj}
	Survivors	Non-survivors			Survivors	Non-survivors		
LPE(0:0/22:6)	9.26	8.64	0.036	0.287	8.00	7.39	4.30E-03	0.068
LPE(22:6/0:0)	11.48	10.78	0.044	0.287	10.53	9.91	1.49E-03	0.068
Docosapentaenoic acid FA(22:5)_2	9.23	8.73	0.034	0.287	8.22	8.24	0.861	0.919
Eicosadienoic acid FA(20:2)	10.53	10.01	0.036	0.287	9.59	9.65	0.590	0.743
Eicosatrienoic acid FA(20:3)	10.69	10.23	0.021	0.287	9.80	9.72	0.404	0.667
Octadecadienoic acid FA(18:2)	14.45	13.80	0.019	0.287	13.78	13.86	0.535	0.728
Octadecatrienoic acid FA(18:3)_1	12.09	11.50	0.035	0.287	11.44	11.55	0.470	0.667
octadecenoic acid FA(18:1)	15.94	15.37	0.045	0.287	15.06	15.26	0.169	0.560
SulfoHexCer(d18:2/16:0(OH))	9.99	9.52	0.021	0.287	9.43	9.30	0.202	0.636
LPE(0:0/20:4)	9.45	8.86	0.057	0.287	8.38	7.81	0.011	0.119
LPI(16:1/0:0)	8.69	8.62	0.765	0.846	7.99	8.22	0.031	0.247
LPI(18:1/0:0)_1	10.89	10.90	0.968	0.984	10.00	10.21	0.030	0.247
LPI(22:5/0:0)	8.55	8.30	0.208	0.338	7.60	7.37	0.049	0.310
PA(36:2);PA(18:0/18:2) PA(18:1/18:1)	9.36	9.41	0.780	0.847	8.98	9.10	8.68E-03	0.109
PE(18:0/22:6)	14.50	14.43	0.741	0.833	13.39	13.09	4.08E-03	0.068
PE(O-16:1/22:6) and/or PE(P- 16:0/22:6)	13.30	12.90	0.069	0.287	12.54	12.28	3.50E-03	0.068

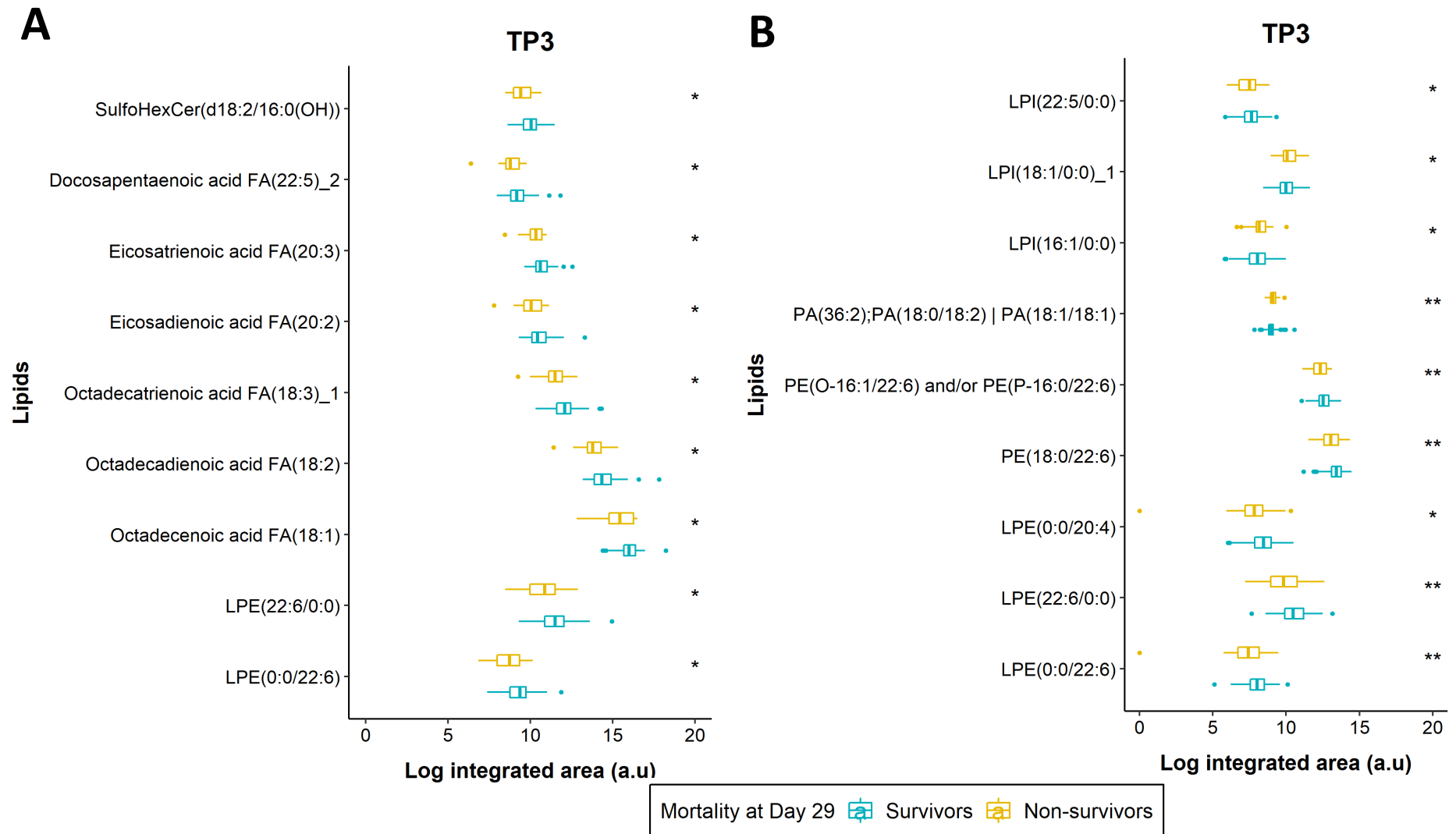


Figure 5.15: Box plots for lipids which were significantly different between survivors and non-survivors at TP3 in VANISH (A) and LeoPARDS (B) LNEG ppR datasets.

5.4.2 Prediction of outcome based on metabolic changes in the first 24 h

Metabolic differences in lipids and lipoproteins were found between survivors and non-survivors from the univariate analysis of lipids obtained from the NMR and UHPLC-MS targeted data. Now, the aim was to validate these findings for both VANISH and LeoPARDS at ICU admission and after 24 h of admission. The purpose was to see whether the results from one cohort can be used to predict mortality if tested on another cohort and vice versa. TP0 and TP1 samples were only used for this analysis as detecting metabolic differences in the first 24 will aid in earlier prognostication of patients who are likely to die or survive.

5.4.2.1 NMR targeted data (BI LISA)

The predictive models were performed on the NMR targeted data from both cohorts at ICU admission and after 24 hrs of admission. The same number of samples were used in this analysis as the mortality analysis of the NMR targeted data discussed previously at TP0 (N = 166 and N = 450 for VANISH and LeoPARDS, respectively) and TP1 (N = 135 and N = 394 for VANISH and LeoPARDS, respectively) (figure 5.1 & figure 5.2).

The performance metrics for the mortality prediction models of BI LISA for the two cohorts for TP0 and TP1 samples are listed in table 5.13. Our results indicated that the mortality validation models for both TP0 (figure A1.12, see appendix 1.5) and TP1 samples do not work that well as there were not many significant differences in the lipoproteins between survivors and non-survivors from both cohorts at these two timepoints. At TP0 none of the lipoproteins were common between the two cohorts whilst at TP1 six lipoproteins (listed in table 5.2) were common between the two cohorts hence the predictive models for TP1 performed slightly better than TP0 models but were still not good.

The predictive models for within cohort validation of VANISH at TP1 (VANISH TP1_{WC}) and validation of VANISH within LeoPARDS dataset at TP1 (VANISH TP1_{EXCO}) had an AUC of 0.63 and 0.58 (for 35 significant metabolites selected from the univariate mortality model of VANISH at TP1 and of those 6 metabolites were common between the two cohorts, table 5.2), respectively (figure 5.16). Survivors and non-survivors were correctly classified by the predictive models with a balanced accuracy of 60.60% and 56.28% for VANISH TP1_{WC} and TP1_{EXCO}, respectively (table 5.13).

The cross validated ROC curves for within cohort validation of LeoPARDS at TP1 (LeoPARDS TP1_{WC}) and validation of LeoPARDS within VANISH dataset at TP1 (LeoPARDS TP1_{EXCO}) had an AUC of 0.62 and 0.65 (for 34 significant metabolites selected from the univariate mortality model of LeoPARDS at TP1 and of those only 6 metabolites were common between the two cohorts, same as before, table 5.2), respectively (figure 5.16). Survivors and non-survivors were correctly classified by the predictive models with a balanced accuracy of 57.21% and 53.93% for LeoPARDS TP1_{WC} and TP1_{EXCO}, respectively, (table 5.13), respectively.

Table 5.13: Performance metrics for mortality prediction models within cohort (WC) and with external cohort (EXCO) for both TP0 and TP1 sample from BI LISA. Note: TP0_{WC} = within cohort for TP0 and TP0_{EXCO} = with external cohort for TP0; TP1_{WC} = within cohort for TP1 and TP1_{EXCO} = with external cohort for TP1.

Parameters	VANISH				LeoPARDS			
	TP0 _{WC}	TP0 _{EXCO}	TP1 _{WC}	TP1 _{EXCO}	TP0 _{WC}	TP0 _{EXCO}	TP1 _{WC}	TP1 _{EXCO}
Accuracy (%)	71.69	67.56	71.11	60.15	65.33	72.29	73.10	74.07
Balanced accuracy (%)	50.95	50.00	60.60	56.82	49.25	49.18	57.21	53.93
Sensitivity (%)	6.82	0.00	40.62	49.53	3.43	0.00	22.43	15.63
Specificity (%)	95.08	100	80.58	64.11	95.06	98.36	91.99	92.23
Positive predictive value (%)	33.33	NaN	39.39	33.97	25.00	0.00	51.06	38.46
Negative predictive value (%)	73.89	67.56	81.37	77.31	67.21	73.17	76.08	77.87
Prevalence of death (%)	26.51	32.44	23.70	27.16	32.44	26.51	27.16	23.70

Table 5.14: Confusion matrices for TP1 mortality predictive models for VANISH and LeoPARDS LPOS ppR datasets. Note: TP1_{WC} = within cohort for TP1 and TP1_{EXCO} = with external cohort for TP1.

Validation of VANISH within cohort			
VANISH TP0 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	83	19
	Non-survivors	20	13
Validation of VANISH in LeoPARDS			
VANISH TP0 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	184	54
	Non-survivors	103	53
Validation of LeoPARDS within cohort			
LeoPARDS TP0 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	264	83
	Non-survivors	23	24
Validation of LeoPARDS in VANISH			
LeoPARDS TP0 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	95	27
	Non-survivors	8	5

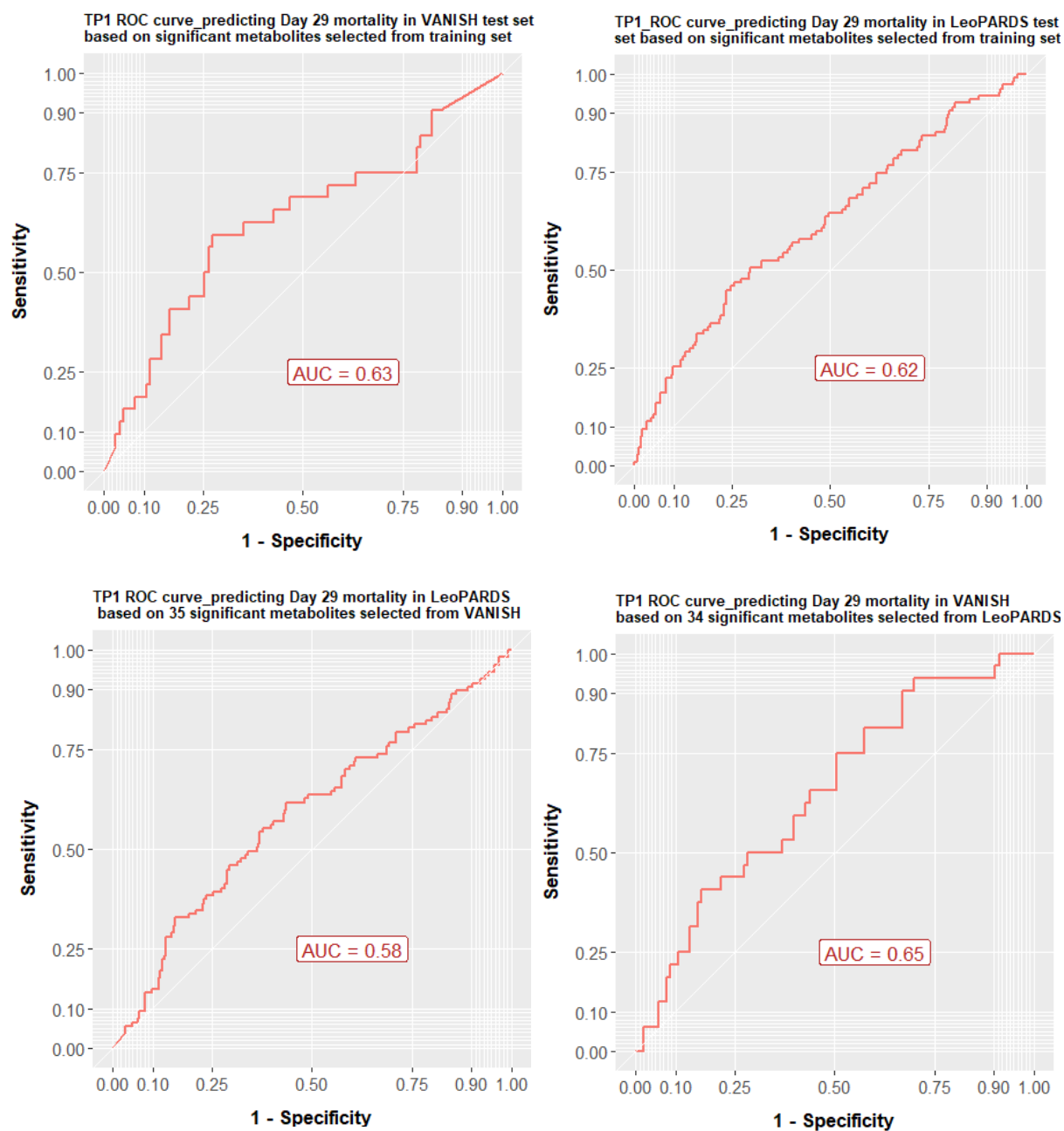


Figure 5.16: ROC curves for the predictive models of mortality on NMR targeted datasets (BI LISA) for VANISH and LeoPARDS at TP1.

5.4.2.2 UHPLC-MS targeted data (LPOS ppR)

The predictive models were performed on the LPOS ppR data from both cohorts at ICU admission and after 24 h of admission. The same number of samples were used in this analysis as the mortality analysis of the LPOS ppR data discussed previously at TP0 (N = 173 and N = 457 for VANISH and LeoPARDS, respectively) and TP1 (N = 137 and N = 408 for VANISH and LeoPARDS, respectively) (figure 5.6 & figure 5.7).

The performance metrics for the mortality prediction models of LNEG ppR data for the two cohorts for TP0 and TP1 samples are listed in table 5.15. Our results indicated that overall, the validation models do not work that well, but TP0 predictive models built with significant metabolites (including 23 common metabolites (table 5.5, figure 5.8) which were different between survivors and non-survivors at baseline) from the two cohorts, did perform slightly better (figure 5.17) than TP1 models (figure A1.12, see appendix 1.5).

The predictive models for within cohort validation of VANISH at TP0 (VANISH TP0_{WC}) and validation of VANISH within LeoPARDS dataset at TP0 (VANISH TP1_{EXCO}) had an AUC of 0.67 and 0.61 (for 38 significant metabolites of those 23 were common in both cohorts as listed in table 5.5, figure 5.8A) selected from the univariate mortality model of VANISH at TP0), respectively (figure 5.17). Survivors and non-survivors were correctly classified by the predictive models with a balanced accuracy of 62.10% and 53.39% for VANISH TP0_{WC} and TP0_{EXCO}, respectively (table 5.15).

The mortality prediction models for within cohort validation of LeoPARDS at TP0 (LeoPARDS TP0_{WC}) and validation of LeoPARDS within VANISH dataset at TP0 (LeoPARDS TP0_{EXCO}) had an AUC of 0.67 and 0.69 (for 96 significant metabolites of those 23 were common in both cohorts as listed in table 5.5, figure 5.8B) selected from the univariate mortality model of LeoPARDS at TP0), respectively (figure 5.17). Survivors and non-survivors were correctly classified by the predictive models with a balanced accuracy of 64.91% and 58.32% for LeoPARDS TP0_{WC} and TP0_{EXCO}, respectively, (table 5.15).

Table 5.15: Performance metrics for mortality prediction models within cohort (WC) and with external cohort (EXCO) for both TP0 and TP1 sample from LPOS ppR. Note: TP0_{WC} = within cohort for TP0 and TP0_{EXCO} = with external cohort for TP0; TP1_{WC} = within cohort for TP1 and TP1_{EXCO} = with external cohort for TP1.

Parameters	VANISH				LeoPARDS			
	TP0 _{WC}	TP0 _{EXCO}	TP1 _{WC}	TP1 _{EXCO}	TP0 _{WC}	TP0 _{EXCO}	TP1 _{WC}	TP1 _{EXCO}
Accuracy (%)	68.79	40.26	68.61	37.50	70.68	73.41	68.38	76.64
Balanced accuracy (%)	62.10	53.39	54.54	53.77	64.91	58.32	61.72	50.00
Sensitivity (%)	47.83	88.89	28.13	89.09	49.31	26.09	47.27	0.00
Specificity (%)	76.38	17.89	80.95	18.46	80.51	90.55	76.17	1.00
Positive predictive value (%)	42.31	33.25	31.03	28.74	53.79	50.00	42.28	NaN
Negative predictive value (%)	80.17	77.78	78.70	82.09	77.54	77.18	79.65	76.64
Prevalence of death (%)	26.59	31.51	23.36	26.96	31.51	26.59	26.96	23.36

Table 5.16: Confusion matrices for TP0 mortality predictive models for VANISH and LeoPARDS LPOS ppR datasets. Note: TP0_{WC} = within cohort for TP0 and TP0_{EXCO} = with external cohort for TP0.

Validation of VANISH within cohort			
VANISH TP0 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	97	24
	Non-survivors	30	22
Validation of VANISH in LeoPARDS			
VANISH TP0 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	56	16
	Non-survivors	257	128
Validation of LeoPARDS within cohort			
LeoPARDS TP0 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	253	71
	Non-survivors	61	71
Validation of LeoPARDS in VANISH			
LeoPARDS TP0 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	115	34
	Non-survivors	12	12

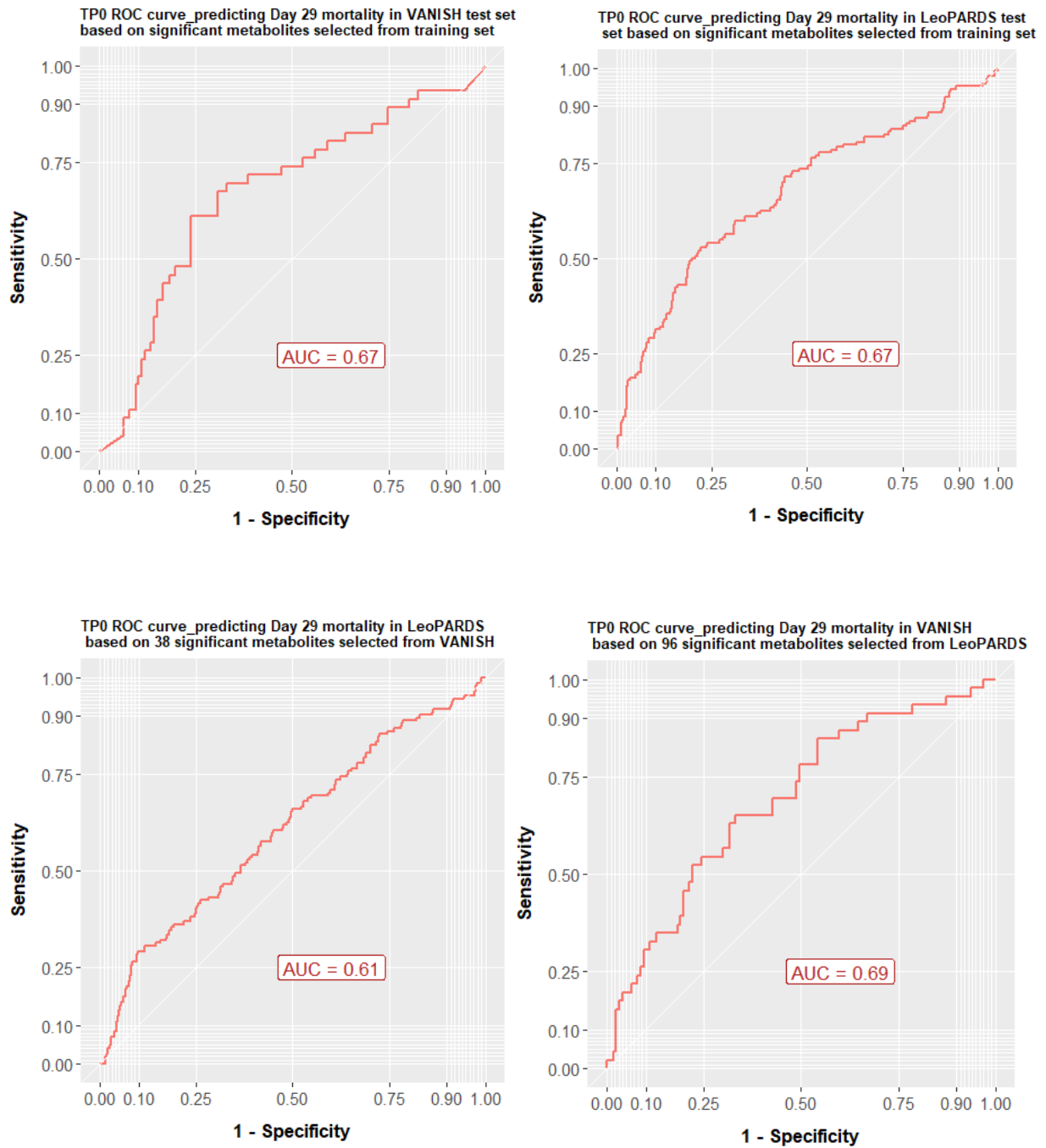


Figure 5.17: ROC curves for the predictive models of mortality on LPOS ppR dataset for VANISH and LeoPARDS at TP0.

5.4.2.3 UHPLC-MS targeted data (LNEG ppR)

The predictive models were performed on the LNEG ppR data from both cohorts at ICU admission and after 24 h of admission. The same number of samples were used in this analysis as the mortality analysis of the LNEG ppR data discussed previously at TP0 (N = 173 and N = 460 for VANISH and LeoPARDS, respectively) and TP1 (N = 137 and N = 409 for VANISH and LeoPARDS, respectively) (figures 5.10 & 5.11).

The performance metrics for the mortality prediction models of LNEG ppR data for the two cohorts for TP0 and TP1 samples are listed in table 5.17. Our results indicated that overall, the validation models built in LeoPARDS at both timepoints performed well and are better models than validation models built in VANISH.

The cross validated ROC curves for within cohort validation of VANISH test set at TP0 (VANISH TP0_{WC}) and validation of VANISH within LeoPARDS dataset at TP0 (VANISH TP0_{EXCO}) had an AUC of 0.57 and 0.62 (for 6 significant metabolites selected from the univariate mortality model of VANISH at TP0 and of those four (LPI(18:0/0:0), LPI(18:1/0:0)_1, PE(O-16:1/22:6) and/or PE(P-16:0/22:6), eicosapentaenoic acid FA(20:5) were common in both cohorts as listed in table 5.9, figure 5.12A), respectively (figure 5.18). The predictive models for within cohort validation of VANISH at TP1 (VANISH TP1_{WC}) and validation of VANISH within LeoPARDS dataset at TP1 (VANISH TP1_{EXCO}) had an AUC of 0.66 and 0.68 (for 8 significant metabolites selected from the univariate mortality model of VANISH at TP1 and of those only four metabolites (LPI(18:1/0:0)_1, LPI(20:3/0:0)_2, PE(18:0/22:5), PE(O-16:1/22:6) and/or PE(P-16:0/22:6) were common between the two cohorts as listed in table 5.10, figure 5.13A), respectively (figure 5.19).

The validation models built in LeoPARDS at TP0 and TP1 performed well, and we found that the cross validated ROC curves for within cohort validation of LeoPARDS at TP0 (LeoPARDS TP0_{WC}) and validation of LeoPARDS within VANISH dataset at TP0 (LeoPARDS TP0_{EXCO}) had an AUC of 0.68 and 0.73 (for 18 significant metabolites selected from the univariate mortality model of LeoPARDS at TP0 and of those same four metabolites VANISH TP0 model were common between the two cohorts as listed in table 5.9, figure 5.12B), respectively (figure 5.18). The predictive models for within cohort validation of LeoPARDS at TP1 (LeoPARDS TP1_{WC}) and validation of LeoPARDS within VANISH dataset at TP1 (LeoPARDS TP1_{EXCO}) had an

AUC of 0.74 and 0.78 (for 18 significant metabolites selected from the univariate mortality model of LeoPARDS at TP1 and of those same four metabolites as VANISH TP1 model were common between the two cohorts as listed in table 5.10, figure 5.13B), respectively (figure 5.19). Survivors and non-survivors were correctly classified by the predictive models with a balanced accuracy of 59.47% and 57.42% for LeoPARDS TP0_{WC} and TP0_{EXCO}, respectively, whilst LeoPARDS TP1_{WC} and TP1_{EXCO} models had a balanced accuracy of 61.35% and 71.52% (table 5.17), respectively indicating that the performance of the two pairs of classification models is better for validation models at TP1 than validation models at TP0.

Table 5.17: Performance metrics for mortality prediction models within cohort (WC) and with external cohort (EXCO) for both TP0 and TP1 sample from LNEG ppR. Note: TP0_{WC} = within cohort for TP0 and TP0_{EXCO} = with external cohort for TP0; TP1_{WC} = within cohort for TP1 and TP1_{EXCO} = with external cohort for TP1.

Parameters	VANISH				LeoPARDS			
	TP0 _{WC}	TP0 _{EXCO}	TP1 _{WC}	TP1 _{EXCO}	TP0 _{WC}	TP0 _{EXCO}	TP1 _{WC}	TP1 _{EXCO}
Accuracy (%)	69.36	65.43	71.53	75.06	70.43	75.14	74.57	72.99
Balanced accuracy (%)	52.01	58.37	51.01	58.29	59.47	57.42	61.35	71.52
Sensitivity (%)	15.22	39.04	12.50	21.62	29.54	19.57	32.43	68.75
Specificity (%)	88.98	77.71	89.52	94.97	89.49	92.28	90.27	74.29
Positive predictive value (%)	33.33	44.88	26.67	61.54	56.58	60.00	55.34	44.90
Negative predictive value (%)	74.34	73.27	77.05	76.49	73.18	76.58	78.20	88.64
Prevalence of death (%)	26.59	31.74	23.36	27.14	31.74	26.59	27.13	23.36

Table 5.18: Confusion matrices for TP0 mortality predictive models for VANISH and LeoPARDS LNEG ppR datasets. Note: TP0_{WC} = within cohort for TP0 and TP0_{EXCO} = with external cohort for TP0.

Validation of VANISH within cohort			
VANISH TP0 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	113	39
	Non-survivors	14	7
Validation of VANISH in LeoPARDS			
VANISH TP0 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	244	89
	Non-survivors	70	57
Validation of LeoPARDS within cohort			
LeoPARDS TP0 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	281	103
	Non-survivors	33	43
Validation of LeoPARDS in VANISH			
LeoPARDS TP0 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	121	37
	Non-survivors	6	9

Table 5.19: Confusion matrices for TP1 mortality predictive models for VANISH and LeoPARDS LNEG ppR datasets. Note: TP1_{WC} = within cohort for TP1 and TP1_{EXCO} = with external cohort for TP1.

Validation of VANISH within cohort			
VANISH TP1 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	94	28
	Non-survivors	11	4
Validation of VANISH in LeoPARDS			
VANISH TP1 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	283	87
	Non-survivors	15	24
Validation of LeoPARDS within cohort			
LeoPARDS TP1 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	269	75
	Non-survivors	29	36
Validation of LeoPARDS in VANISH			
LeoPARDS TP1 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	78	10
	Non-survivors	27	22

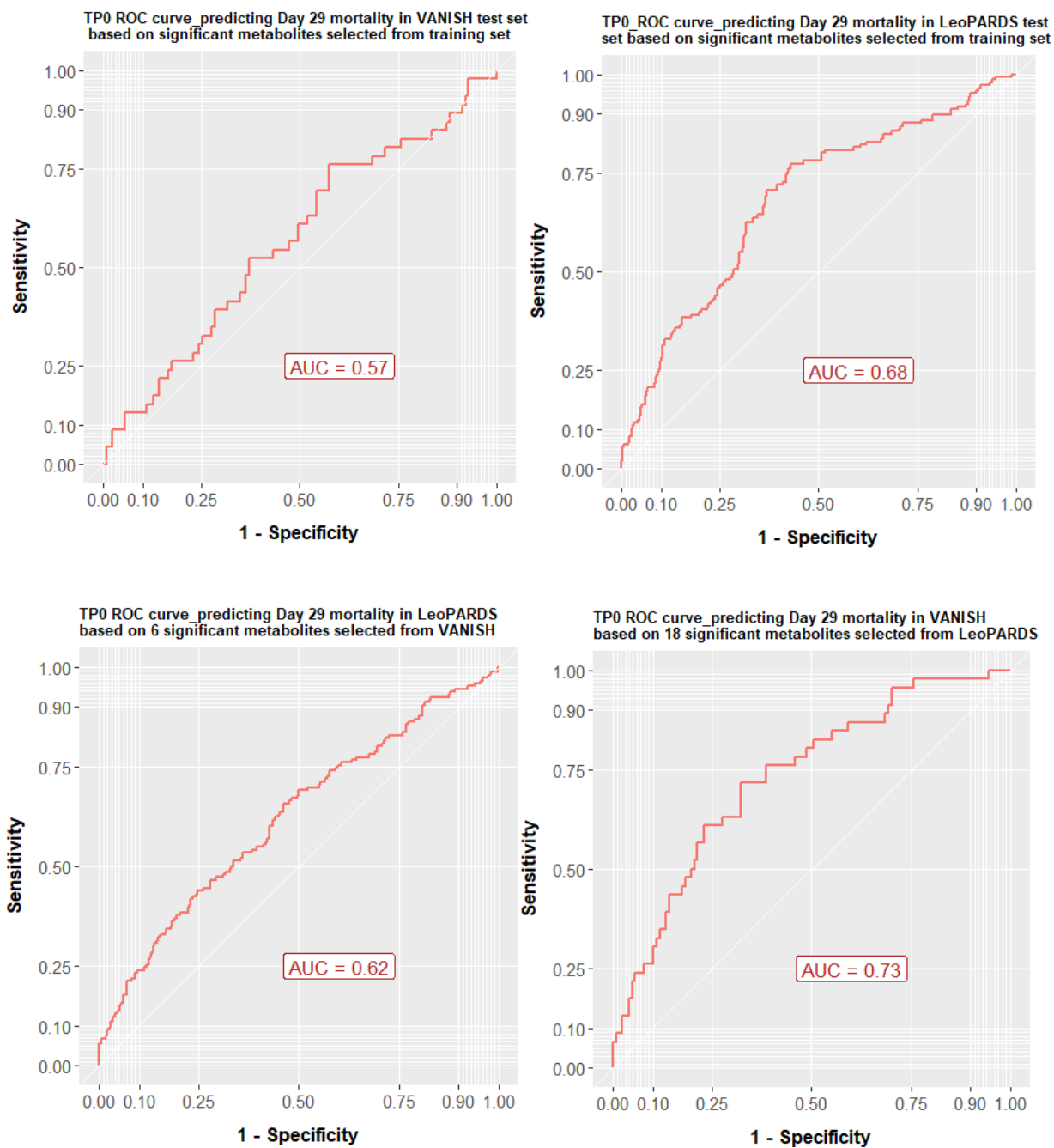


Figure 5.18: ROC curves for the predictive models of mortality on LNEG ppR dataset for VANISH and LeoPARDS at TP0.

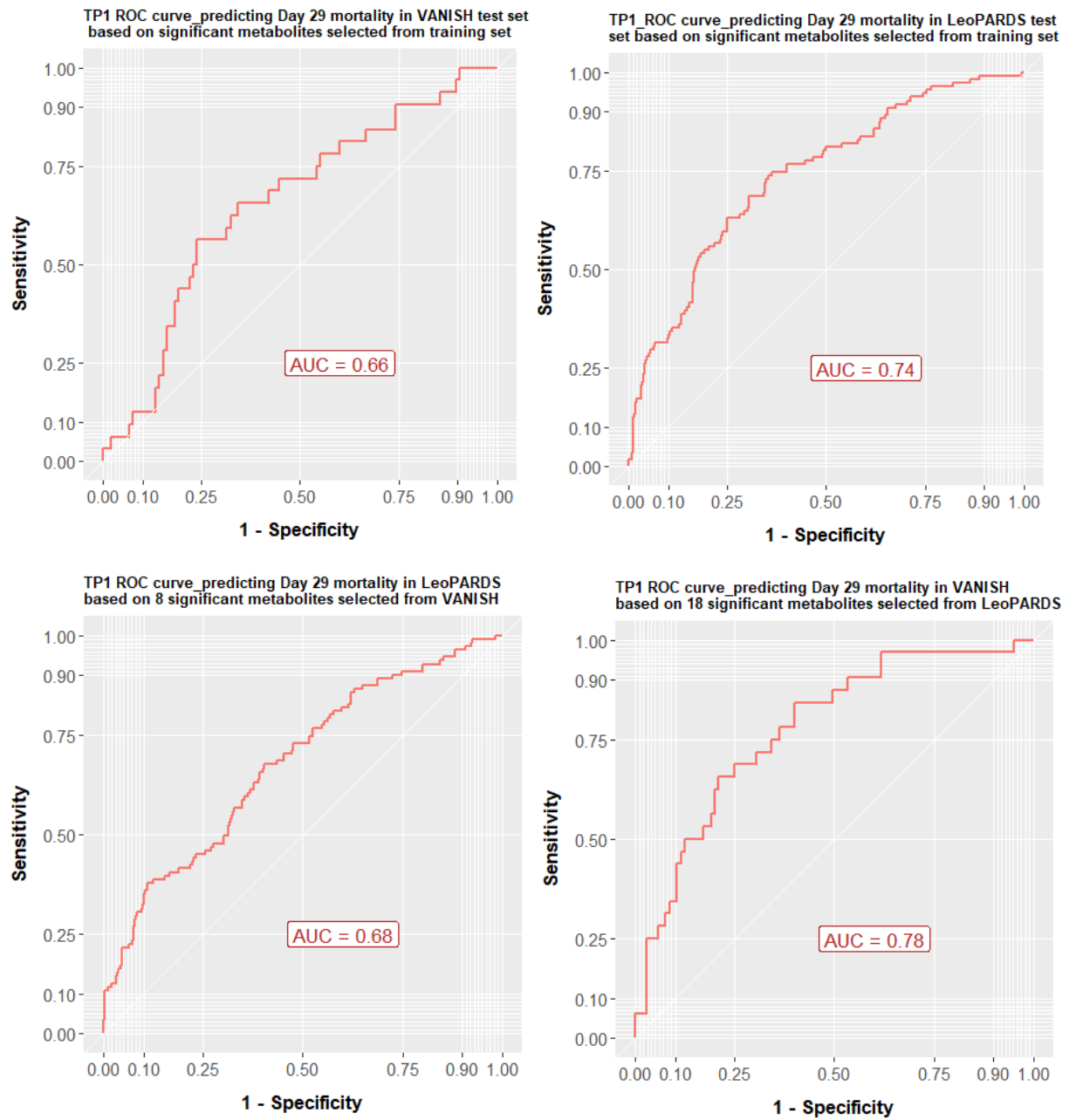


Figure 5.19: ROC curves for the predictive models of mortality on LNEG ppR dataset for VANISH and LeoPARDS at TP1.

5.5 Discussion

It is well known that sepsis is a global health condition in which lipid metabolism and lipoprotein composition is greatly affected (296). Metabolomic techniques combined with bioinformatic analysis have a great potential to help us understand these metabolic derangements at a molecular level to aid us in determining the underlying biological processes in patients with sepsis.

In this study metabolomics was used to investigate the metabolic differences in lipids and lipoproteins of septic shock survivors and non-survivors at ICU admission and on the days after ICU admission using NMR and UHPLC-MS methodologies in the two cohorts. Our validation models for mortality based on the results from univariate analysis of targeted data were not fully validated in the two cohorts.

In this study, we found that septic shock non-survivors had higher levels of acyl carnitines (medium-and long-chain), glycerophospholipids (LPGs, LPIs, and PA(36:2);PA(18:0/18:2) | PA(18:1/18:1)) whilst lipids such as FAs, TGs and lipoprotein bound TGs, glycerophospholipids (such as PEs, PCs, and LPCs), sphingolipids (such as SMs, Cers) and apolipoproteins, cholesterol and lipoprotein bound cholesterol were lower in comparison to septic shock survivors in both cohorts across timepoints.

Fatty acid oxidation is one of the main fuel source for cells (297) and this process seems to be disrupted in patients with sepsis as our study showed that fatty acyls as medium and long chain acylcarnitines (including octanoylcarnitine decanoylcarnitine, hydroxydodecanoylcarnitine, arachidyl carnitine and eicoseneoylcarnitine) were significantly elevated in patients who went on to die than those who didn't in both cohorts at ICU admission and after 24H of admission. These findings were also in accordance with other metabolomic studies (146-149, 163) and suggest that patients with septic shock who went on to die have enhanced incomplete fatty acid oxidation (i.e., β -oxidation) due to mitochondrial dysfunction (162, 253). Carnitine also takes part in the transportation of long-chain FAs (267) and the accumulation these acyl carnitines might also suggest increased uptake of FAs from cytoplasm into mitochondria(267).

Polyunsaturated fatty acids (PUFAs) especially C-20 PUFAs including arachidonic acid, homo- γ -linolenic acid and eicosapentaenoic acid (EPA) are involved in the synthesis of lipid

mediators such as eicosanoids, a subgroup of oxylipins (298) with immunomodulatory role and also other physiological processes (298, 299) such as vasodilation and inflammation. Our results showed that PUFAs including EPA (i.e., FA(20:5)) and eicosatrienoic acid (ETA) (i.e., FA(20:3)), eicosatetraenoic acid FA(20:4) also known as arachidonic acid, eicosadienoic acid (i.e., FA(20:2)) and docosapentaenoic acid (DPA) (i.e., FA(22:5)_1) were reduced in non-surviving patients in both cohorts at ICU admission and after 24 h of treatment. Two PUFAs (including EPA and ETA) remained significant after correcting for multiple comparisons but only in LeoPARDS. PUFAs, such as EPA and docosahexanoic acid (DHA) are precursors for specialized pro-resolving mediators (SPMs) such as resolvins, protectins and maresins (300, 301). As our results showed that patients who went on to die had low levels of EPA and this might indicate that EPA is quickly metabolized to exhibit anti-inflammatory activity via the production of SPMs, which are biologically active during organ damage and tissue inflammation (300). All of these FAs have not been previously associated with sepsis mortality however, Lin et al. reported that two PUFAs (including EPA and DHA) were decreased in septic patients with respect to healthy controls (302) and in our case lower non-survivors had lower EPA than survivors which suggests disease continuum in these patients.

Sphingolipids such as SMs and Cers have important roles in the regulation of cellular processes, such as lymphocyte trafficking, mediating pro- and anti-inflammatory effects, and maintaining the integrity of the endothelial barrier (303). Our study revealed that sphingolipids including SMs and Cers (which are breakdown products of SMs) were lower in patients who went on to die than those who didn't at all timepoints (TP0- TP3) in both cohorts. Our results suggest that patients who went on to die might have a reduction in the precursor SMs and hence lower production of Cers via sphingomyelinase during inflammation (304). Cers, have similar structure to lps and may act as receptor agonists (305) for the CD14 receptor (306). The results related to SMs were in accordance with literature findings (151) whilst low levels of Cers in the serum of non-surviving patients were not in accordance with the literature and were higher in the non-surviving patients (167). Our results suggest that Cers are mostly acting as a ligand for the CD14 receptor (306) and will be mostly bound to the receptor therefore reduced Cers in the serum.

Phospholipids, the principal component of the biological cell membranes are crucial for the synthesis and stability of lipoproteins. (307). PCs and PEs are the two most abundant phospholipids (307) and our results showed reduced levels of PCs and PEs in patients who went on to die than those who didn't in both cohorts at all timepoints. Similarly, LPCs generated by the cleavage of PC from HDL via endothelial lipase or from the hydrolysis of PC on the cell membranes by phospholipases A1 or A2 (308) were also lower in non-survivors with respect to survivors at all timepoints in both cohorts and these results were also supported by other metabolomic studies (149, 151, 156, 158). LPCs have several roles ranging from both anti and pro-inflammatory (308) to immuno-modulation (such as regulating the function of monocytes and macrophages (309), inhibiting platelet aggregation and promoting the secretion of NO in endothelial cells (308). Additionally, LPEs which are also produced from the hydrolysis of PEs by phospholipase A2 (308) were also reduced in patients who went on to die than those who survived at all timepoints in both cohorts, however these were not consistently statistically significant in both cohorts. Our results suggest there is increased hydrolysis of PCs, and PEs into their catabolic products in non-survivors in comparison to survivors whilst low levels of LPC in non-surviving patients also indicate increased hydrolysis of LPC into LPA via phospholipase D or autotaxin (303).

Phospholipids such as phosphatidylglycerols (PGs) are inherent component of lung surfactant with anti-inflammatory roles (310). Our results from the univariate analysis of LNEG ppR data showed that LPGs, bioactive lipids formed from the hydrolysis of PGs via phospholipase A2 (308) were higher in patients who die before day 29 than those who didn't at ICU admission and after 24 h of treatment in LeOPARDS and the trend was also the same in VANISH. High levels of LPGs in non-survivors might suggest increased hydrolysis of PGs into LPGs, increased modulation of endothelial cell activity as well as enhanced activation of natural killer cell trafficking probably (308).

LPs produced from the hydrolysis of phosphatidylinositols (PIs) (i.e., phospholipids which are important in intracellular signalling (149)) by phospholipases A1 and A2 (308) were elevated in patients who went on to die than those who didn't in both cohorts at all timepoints. These bioactive lipids have several roles ranging from regulating fat deposition, glucose homeostasis, stimulating insulin secretion (whereby both LPI and insulin work in

synergy to increase cellular proliferation) as well as stimulating an increase in the concentration of calcium ions and alterations in the membrane potential of endothelial cells (308). High levels of LPIs in non-survivors might suggest increased stimulation of insulin release from islets of Langerhans (308). LPIs have not been previously linked with mortality associated with sepsis. Interestingly, only LPI(22:5/0:0) was reduced in septic shock non-survivors than survivors at TP3 in both cohorts but the results were not significant after correction.

Phosphatidic acid (PA), is a precursor for DG and LPA and is involved in enhancing thrombosis mediated via the exposure of phosphatidylserine (PS) on human erythrocytes (311). Two PAs including PA(36:2); PA(18:0/18:2) | PA(18:1/18:1) at all timepoints (i.e., TP0-TP3) and PA(16:0/18:1) at TP1 were elevated in patients who went on to die than those who didn't in both cohorts. These results might suggest increased thrombosis in non-survivors than survivors. LPA, a bioactive lipid mediator of inflammation can regulate thrombotic stability by stimulating the release of neutrophil extracellular traps (NETs) from neutrophils through lysophosphatidic acid receptors 1-6 (312). In sepsis, increased release of NETs have been shown to contribute towards the pathogenesis of this heterogeneous condition (312) by causing increased intravascular thrombosis and DIC which eventually leads to organ damage (312). Our results showed that LPA (22:6/0:0), a catabolic product of PA and LPC was present in higher concentration in septic shock non-survivors with respect to survivors in both cohorts at ICU admission and these results might suggest increased NET production via LPA receptors which will lead to increased thrombosis in patients who went on to die than those who didn't. Increased levels of LPA might reflect increased activity of Autotaxin (ATX), a lysophospholipase D enzyme which is involved in the synthesis of LPA via hydrolysis of LPC (313). It has been reported that enhanced activity of ATX is associated with increased 30-day mortality in patients with sepsis. It also correlates with platelet count and the ratio of angiopoietin-2/-1 suggesting a link between platelets, ATX and vascular dysfunction (314). The ATX/LPA axis has a pathological role in diseases such as chronic inflammation and cancer (313, 315, 316) and patients with sepsis have been found to have increased serum levels of ATX upon ICU admission (313). Elevated LPA levels in sepsis non-survivors might also indicate enhanced activity of phospholipases A1 or A2, thereby resulting in increased

conversion of PA into LPA (317). This is supported by literature showing that patients with septic shock have enhanced activity of serum phospholipase A2 (318, 319).

Similarly, the results from the univariate analysis of NMR targeted data revealed that all lipoprotein classes (including HDL, LDL, IDL and VLDL) carrying phospholipids, also followed the same trend as all the measured phospholipid classes (except for PA) from UHPLC-MS data. In brief, lipoprotein bound phospholipids were present in significantly low concentrations in non-survivors than survivors in LeoPARDS. These results were in accordance with the findings from de la Llera Moya et al. who revealed that decreased expression of phospholipids and Apo-A1 causes alterations in HDL composition in human endotoxemia (320).

Apolipoproteins, protein components of lipoproteins, play important roles in lipid transport and metabolism (321). These protein portions have several roles ranging from activation and inhibition of enzymes involved in lipid metabolism, maintaining the stability of lipoproteins as well as enhancing solubility in aqueous environment (321). Apo-A1 and Apo-A2 are the two most abundant apolipoproteins (321). About 20% of HDL contains Apo-A2, which is involved in regulating the activity of extracellular enzyme, lipoprotein lipase (LPL) (322) which is present on vascular endothelial surface and is responsible for the degradation of circulating TGs in the bloodstream (323). Apo-A2 is also a cofactor for lecithin cholesterol acyltransferase (LCAT), an important enzyme involved in the metabolism of lipoproteins (322) with both phospholipase A2 and an acyltransferase action (322). LCAT is also crucial for the interconversion, rearrangements and enabling normal maturation of all lipoproteins (322) and it also takes part in the reverse cholesterol transport(322). Our results showed that HDL Apo-A2 at TP2 and its subfractions (including subfraction 1 at both TP1 & TP3, subfraction 2 at three timepoints (i.e.,TP1-TP3), and subfractions 3 at TP1 & TP2) were significantly reduced in non-survivors with respect to survivors in LeoPARDS and the trend for these lipoproteins was also the same in the alternate cohort. These results might suggest reduced activity of LPL in patients who went on to die than those who didn't. This level of lipoprotein detail has not been previously described in the literature and HDL-Apo A2 has not been previously associated with mortality in sepsis which suggests this needs to be explored further.

Apo-B100, a ligand for the LDL receptor (324), is the main component of three lipoprotein classes including LDL, VLDL & IDL (which play a role in atherosclerosis development) (325) and this apolipoprotein is crucial for the assembly and secretion of VLDL (322). Our results showed that LDL Apo-B100 subfraction 1 (at both TP1 & TP2), and IDL Apo-B100 (at TP2) were lower in patients who went on to die than those who didn't in both cohorts. It has been reported that lps-binding protein interacts with Apo-B100 of LDL and VLDL in patients with sepsis thus increasing the lps-LDL/VLDL interaction (326). In our case the results suggest that these lipoproteins have decreased scavenging capacity for the bacterial toxin (i.e., lps) in non-survivors with respect to survivors.

Cholesterol, one of the important lipid molecule present in plasma cell membranes is required for normal cell function (327). It exists in the plasma in two main forms, free cholesterol and CEs, both of which are part of all lipoprotein classes including HDL, LDL, IDL, VLDL and chylomicrons (328). Some studies have shown that there is an important association between innate immunity and cholesterol metabolism(329-331). Our study revealed that patients who went on to die had reduced levels of cholesterol in both cohorts at TP1 and the results were significant after correction but only in LeoPARDS. Our findings were also in accordance with other metabolomic studies (151, 170, 181) and suggest increased cholesterol metabolism in septic shock non-survivors which will lead to increased formation of other essential compounds such as bile acids and adrenal and gonadal steroids hormones (327). Bilirubin was found to elevated in non-surviving patients from both cohorts at TP1 and TP2, but the results were only significant in LeoPARDS and this finding reflects increased liver dysfunction (332) in patients who went on to die than those who didn't.

The results from the lipoprotein analysis showed that lower density subfractions of LDL and VLDL (including LDL cholesterol subfraction 1 at both TP1 & TP2 and VLDL cholesterol subfractions 2 and 3 at TP3) were significantly lower in non-surviving patients than those who didn't in LeoPARDS and these lipoproteins had the same pattern in the alternate cohort. Lee et al. reported higher LDL levels in non-surviving patients with respect to non-survivors on days 0, 1, 3 and 7 (170) and our results were also in accordance with this study. Interestingly, increased levels of higher density subfractions of HDL and LDL (including HDL cholesterol subfraction 4, and LDL cholesterol subfractions 4 & 5) were found in non-survivors with respect to survivors in LeoPARDS at TP1 and the trend for this was the

opposite in VANISH and as LeoPARDS has larger sample size than VANISH, hence higher statistical power therefore we will be trusting the results from this cohort. This level of lipoprotein detail for mortality associated with sepsis has not been previously reported in the literature. Low levels of HDL cholesterol have been associated with higher risk of multiorgan failure as well as longer hospital and ICU stay, increased mortality, and higher rates of hospital acquired infections (171, 172, 333). As our results indicated increased levels of HDL cholesterol subfraction 4 at TP1 not in accordance with the literature findings (171, 172) which might suggest there is some complex mechanism taking place in patients who die which is still not fully known. Our results might indicate that non-survivors might lack or have reduced activity of cholesteryl ester transfer protein (CETP), a glycoprotein made up of 476 amino acids which transfers CE from HDL to LDL (334) resulting in increased HDL cholesterol levels in the plasma (334). CETP has been previously reported to interrupt the interaction between bacterial toxin lps and toll-like receptor 4 (TLR4), thus reducing the uncontrolled inflammatory response in patients with sepsis (335).

Similarly, our results also revealed that the levels of free cholesterol including HDL free cholesterol (at TP1 & TP2) and its subfractions 3 & 4 (at TP1) were elevated in patients who went on to die than those who didn't in LeoPARDS and pattern for these lipoproteins was the same in VANISH except for HDL free cholesterol (at TP2) and its subfractions 3 & 4 (at TP1). Moreover, low density subfractions of LDL and VLDL free cholesterol (including LDL free cholesterol subfraction 1 at TP1 and VLDL free cholesterol subfraction 1 at TP3) were significantly lower in non-surviving patients than those who didn't in LeoPARDS ($p < 0.05$) whilst LDL free cholesterol and its higher density subfractions 4-6 were significantly higher in non-survivors than survivors in LeoPARDS at TP1. The pattern for these lipoproteins was the same in the alternate cohort except for LDL free cholesterol subfractions 5 at TP1. These findings were also in accordance with our cholesterol results mentioned previously and suggest that patients with septic shock who went on to die might have a complex mechanism taking place which is not yet fully known. The conversion of both cholesterol and free cholesterol into CE takes place via enzyme acyl-coenzyme A :cholesterol acyltransferase (ACAT) (336) and LACT (324), respectively. Our study revealed that CEs (including CE(20:4) at TP1 & TP2, CE(20:5) at all timepoints) were significantly reduced in

non-surviving patients in both cohorts and only CE(20:5) at ICU admission did not remain significant after correction in LeoPARDS.

The results of univariate analysis of lipoproteins and lipids from BI LISA, LPOS ppR and LNEG ppR data showed that patients who went on to die had reduced levels of triglycerides than those who did not in both cohorts almost at all timepoints. Lipoprotein bound triglycerides (including HDL, IDL, LDL, VLDL triglycerides) were lower in septic shock non-survivors with respect to survivors in LeoPARDS and these triglycerides mostly had the same trend in the alternate cohort across timepoints. Our results were in accordance with the findings from Lee and co-workers who reported low levels of triglycerides in non-survivors with respect to survivors on days 0, 1, 3 and 7. (170) These results suggest patients who went on to die might have increased lipolysis which leads to hydrolysis of TGs into DGs and FAs via adipose triglyceride lipase. In general, fasting-induced lipolysis could primarily be activated by catecholamines such as noradrenaline, but other hormones (including cortisol, glucagon, growth hormone and adrenocorticotrophic hormone) and dietary compounds (including caffeine and calcium) could also stimulate lipolysis (337). Hypcholesterolemia and low level of TGs have also been linked with a poor prognosis in patients with sepsis (338) and this also supports our findings in relation with mortality outcome. Similarly, DGs were also significantly lower in patients who went on to die in comparison to those who didn't in LeoPARDS across timepoints and the pattern for the DGs was mostly the same in the alternate cohort. Lower levels of DGs in non-survivors might suggest decreased hydrolysis of TGs and hence decreased lipolysis in patients who went on to die.

The mortality validation analysis revealed that the predictive models generated using lipids and lipoproteins which were significantly different between patients who went on to die and those who went on to survive in both cohorts did not perform well except for one model (LeoPARDS TP1_{EXCO} from LNEG ppR data based on 18 significant lipids (from which four lipids were common (LPI(18:1/0:0)_1, LPI(20:3/0:0)_2, PE(18:0/22:5), & PE(O-16:1/22:6) and/or PE(P-16:0/22:6)) in both cohorts) at both timepoints. As the validation models incorporated all the significant metabolites with fewer number of metabolites common between the two cohorts thus this will contribute towards the poor performance of these predictive models. LeoPARDS TP1_{EXCO} had some potential to discriminate survivors

from non-survivors and in future this might be useful in earlier prognostication of sepsis patients who are likely to become worse and this might also guide the clinicians in making clinical decisions.

5.6 Conclusion

Targeted data analysis of serum from septic shock survivors and non-survivors using NMR and UHPLC-MS platforms showed that there are some differences in the lipid and lipoprotein profiles of these two groups across timepoints in both cohorts. Alterations in lipoproteins were mostly observed at later timepoints whilst lipid profiles showed significant differences between survivors and non-survivors at baseline and at later timepoints in both cohorts. Lipid classes including acylcarnitines, and glycerophospholipids (LPGs, LPIs, and PAs) were accumulated in patients who went on to die whilst other lipids such as FAs, TGs, glycerophospholipids (including PEs, PCs, and LPCs) and sphingolipids (SMs and Cers) were lower in non-survivors with respect to survivors in both cohorts across timepoints.

Overall, it can be concluded that this analysis showed that most of the lipids and lipoproteins which were significantly different between patients who went on to die and those who survived changed in the same direction in the two cohorts and these changes were also consistent across timepoints. However, the validation models focusing on mortality did not perform that well as the metabolites incorporated in these predictive models had less commonality between the two cohorts. To conclude, this study revealed baseline differences in the lipid and lipoprotein profiles of septic shock survivors and non-survivors which will aid in understanding the metabolic trajectories of these two clinical groups later in the analysis.

Chapter 6 – Metabolic Trajectories of lipids and lipoproteins in Septic Shock

6.1 Introduction

A metabolomic approach has been used in several studies to investigate the metabolic derangements of lipids and lipoproteins in patients with sepsis and these studies have provided some understanding of the metabolic alterations which occur in sepsis patients based on mortality (144). These metabolic derangements have been previously described in chapter 5 and most of the studies focused on the differences in the lipid and lipoprotein levels between survivors and non-survivors at static timepoints (145, 148, 150, 163, 170). Only one study has investigated, how the lipoprotein and lipid profiles evolve with time within each group (156). This study revealed that 28 and 90 day survivors had higher levels of glycerophospholipids (including 10 LPCs, 17 diacyl-PCs, 30 acyl-alkyl PCs, 2 acyl carnitines (carnitine and butyrylcarnitine), 4 long-chain SMs on day 7 in comparison to day 1 survivors whereas kynurenine and two polyunsaturated diacyl-PCs (PC aa C38:6, PC aa C40:6) reduced in survivors from day 1 to day 7 (156). In this study, patients who went on to die had an overall increase of glycerophospholipids (such as LPCs and PCs) and sphingolipids (such as SMs) from day 1 to day 7 based on 28-and 90-day mortality (156). This study had a relatively small sample size (N = 20; 11 survivors and 9 non-survivors for 28-day mortality; 9 survivors and 11 non-survivors for 90-day mortality) (156), was based on a small panel of lipids classes and did not have a validation cohort to confirm their findings. At present, lipoprotein and lipid changes have not yet been fully understood in patients with sepsis. Understanding the baseline lipid and lipoprotein changes in these patients is extremely useful but knowing how the lipid and lipoprotein profiles of these patients evolve over time will allow us to understand the complex biological mechanisms changing in patients who go on to survive and those who go on to die. This information will be extremely useful as it will allow clinicians to determine if the patients are getting better or worse and they will be able to adjust their treatment accordingly.

This chapter focusses on investigating the metabolic trajectories of lipids and lipoproteins for survivors and non-survivors of septic shock from two independent cohorts, with the purpose of understanding the metabolic pathways involving lipids and lipoproteins which change over time in sepsis. In future this might help clinicians in making clinical decisions as knowing the direction of change for lipid and lipoprotein levels in patients with sepsis will allow clinicians to determine whether the patients are getting better or worse and adjust

their treatment accordingly. In this study, metabolic trajectories related to clinical patient outcomes i.e., mortality and survival will be explored in both cohorts and whether the trajectories are different among patients who went on to survive and those who did not. This will be taken a step further where the findings from one cohort will be validated in the other cohort and vice versa. To date this type of validation of trajectory results focusing on mortality has not been previously done in other metabolic comparisons of survivors and non-survivors. There has not been any work done on how the lipids and lipoproteins change with organ failure scores (i.e., SOFA scores) in sepsis patients, so this study also investigated the metabolic changes of lipids and lipoproteins with SOFA scores with the purpose of understanding the molecular biology of sepsis patients with organ dysfunction.

6.2 Aims

In this study we investigated whether the metabolic profiles of patients with septic shock evolve with time based on mortality and if there are any metabolic differences in septic shock patients based on organ dysfunction.

The aims of this chapter are as follows:

- 1) To determine whether there are differences in the metabolic trajectories of survivors and non-survivors and if these could be used to track patient course.
- 2) To assess whether the metabolic trajectories of septic shock patients from one cohort can validate mortality results in another cohort.
- 3) To determine if the metabolic signatures of septic shock patients vary with organ failure.

6.3 Statistical Analysis

The trajectory analysis on outcome and validation of its results as well as metabolic changes with organ failure scores was carried out on the combined targeted data of a total of 419 lipids and lipoproteins from the two cohorts and the complete methods for this are listed in chapter 2, section 2.3.5.

6.4 Results

6.4.1 Metabolic trajectories focusing on outcome

6.4.1.1 Metabolic trajectories focusing on outcome

Trajectory analysis was performed on a total of 475 samples from VANISH and 1404 samples from LeoPARDS, as shown in figures 6.1 and 6.2, to determine the trajectories of lipids and lipoproteins for survivors and non-survivors from both cohorts. In VANISH, the samples used for the trajectory analysis were from the combined BI LISA (N = 475), LPOS ppR (N = 488) and LNEG ppR (N = 488) data. Sample exclusions from both VANISH and LeoPARDS, were performed in the same way as mentioned in chapter 2, section 2.2.1.3 and 2.2.2.3. However, some additional exclusions were performed during the analysis. In VANISH, an additional 18 samples from BI LISA and 19 samples from both LPOS and LNEG ppR which had no sample timings recorded and 4 samples which belonged to a patient whose samples were collected after several days of receiving the study drug from all three datasets were excluded whilst in LeoPARDS, an additional 10 samples which had no sample timings recorded were excluded from all three datasets.

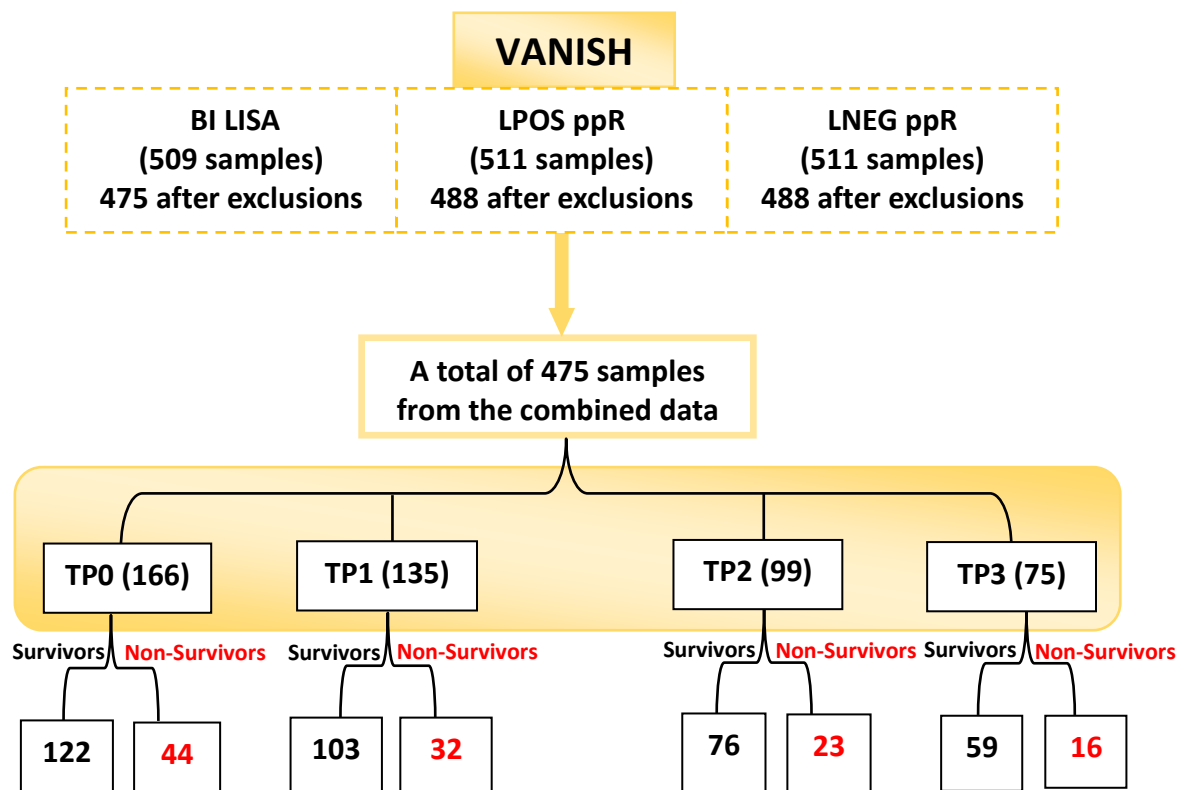


Figure 6.1: Flow chart for the samples included in the trajectory analysis of combined BI LISA, LPOS ppR and LNEG ppR data from VANISH.

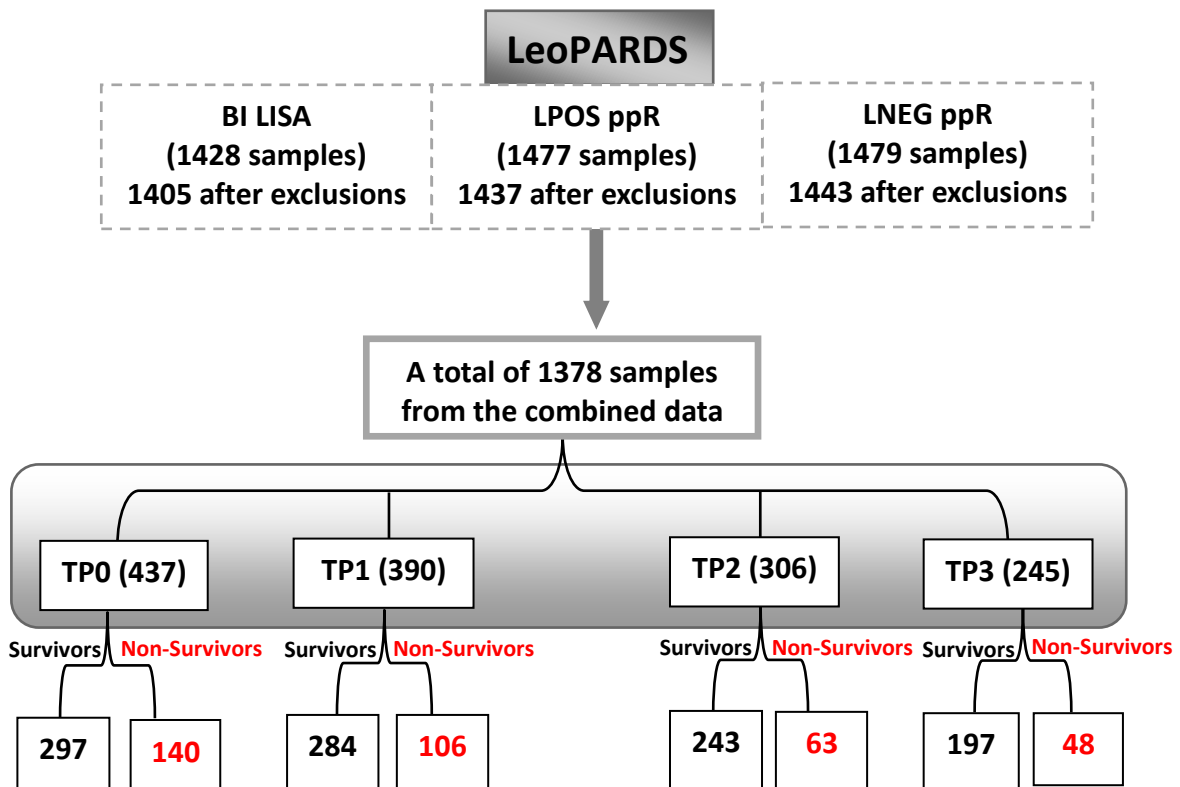


Figure 6.2: Flow chart for the samples included in the trajectory analysis of combined BI LISA, LPOS ppR and LNEG ppR data from LeoPARDS.

The results from the LME models of lipids and lipoproteins for survivors and non-survivors with and without BMI as a confounding factor were the same with few exceptions which will be addressed later. A summary of the results for the trajectory analysis of 419 lipids and lipoproteins for survivors and non-survivors for both cohorts is presented in figure 6.3 and figure A1.13 (see appendix 1.6).

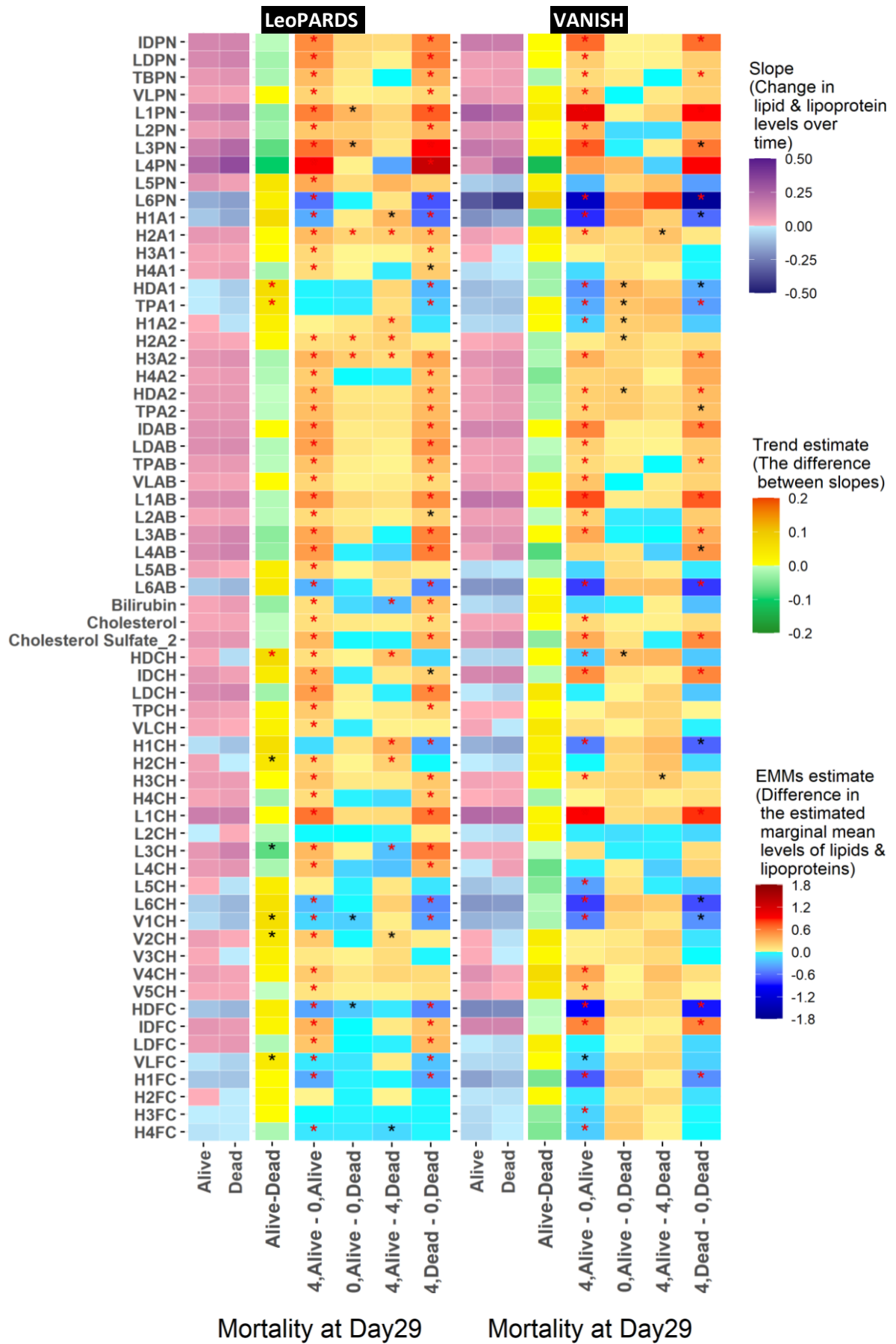
In brief, there was a lack of significant difference between the overall metabolic trajectories of survivors and non-survivors from the two cohorts according to the adjusted p-values suggesting that the trajectories were similar for the two groups no matter what the outcome. Only two fatty acyl carnitines such as palmitoylcarnitine CAR (16:0) and eicosenoylcarnitine CAR (20:1) had significantly different overall trajectories for survivors and non-survivors based on the unadjusted p-values (Trend estimate $p < 0.05$) and these long-chain acyl carnitines remained significant after correction but only in LeoPARDS. These fatty acyl carnitines increased over time in patients who survived and decreased over time

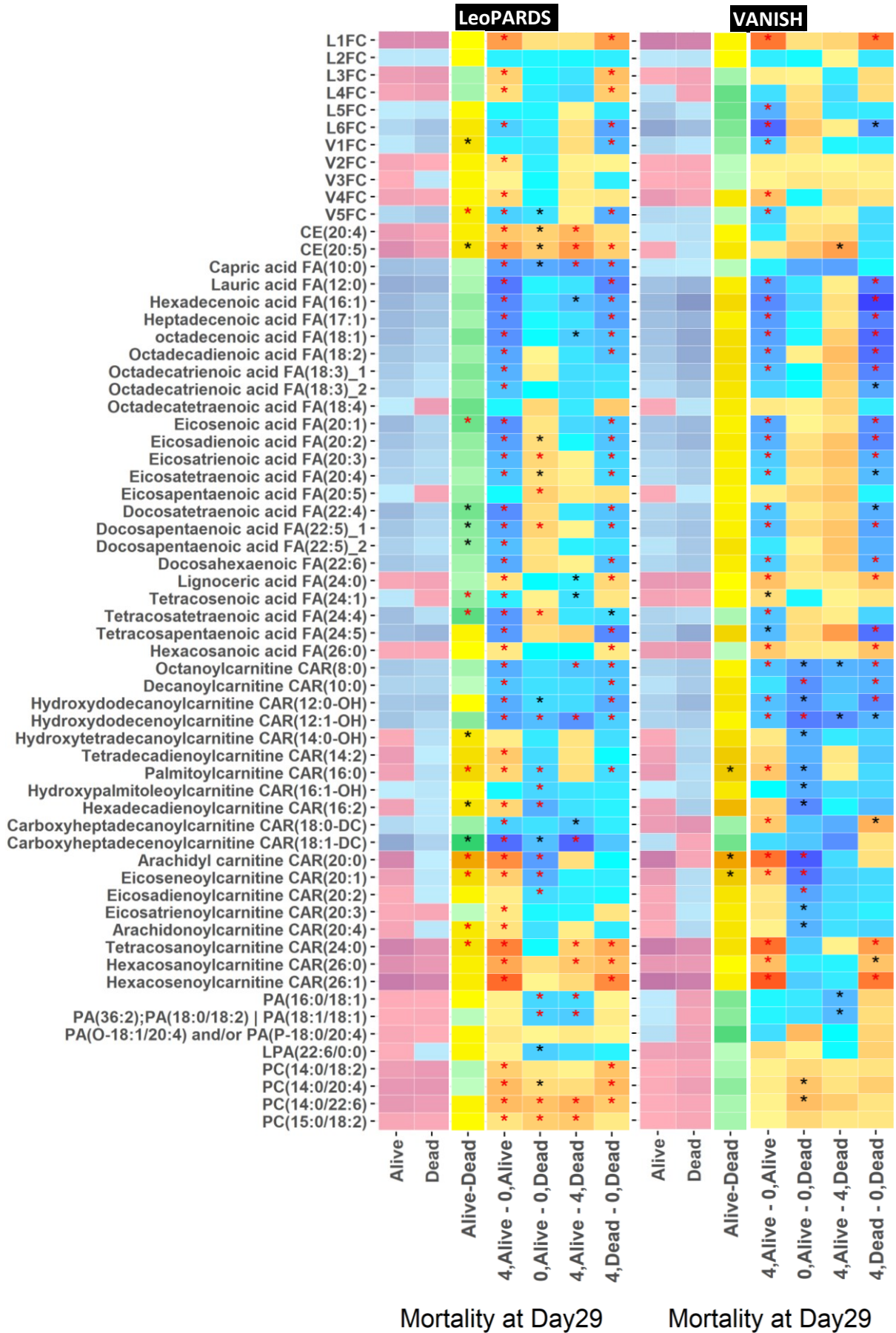
in patients who went on die in both cohorts (Trend estimate $p_{Adj} < 0.05$ shown by red asterisks in LeoPARDS and $p < 0.05$ shown by black asterisk in VANISH) (figure 6.3).

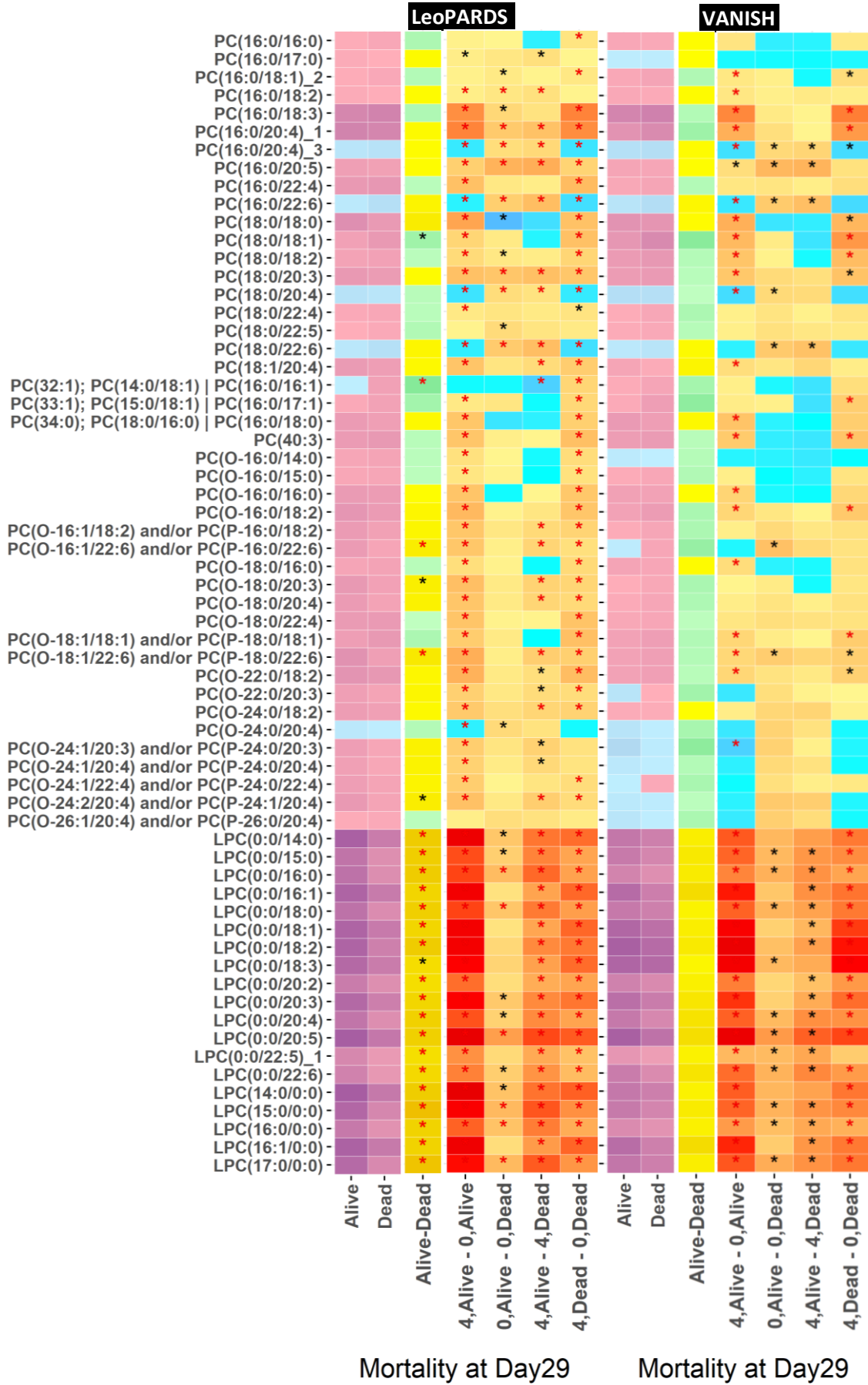
The levels of lipids and lipoproteins change over time in patients with sepsis and it was found that in both cohorts a total of 110 lipids and lipoproteins were significantly different in both survivors and non-survivors at day 0 in comparison to day 4 ($p_{Adj} < 0.05$) (figure 6.3) which suggests a very dynamic trajectory for these two clinical groups. Of these 94 lipids and lipoproteins including particle numbers of several lipoproteins (TBPN, IDPN, , L1PN), apolipoproteins (HDA2, H3A2, TPAB, IDAB, L1 & L3AB), cholesterol Sulfate_2, lipoprotein bound cholesterol and free cholesterol (L1CH, IDFC, L1FC), two fatty acids (lignoceric acid FA(24:0), hexacosanoic acid FA(26:0)), two long chain acyl carnitines (tetracosanoylcarnitine CAR(24:0), hexacosenoylcarnitine CAR(26:1)), LDL phospholipid subfraction 1 (L1PL), glycerophospholipids (7 PCs, 42 LPCs, 5 PEs, 7 LPEs (LPE(0:0/18:1), LPG(18:1/0:0)), sphingolipids (2 Cers (Cer(d18:1/24:0), Cer(t18:0/24:1)), SulfoHexCer(d18:1/24:1(OH)) and SM(d18:1/24:1)), 7 free triglycerides, lipoprotein bound triglycerides (LDTG, L1-L5TG, H4TG), two monoacylglycerols (MGs) (MG(18:1)_1, MG(18:1)_2) and retinol were significantly higher in both survivors and non-survivors at day 4 ($p_{Adj} < 0.05$) than at day 0 in both cohorts. 16 lipids and lipoproteins including L6PN, L6AB, HDLC, H1FC, 10 fatty acids (one SFA, 4 MUFAs and 5 PUFAs) and two fatty acyl carnitines (octanoylcarnitine CAR(8:0), hydroxydodecanoylcarnitine CAR(12:0-OH)) decreased over time in the two clinical groups from both cohorts ($p_{Adj} < 0.05$) (figure 6.3).

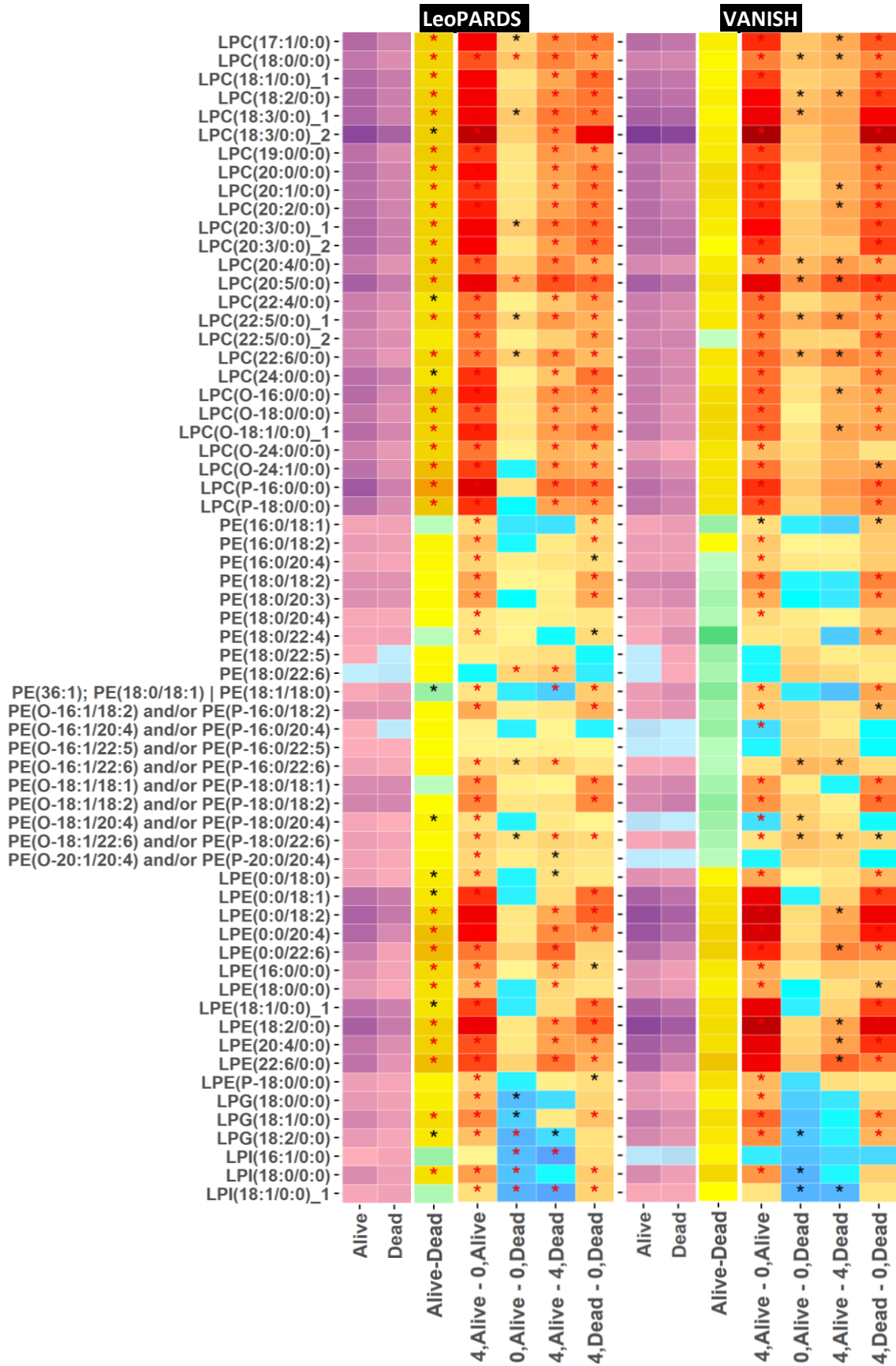
In addition to the metabolic trajectories, the metabolic differences between survivors and non-survivors at day0 were also determined using the LME models. At day 0, only 4 fatty acyl carnitines (hydroxydodecenoylcarnitine CAR(12:1-OH), eicoseneoylcarnitine CAR(20:1), eicosadienoylcarnitine CAR(20:2), arachidyl carnitine CAR(20:0)) had significantly higher EMMs in non-survivors ($p_{Adj} < 0.05$) with respect to survivors in both cohorts (figure 6.3) and these findings were also consistent with the univariate results of LPOS ppR data (chapter 5, section 5.4.1.2.1, table 5.5). At day 4, there were not any significant difference in the lipids and lipoproteins levels between survivors and non-survivors from the two cohorts based on the adjusted p-values, but the trend for most of the lipid species for the clinical groups was the same in both cohorts (figure 6.3).

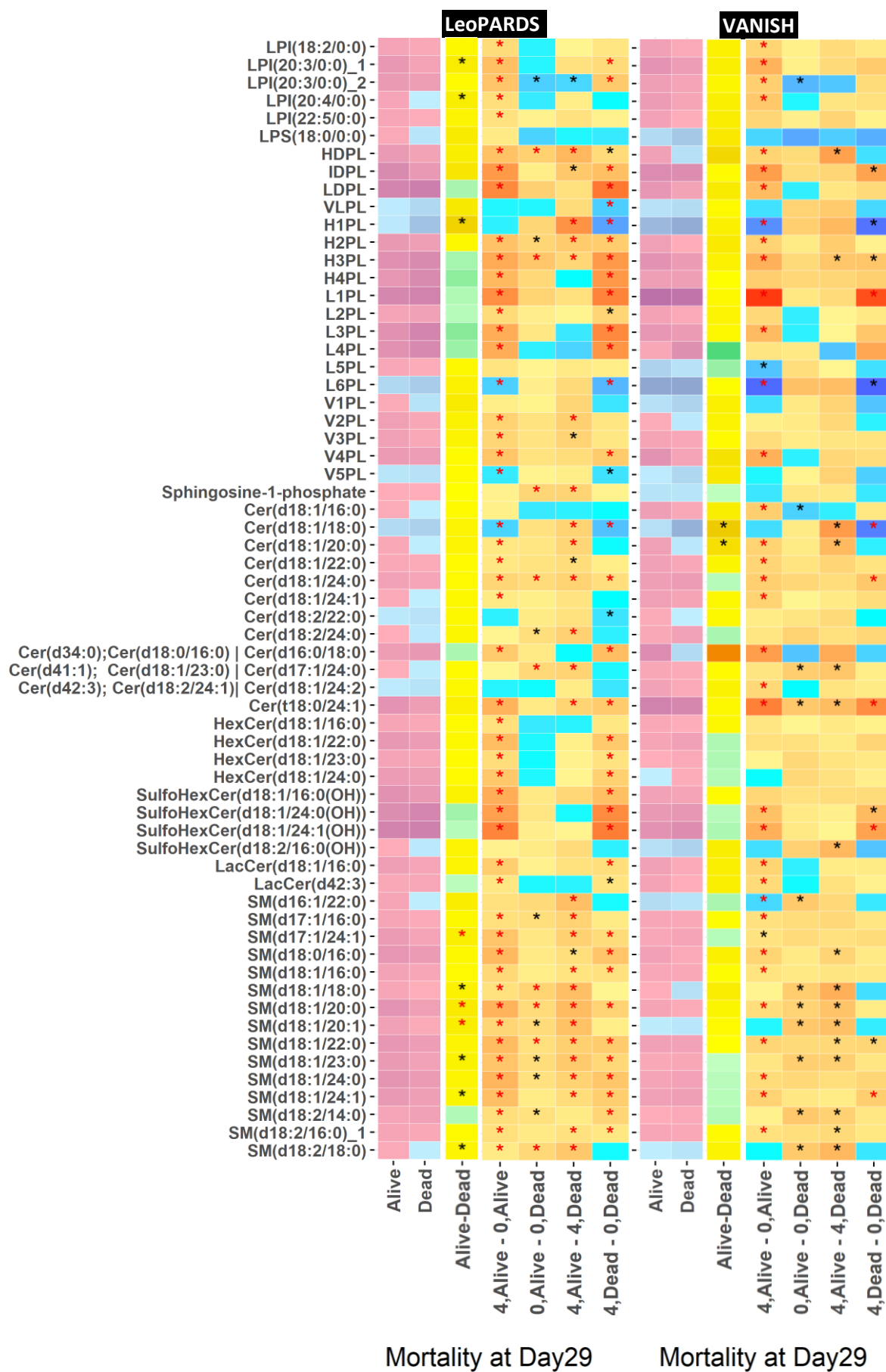
The trajectory model of mortality with BMI (figure A1.13, see appendix 1.6) also showed the same results as the model without BMI, but 18 lipids and lipoproteins (including HDA1, TPA1, HDCH, arachidonylcarnitine CAR(20:4), PC(O-16:1/22:6) and/or PC(P-16:0/22:6), 8 LPCs, LPE(0:0/18:2), 3 SMs (SM(d17:1/24:1), SM(d18:1/20:0), SM(d18:1/20:1), DG(18:2/18:1)) did not remain significant after B-H correction in LeoPARDS in the model with BMI (figure A1.13, see appendix 1.6) whilst they remained significant after correction in the LME model without BMI (figure 6.3). However, the trend for all these metabolites was in the same direction for survivors and non-survivors in both LME models with and without BMI for LeoPARDS. Additionally, L3CH was significant after BH correction in the model with BMI, but not in the model without BMI though the trend for survivors and non-survivors was the same in both models. Besides this there were not any other major differences between the results obtained from two LME models for both cohorts.

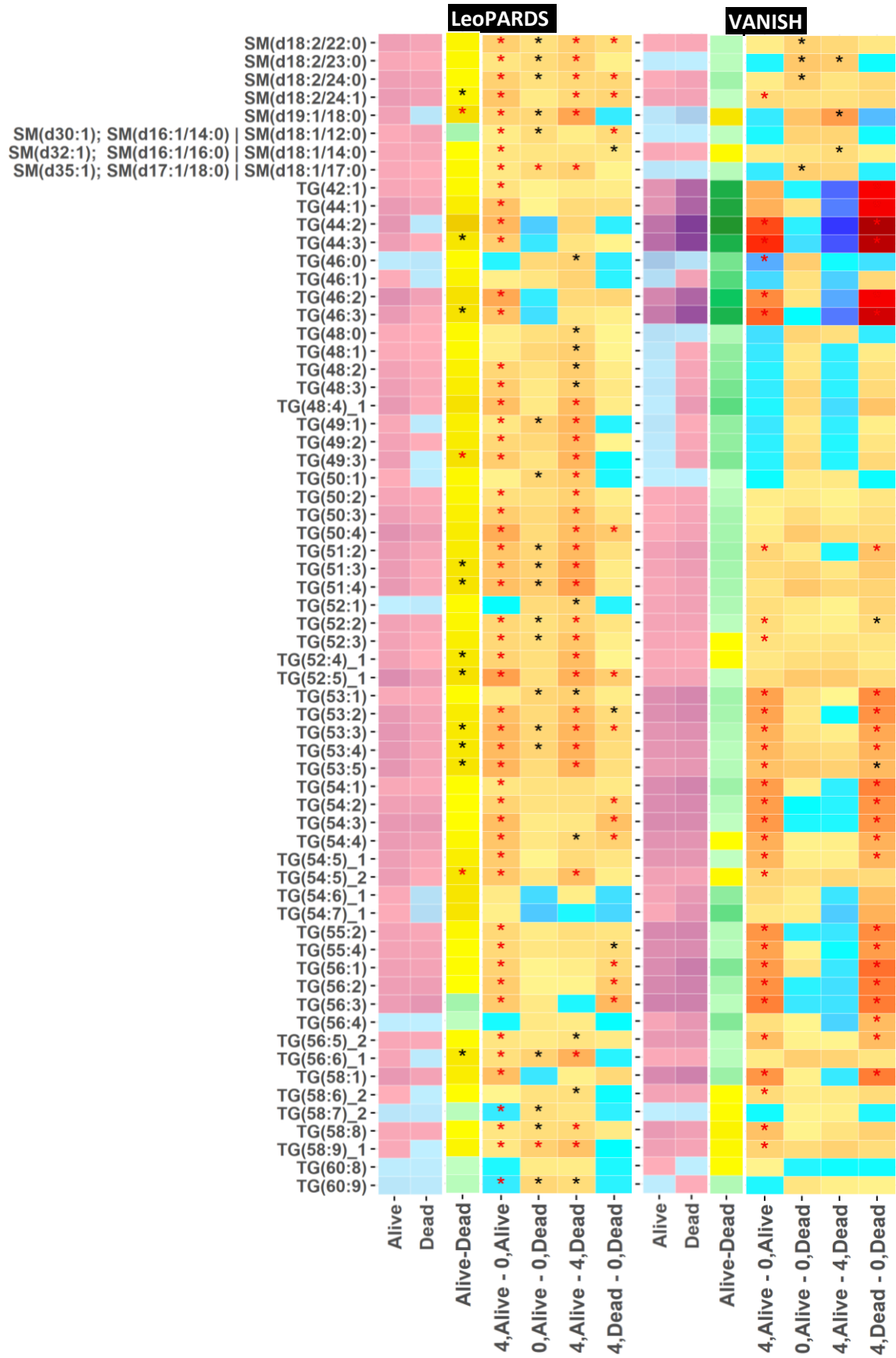












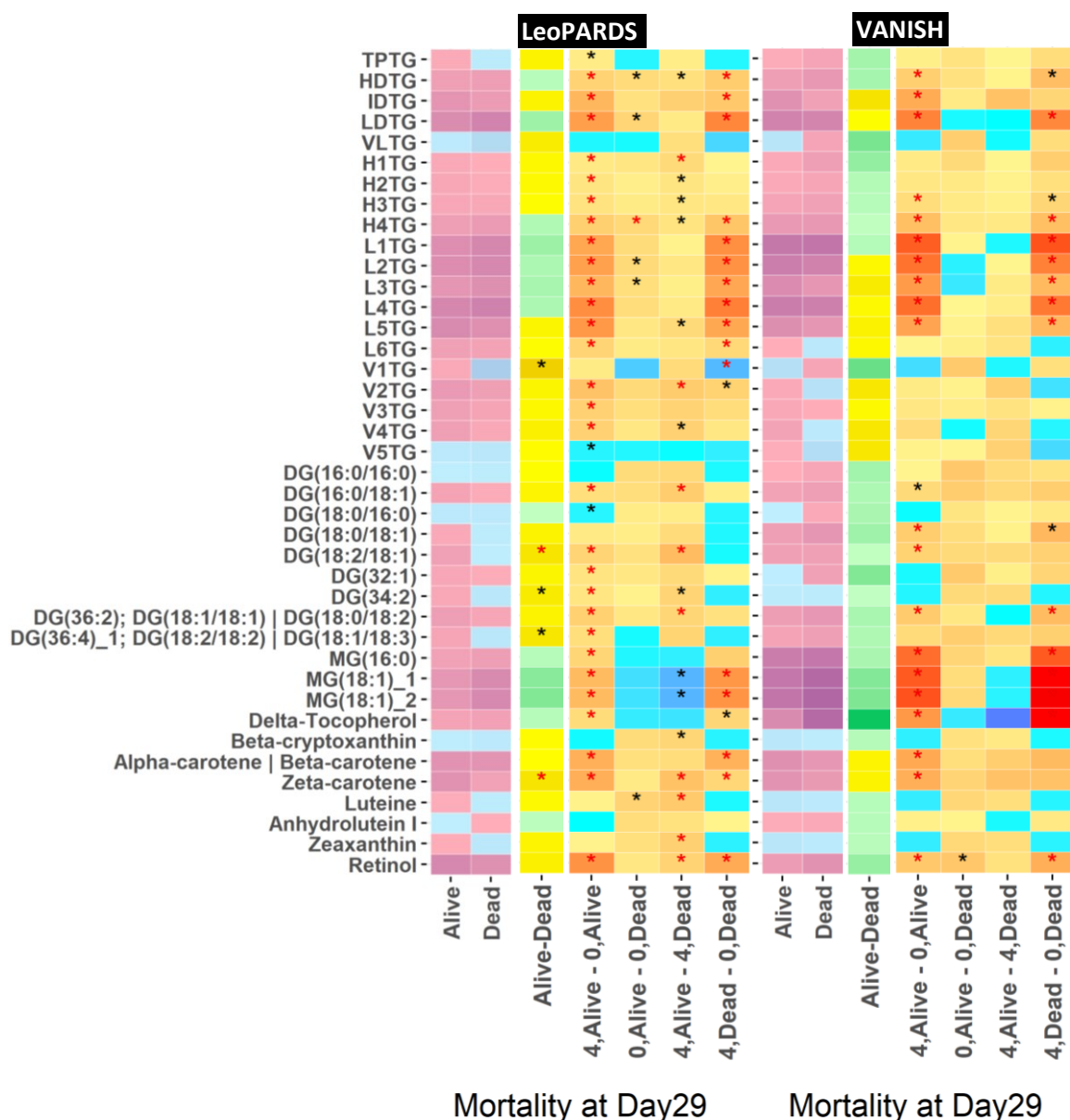


Figure 6.3: The results of LME models focusing on mortality for combined BI LISA, LPOS ppR and LNEG ppR log transformed data with age and sex as confounders for VANISH and LeoPARDS. Various contrasts were performed via contrast coding during the trajectory analysis and their outcomes are shown in this figure. LME model outputs including slope (i.e., change in lipid and lipoprotein levels over time), estimate (i.e., the difference between the estimated marginal mean levels of lipids and lipoproteins) are presented in different colour scales. Lipid and lipoproteins which are significantly different between groups from various pairwise comparisons are highlighted in black asterisk whilst those that remained significant after correction are shown in red. The slopes for alive (i.e., survivors) and dead (i.e., non-survivors) are shown as well as the difference between their slopes and whether this difference in the overall slopes is significant between the two groups. Positive values for slope indicate that the metabolites increase with time whilst the negative values indicate that the metabolites decrease over time and the stronger the colour the bigger the magnitude. The difference between the slopes for alive and dead is presented in different colour scale. Estimated marginal means were also calculated for each group at days 0 and day 4 using the LME model and the difference between the EMMs for four pairwise comparisons are presented here. The four pairwise comparisons include: 4, Alive-0, Alive = Difference between the EMMs of survivors at day 4 and day 0 values; 4,Dead-0,Dead = Difference between the EMMs of non-survivors at day 4 and day 0 values; 0,Alive-0,Dead = Difference between the EMMs of survivors and non-survivors at day 0; 4,Alive-4,Dead = Difference between the EMMs of survivors and non-survivors at day 4. Abbreviations: CH = Cholesterol; FC = Free

Cholesterol; PL = Phospholipid; TG = Triglycerides; A1 = Apo-A1; A2 = Apo-A2 , AB = Apo-B; HD = High density lipoprotein (HDL); VL = Very low density lipoprotein (VLDL); ID = Intermediate density lipoprotein (IDL); LD = Low density lipoprotein; TPCH = Total plasma Cholesterol; VLCH = VLDL Cholesterol; V1-V5CH = VLDL Cholesterol subfractions 1-5; IDCH = IDL Cholesterol; LDCH = LDL Cholesterol; L1-6CH = LDL Cholesterol subfractions 1-6; HDCH = HDL Cholesterol; H1-4CH = HDL Cholesterol subfractions 1-4; VLFC = VLDL Free Cholesterol; V1-V5FC = VLDL Free Cholesterol subfractions 1-5; IDFC = IDL Free Cholesterol; LDFC = LDL Free Cholesterol; L1-L6FC = LDL Free Cholesterol subfractions 1-6; HDFC = HDL Free Cholesterol; H1-H4FC = HDL Free Cholesterol subfractions 1-4; TPA1 = Total Plasma A1; HDA1 = HDL Apo-A1; H1-H4A1 = HDL Apo A1 subfractions 1-4; TPA2 = Total plasma A2; H1-H4A2 = HDL Apo A2 subfractions 1-4; TPAB = Total plasma Apo-B; LDAB = LDL Apo-B; L1-L6AB = LDL Apo-B subfractions 1-6; IDAB = IDL Apo-B; TPBN: Total blood particle number; VLPN = VLDL Particle Number; IDPN = IDL Particle Number; LDPN = LDL Particle Number; L1-L6PN = LDL Particle number subfractions 1-6; TPTG = Total Plasma Triglycerides; VLTG = VLDL Triglycerides; V1-V5TG = VLDL Triglycerides subfractions 1-5; IDTG = IDL Triglycerides; LDTG = LDL Triglycerides; L1-L6TG = LDL Triglycerides subfractions 1-6; HDTG = HDL Triglycerides; H1-H4TG = HDL Triglycerides subfractions 1-4; VLPL = HDL Phospholipids; V1-V5PL = VLDL Phospholipid subfractions 1-5; IDPL = IDL Phospholipids; LDPL = LDL Phospholipids; L1-L6PL = LDL Phospholipids subfractions 1-6; HDPL = HDL Phospholipids; H1-H4PL = HDL Phospholipids subfractions 1-4; PC = Phosphatidylcholine; LPC = Lysophosphatidylcholine; PE = Phosphatidylethanolamine; LPE = Lysophosphatidylethanolamine; PI = Phosphatidylinositol; LPI = Lysophosphatidylinositol; LPG = Lysophosphatidylglycerol; PA = Phosphatidic acid; LPA = Lysophosphatidic acid; LPS = Lysophosphatidylserine; SM = Sphingomyelin; Cer = Ceramide; LacCer = Lactosylceramide; HexCer = Hexosylceramide; SulfoHexCer = Sulfohexsoylceramide; CE = Cholesteryl esters; DG = Diacylglycerol; MG = Monoacylglycerols; TG = Triglycerides; CAR = Carnitine; FA = Fatty acid. Note: Carbon atoms and double bonds in all lipid species are represented as (C atoms:double bond); d indicates 2 hydroxyl groups in the sphenoid base of ceramides and sphigomyelins; DC denotes for dicarboxylic acid; Prefixes "OH", "O" and "P" represents different substituents/linkages in the lipid species: "OH" denotes for hydroxyl group, "O" represents an alkyl ether linkage whilst "P" refers to the plasmalogen link. Lipid species with abbreviations _1 and _2 indicate same m/z value but different retention times for those lipids.

6.4.1.2 Prediction of outcome based on metabolic changes from baseline

The differences between the trajectories of lipids and lipoproteins for survivors and non-survivors were found from the trajectory analysis of combined targeted data (both BI LISA, LPOS ppR and LNEG ppR). The aim was to validate these findings for both VANISH and LeoPARDS and see whether the results from one cohort can be used to predict outcome in the other. The predictive models were not only performed on paired samples of TP0 and TP1 with fold changes (TP0TP1) (N = 128 and N = 367 for VANISH and LeoPARDS, respectively) but also on paired samples of TP0 and TP2 with fold changes (i.e., TP0TP2) (N = 93 and N = 284 for VANISH and LeoPARDS, respectively) from combined BI LISA, LPOS ppR and LNEG ppR data from both cohorts as shown in figure 6.4 and figure 6.5. The same number of samples were excluded from all the datasets (BI LISA, LPOS and LNEG ppR data) as the trajectory analysis for outcome discussed previously in this chapter. In this analysis, TP0TP1 and TP0TP2 matched samples with fold-changes were used to see whether the lipids and lipoproteins with significantly different trajectories for survivors and non-survivors could be used to aid earlier identification patients who were likely to die or survive. In the predictive models for validation within the study cohort, LME models were built on samples which had matching patient IDs with TP0TP1 and TP0TP2 samples with fold changes from VANISH (N = 343 and N = 282 for TP0TP1 and TP0TP2 matched samples with fold changes, respectively) and LeoPARDS (N = 1039 and N = 882 for TP0TP1 and TP0TP2 matched samples with fold-changes, respectively).

The performance metrics for the mortality prediction models of the combined targeted data for the two cohorts for TP0TP1 and TP0TP2 samples with fold-changes are listed in table 6.1. The predictive models for TP0TP1 samples with fold changes for both cohorts performed slightly better than the predictive models for TP0TP2 samples (figure A1.14, see appendix 1.6) with fold changes but still the models were not good overall. In brief, the cross validated ROC curve for within cohort validation of VANISH for TP0TP1 samples with fold-changes (VANISH TP0TP1_{WC}) for VANISH test set and validation of VANISH within LeoPARDS dataset for TP0TP1 samples with fold-changes (VANISH TP0TP1_{EXCO}) had an AUC of 0.59 and 0.64, respectively (figure 6.6, table 6.1) (for five significant lipids (two lipids (eicoseneoylcarnitine CAR (20:1) and arachidonoylcarnitine CAR (20:4)) common in both

cohorts) selected from LME model based on mortality for VANISH with age and sex as confounders, figure 6.3).

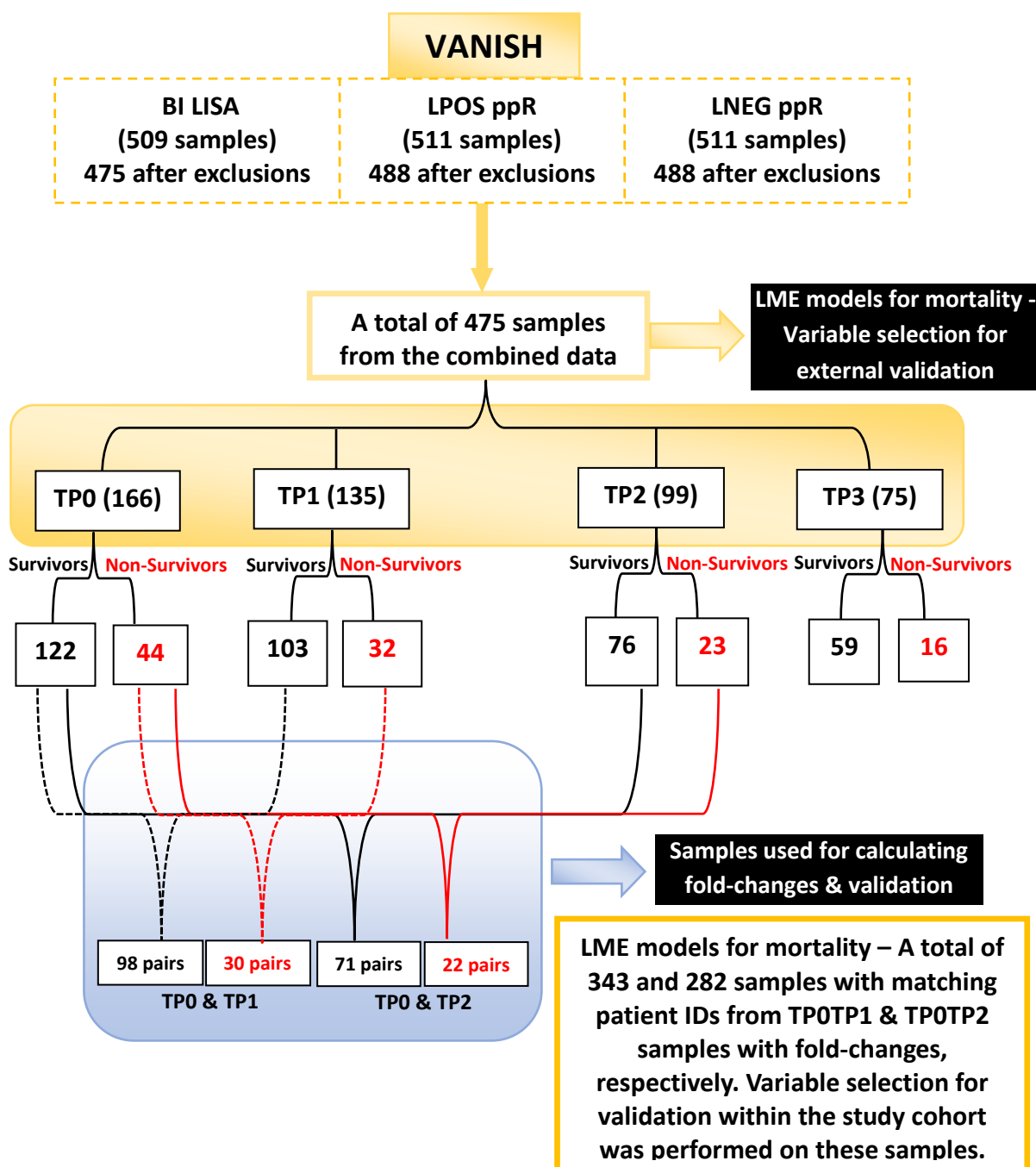


Figure 6.4: Flow chart representing samples included in the validation of mortality results from the trajectory analysis of combined BI LISA, LPOS ppR and LNEG ppR data from VANISH.

The predictive models for within cohort validation of LeoPARDS for TP0TP1 samples with fold-changes (LeoPARDS TP0TP1_{WC}) from LeoPARDS test set and validation of LeoPARDS within VANISH dataset for TP0TP1 samples with fold-changes (LeoPARDS TP0TP1_{EXCO}) had an AUC of 0.56 and 0.72, respectively (figure 6.6) (for 120 significant metabolites (two lipids

common in both cohorts, same as previous validation models) selected from LME model based on mortality for LeoPARDS with age and sex as confounders figure 6.3). Both of our validation models did not work as there were only two common lipids between the two cohorts which had significant different overall trajectories for survivors and non-survivors.

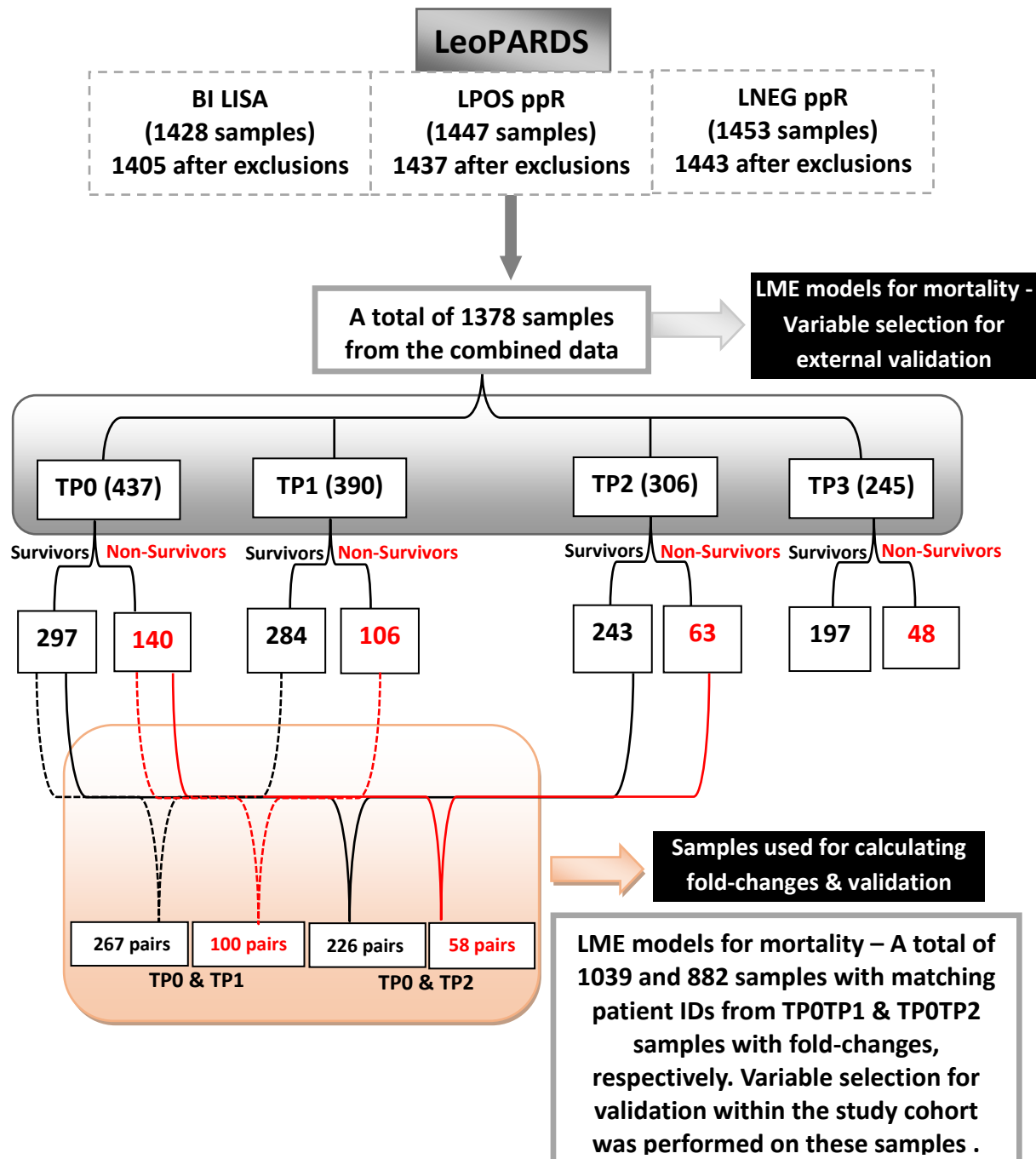


Figure 6.5: Flow chart representing samples included in the validation of mortality results from the trajectory analysis of combined BI LISA, LPOS ppR and LNEG ppR data from LeoPARDS.

Table 6.1: Performance metrics for mortality prediction models within cohort (WC) and with external cohort (EXCO) for TPOTP1 and TPOTP2 foldchange samples from combined BI LISA, LPOS and LNEG ppRa data. Abbreviations: TPOTP1_{WC} = within cohort for TPOTP1 samples with fold changes, TPOTP1_{EXCO} = with external cohort for TPOTP1 samples with fold changes; TPOTP2_{WC} = within cohort for TPOTP2 samples with fold changes; TPOTP2_{EXCO} = with external cohort for TPOTP2 samples with fold changes.

Parameters	VANISH				LeoPARDS			
	TPOTP1 _{WC}	TPOTP1 _{EXCO}	TPOTP2 _{WC}	TPOTP2 _{EXCO}	TPOTP1 _{WC}	TPOTP1 _{EXCO}	TPOTP2 _{WC}	TPOTP2 _{EXCO}
Accuracy (%)	74.22	70.57	68.82	74.65	65.67	70.31	69.01	66.67
Balanced accuracy (%)	60.03	49.44	51.34	49.47	56.70	67.89	56.82	53.07
Sensitivity (%)	33.33	3.00	18.18	6.90	37.00	63.33	36.21	27.27
Specificity (%)	86.74	95.88	84.51	92.03	76.40	72.45	77.43	78.87
Positive predicted value (%)	43.48	21.43	26.67	18.18	37.00	41.30	29.17	28.57
Negative predicted value (%)	80.95	72.52	76.92	79.39	76.40	86.59	82.55	77.78
Prevalence of death (%)	23.44	27.24	23.66	20.42	27.25	23.44	20.42	23.66

Table 6.2: Confusion matrices for TPOTP1 foldchange mortality predictive models for VANISH and LeoPARDS BI LISA, LPOS and LNEG ppR combined datasets. Note: TPOTP1_{WC} = within cohort for TPOTP1 samples with fold changes and TPOTP1_{EXCO} = with external cohort for TPOTP1 samples with fold changes.

Validation of VANISH within cohort			
VANISH TPOTP1 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	85	20
	Non-survivors	13	10
Validation of VANISH in LeoPARDS			
VANISH TPOTP1 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	256	97
	Non-survivors	11	3
Validation of LeoPARDS within cohort			
LeoPARDS TPOTP1 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	204	63
	Non-survivors	63	37
Validation of LeoPARDS in VANISH			
LeoPARDS TPOTP1 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	71	11
	Non-survivors	27	19

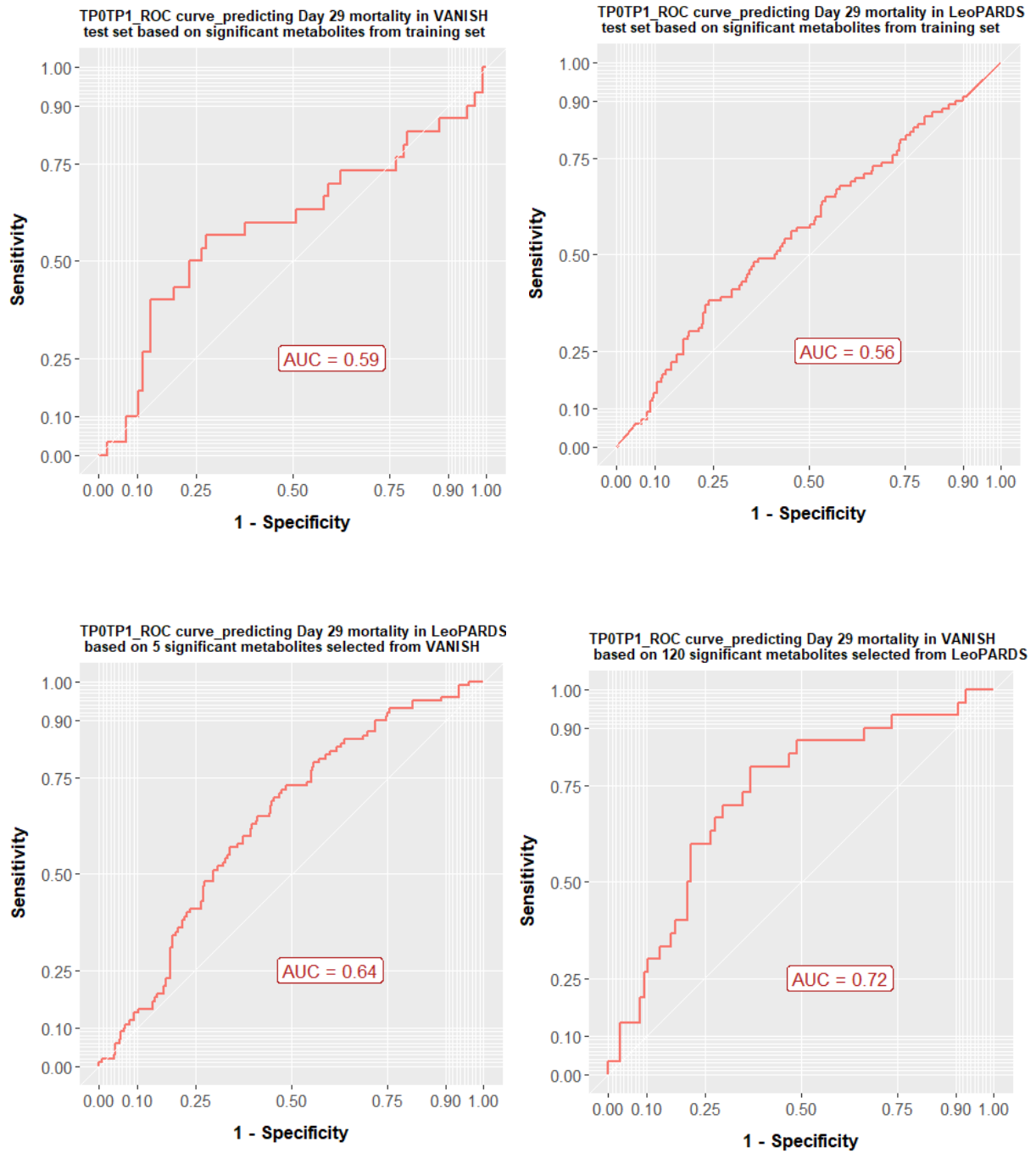


Figure 6.6: ROC curves for the predictive models of mortality on combined BI LISA, LPOS and LNEG ppR dataset for VANISH and LeoPARDS for TP0TP1 samples with fold-changes.

6.4.2 Metabolic changes focusing on organ failure

Combined NMR and UHPLC-MS targeted data (BI LISA, LPOS ppR and LNEG ppR) was used to determine how the metabolic profiles of patients with septic shock vary with organ failure in both cohorts. The metabolic changes with SOFA scores were determined by generating LME models on a total of 458 samples from VANISH and 1345 samples from LeoPARDS, as shown in figure 6.7 and figure 6.8. Sample exclusions from both VANISH and LeoPARDS, were performed in the same way as mentioned in chapter 2, (sections 2.2.1.3 and 2.2.2.3). However, some additional exclusions were performed during this analysis. In VANISH, an additional 39 samples from all three datasets were excluded (35 samples had no sample timings recorded whilst four samples did not have the SOFA scores recorded for the days corresponding to the samples). In LeoPARDS, additional samples (43 samples from both LPOS and LNEG ppR data and 44 from BI LISA) were excluded from all the datasets as these samples had no sample timings recorded. The results for LME models with and without BMI are shown in figure A1.15 (see appendix 1.7) and figure 6.9, respectively.

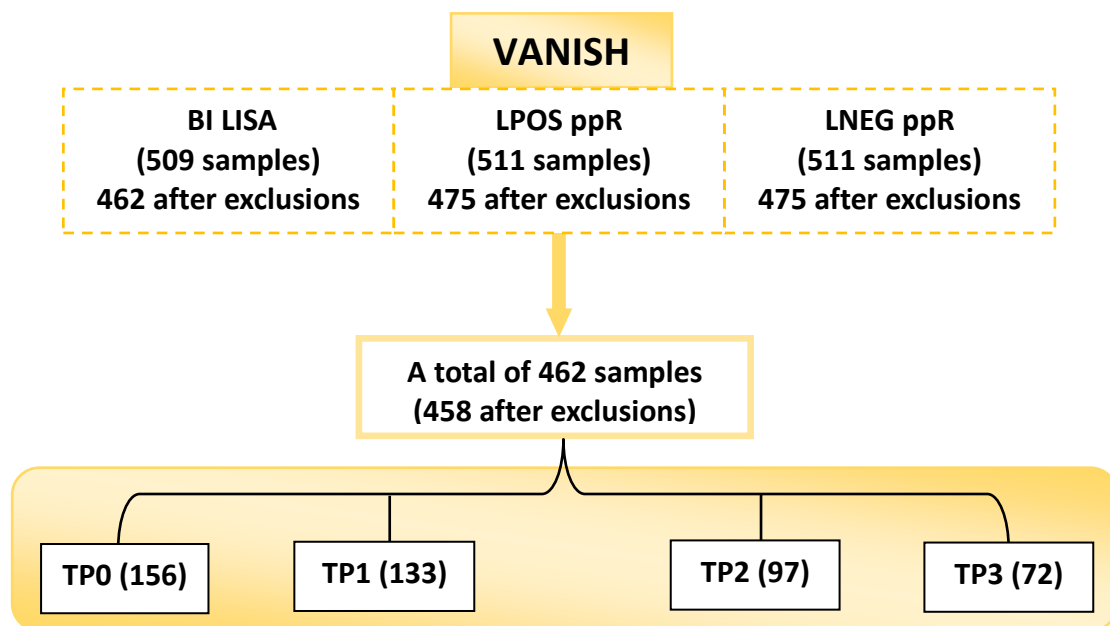


Figure 6.7: Flow chart representing samples included in the trajectory analysis of combined BI LISA, LPOS and LNEG ppR data from VANISH to determine the metabolic changes based on organ failure scores.

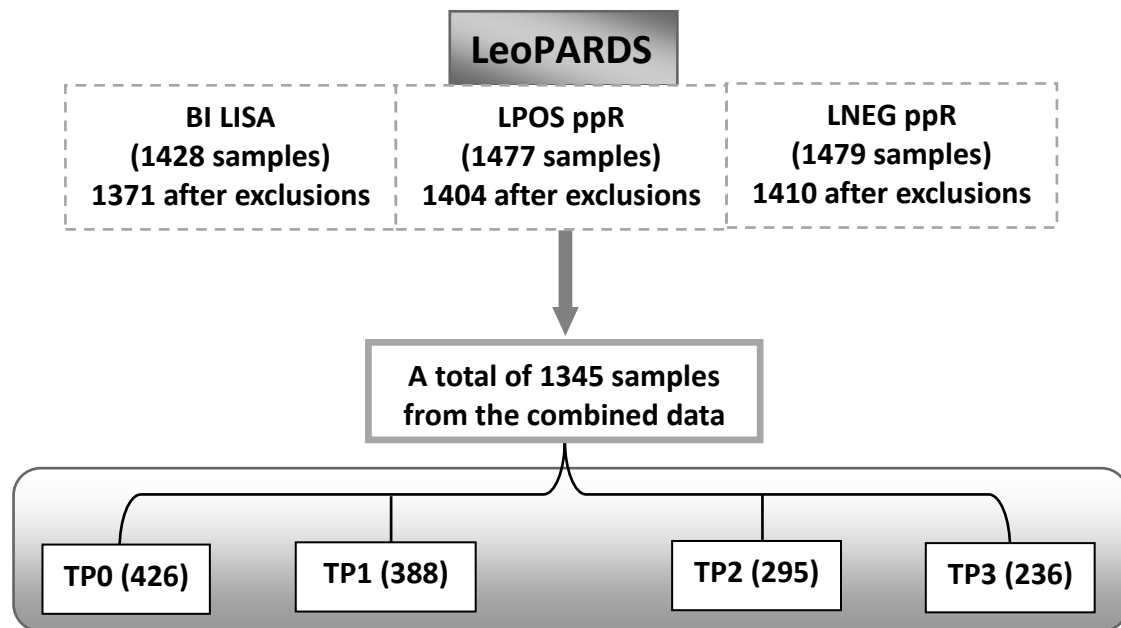


Figure 6.8: Flow chart representing samples included in the trajectory analysis of combined BI LISA, LPOS and LNEG ppR data from LeoPARDS to determine the metabolic changes based on organ failure scores.

The results from the LME models of SOFA scores without BMI (figure 10) as a confounder revealed that the higher the total SOFA score the higher the 22 lipids including three fatty acids (capric acid FA(10:0), tetracosenoic acid FA(24:1), tetracosatetraenoic acid FA(24:4)), 6 medium- and long-chain acyl carnitines, glycerophospholipids (2 PAs, PC(32:1); PC(14:0/18:1) | PC(16:0/16:1), 2 PEs, 2 LPGs), 4 free triglycerides (TG(44:2), TG(46:3), TG(54:6)_1, TG(54:7)_1)) and bilirubin in patients with sepsis from both cohorts ($p_{Adj} < 0.05$). Additionally, 100 lipids and lipoproteins including apolipoproteins, free triglycerides and lipoprotein bound triglycerides (TG(54:5)_2, HDTG, , H1-H4TG, L3TG), lipoprotein bound cholesterol and free cholesterol, glycerophospholipids (PC(16:0/22:6), 44 LPCs, 9 LPEs, LPI(20:3/0:0)_1)), sphingolipids (2 Cers and 13 SMs) and lipoprotein bound phospholipids (HDPL, H1-H3PL, L1PL) decrease as the total SOFA score increases for both cohorts ($p_{Adj} < 0.05$).

Changes in the lipids and lipoproteins linked with the cardiac SOFA score (figure 6.9) were observed in both cohorts with capric acid FA(10:0) increasing as the cardio SOFA score increases ($p_{Adj} < 0.05$) whilst 69 lipids and lipoproteins (including apolipoproteins (H3A2, L1AB), lipoprotein bound cholesterol and free cholesterol, glycerophospholipids (PC(O-16:1/18:2) and/or PC(P-16:0/18:2), 41 LPCs, 6 LPEs), lipoprotein bound phospholipids (HDPL,

H2 & H3PL, L1PL), sphingolipids (8 SMs), LDL triglyceride subfraction decreased in patients who progress towards cardiac failure in both cohorts ($p_{Adj} < 0.05$).

As the coagulation SOFA score increases, 24 lipids species including three fatty acids (lignoceric acid FA(24:0), tetracosenoic acid FA(24:1), tetracosatetraenoic acid FA(24:4)), 2 fatty acyl carnitines (carboxyheptadecanoylcarnitine CAR(18:0-DC), carboxyheptadecenoylcarnitine CAR(18:1-DC)), glycerophospholipids (2 PAs, 6PCs, 3 PEs), Cer(d18:1/16:0), six free triglycerides and bilirubin also increase in both cohorts ($p_{Adj} < 0.05$). However, the levels of 76 lipids and lipoproteins including HDL apolipoproteins, lipoprotein bound cholesterol and free cholesterol, two CEs (CE(20:4), CE(20:5)), 3 fatty acyl carnitines (tetradecadienoylcarnitine CAR(14:2), arachidyl carnitine CAR(20:0), hexacosanoylcarnitine CAR(26:0)), glycerophospholipids (42 LPCs, 6 LPEs), sphingolipids (8 SMs) HDL bound phospholipids, sphingosine-1-phosphate, and LDL triglyceride subfraction 6 decrease with the coagulation SOFA score in both cohorts ($p_{Adj} < 0.05$) (figure 6.9).

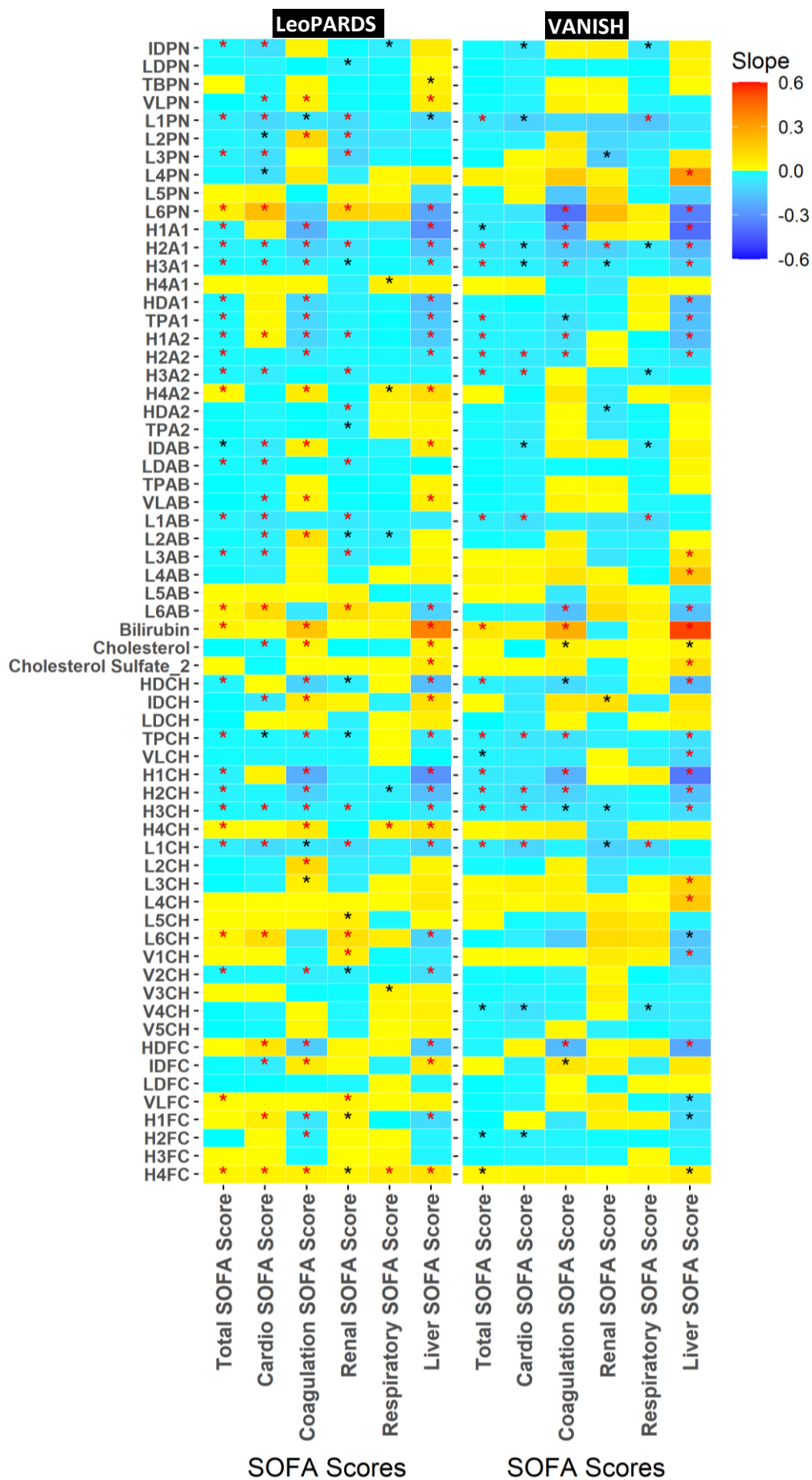
The results from this analysis also revealed that higher the renal SOFA score the higher the levels of 5 medium- and long chain acyl carnitines (carboxyheptadecenoylcarnitine CAR(18:1-DC), hydroxydodecenoylcarnitine CAR(12:1-OH), octanoylcarnitine CAR(8:0), hydroxydodecanoylcarnitine CAR(12:0-OH), decanoylcarnitine CAR(10:0)) ($p_{Adj} < 0.05$) whilst H2A1 and H3PL decrease as the renal SOFA increases ($p_{Adj} < 0.05$) (figure 6.9).

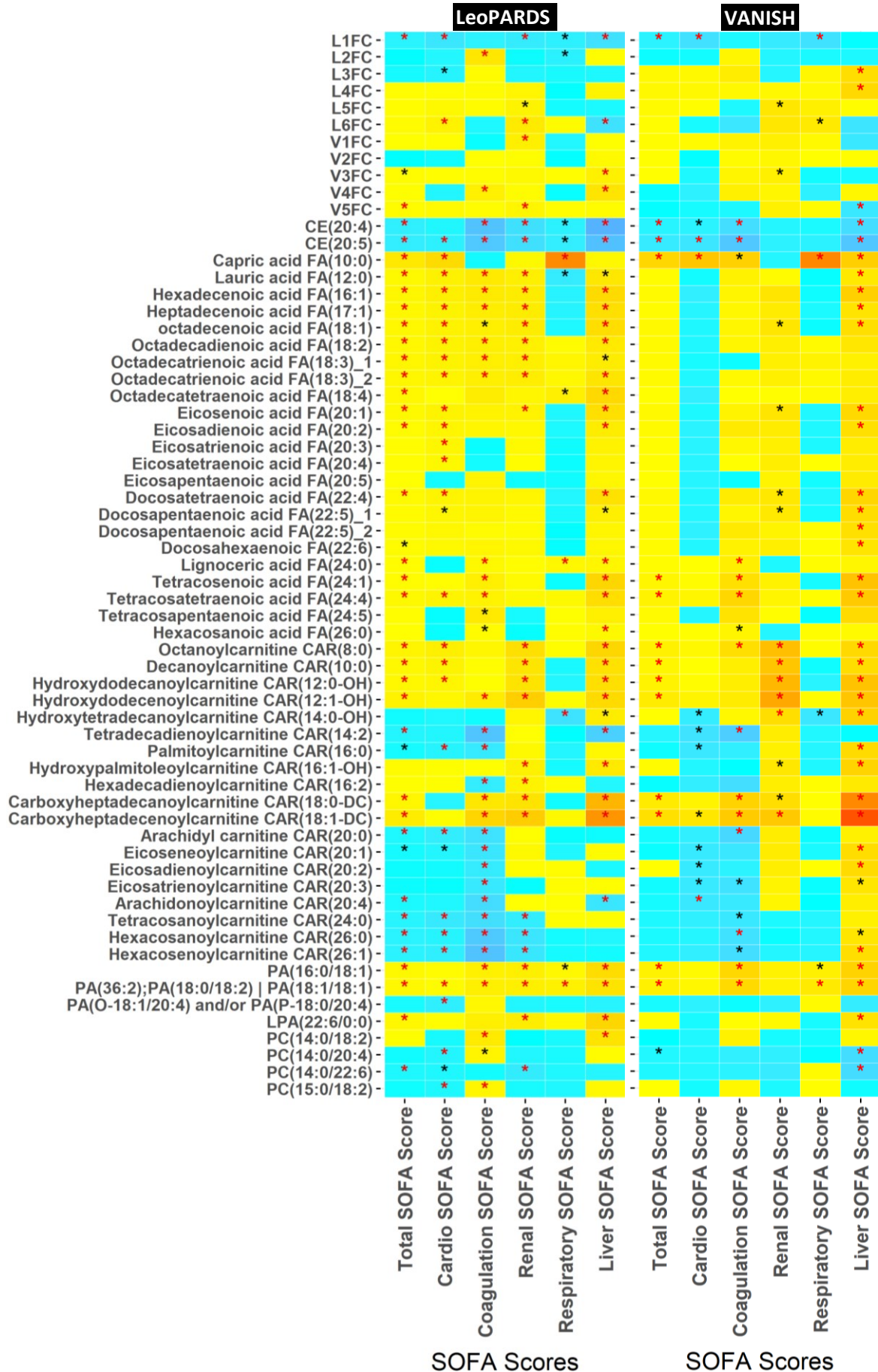
Alterations in lipids and lipoproteins related to respiratory SOFA score were also observed, and it was found that the levels of 9 lipids (including capric acid FA(10:0), glycerophospholipids (such as PA(36:2);PA(18:0/18:2) | PA(18:1/18:1) and PE(36:1); PE(18:0/18:1) | PE(18:1/18:0)), 5TGs), and delta-tocopherol) increase as the respiratory SOFA score increases ($p_{Adj} < 0.05$) in both cohorts. However, the levels of sphingolipid (SM(d18:1/18:0)) and HDL triglyceride subfraction 1 and 3 decreased as the as the respiratory SOFA score increased ($p_{Adj} < 0.05$) in both cohorts (figure 6.9).

The changes in the lipids and lipoproteins focusing on the liver SOFA score were also observed, but none of the lipids and lipoproteins were common in both cohorts after BH correction. However, it was found that the levels of 31 lipids and lipoproteins including bilirubin, cholesterol Sulfate_2, 8 fatty acids (including long-chain monounsaturated fatty acids (MUFAs) and PUFAs, 7 medium- and long-chain acyl carnitines, glycerophospholipids

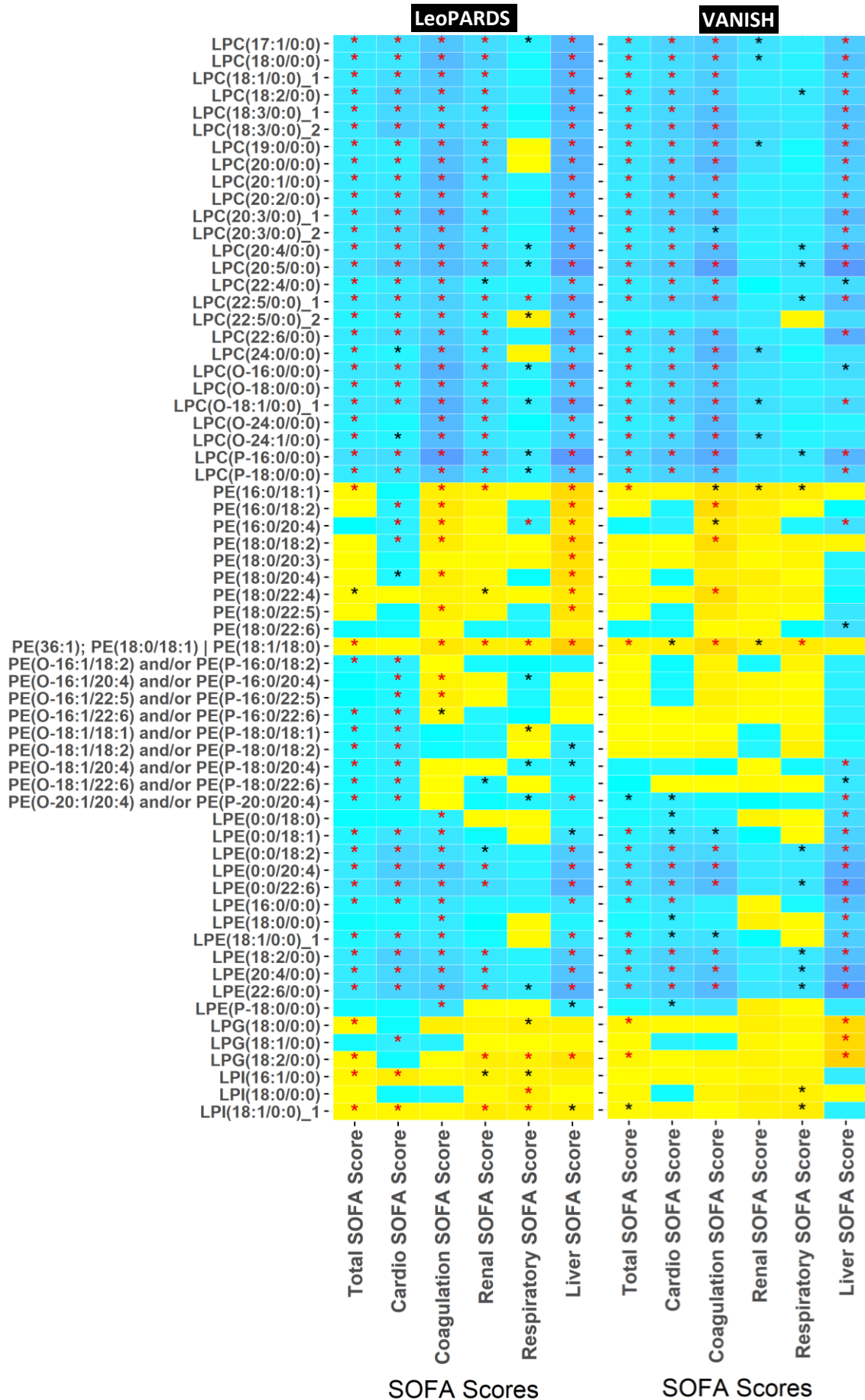
(2PAs, LPA(22:6/0:0), 9 PCs, LPG(18:2/0:0)) and sphingolipid (HexCer(d18:1/16:0)) increase as the Liver SOFA score increases ($p_{Adj} < 0.05$). The level of phospholipid PE(16:0/20:4) increases as the liver SOFA score increases in septic shock patients from LeoPARDS ($p_{Adj} < 0.05$) whilst it decreased as the SOFA score increases in sepsis patients from VANISH ($p_{Adj} < 0.05$) and this might be due to the different patient population. Moreover, 89 lipids and lipoproteins including, L6PN, apolipoproteins, HDL bound cholesterol and free cholesterol, 2CEs (CE(20:4), CE(20:5)), glycerophospholipids (2 PCs, 38 LPCs, PE(O-20:1/20:4) and/or PE(P-20:0/20:4), 8 LPEs, 2LPIs), sphingosine-1-phosphate, sphingolipids (10 SMs), HDL triglyceride and subfractions 1-3 and lipoprotein bound phospholipids (HDPL, H1-H3PL, L6PL), decrease with the increase in the Liver SOFA score ($p_{Adj} < 0.05$) in both cohorts (figure 6.9).

All changes in the lipids and lipoproteins from LME model with (figure A1.15, see appendix 1.7) and without BMI (figure 6.9) were the same for the lipids and lipoproteins discussed earlier except for some small differences where some lipids and lipoproteins become significant after B-H correction in the LME model with BMI and some do not remain significant at all. The direction of change in almost all the lipids and lipoproteins remain the same in the LME model with BMI.





	LeoPARDS						VANISH					
PC(16:0/16:0)-			*		*	*	*		*	*		*
PC(16:0/17:0)-					*	*						*
PC(16:0/18:1)_2-			*			*			*			
PC(16:0/18:2)-		*	*			*			*			
PC(16:0/18:3)-		*	*			*			*			
PC(16:0/20:4)_1-	*	*		*								
PC(16:0/20:4)_3-	*			*	*							
PC(16:0/20:5)-	*	*		*	*		*					*
PC(16:0/22:4)-												*
PC(16:0/22:6)-	*		*	*		*	*	*				*
PC(18:0/18:0)-		*										*
PC(18:0/18:1)-	*		*		*	*	*		*		*	*
PC(18:0/18:2)-		*	*			*	*		*			*
PC(18:0/20:3)-	*	*	*	*								*
PC(18:0/20:4)-				*								*
PC(18:0/22:4)-			*									
PC(18:0/22:5)-			*		*	*				*		
PC(18:0/22:6)-	*		*	*	*	*						*
PC(18:1/20:4)-	*	*		*								*
PC(32:1); PC(14:0/18:1)	*		*			*	*		*			*
PC(33:1); PC(15:0/18:1)			*			*	*		*			*
PC(34:0); PC(18:0/16:0)		*	*			*	*		*			*
PC(16:0/18:0)-			*		*	*			*			*
PC(40:3)-			*	*	*	*	*		*			*
PC(O-16:0/14:0)-					*	*	*		*			*
PC(O-16:0/15:0)-					*	*	*		*			*
PC(O-16:0/16:0)-		*				*	*		*			*
PC(O-16:0/18:2)-	*	*			*	*			*			*
PC(O-16:1/18:2) and/or	*	*			*	*	*	*		*		
PC(O-16:1/22:6) and/or	*	*	*	*	*	*	*					
PC(P-16:0/22:6)-	*	*	*	*	*	*	*					
PC(O-18:0/16:0)-		*				*	*		*			*
PC(O-18:0/20:3)-	*		*	*		*			*			
PC(O-18:0/20:4)-	*		*	*		*						
PC(O-18:0/22:4)-					*							
PC(O-18:1/18:1) and/or	*	*		*								
PC(O-18:1/22:6) and/or	*	*	*	*			*	*				
PC(O-22:0/18:2)-	*	*	*	*					*			
PC(O-22:0/20:3)-	*		*	*		*						
PC(O-24:0/18:2)-	*		*	*	*	*	*					
PC(O-24:0/20:4)-	*				*	*			*			
PC(O-24:1/20:3) and/or	*		*			*						
PC(O-24:1/20:4) and/or	*		*									
PC(O-24:1/22:4) and/or	*		*									
PC(O-24:2/20:4) and/or	*		*									
PC(O-26:1/20:4) and/or	*	*	*						*			
PC(P-26:0/20:4)-	*	*	*	*		*	*	*	*	*		*
LPC(0:0/14:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(0:0/15:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(0:0/16:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(0:0/16:1)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(0:0/18:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(0:0/18:1)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(0:0/18:2)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(0:0/18:3)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(0:0/20:2)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(0:0/20:3)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(0:0/20:4)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(0:0/20:5)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(0:0/22:5)_1-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(0:0/22:6)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(14:0/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(15:0/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(16:0/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(16:1/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(17:0/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
	Total SOFA Score	Cardio SOFA Score	Coagulation SOFA Score	Renal SOFA Score	Respiratory SOFA Score	Liver SOFA Score	Total SOFA Score	Cardio SOFA Score	Coagulation SOFA Score	Renal SOFA Score	Respiratory SOFA Score	Liver SOFA Score
	SOFA Scores						SOFA Scores					



	LeoPARDS						VANISH					
LPI(18:2/0:0)-	*	*	*		*	*	*	*	*			*
LPI(20:3/0:0)_1	*	*	*				*	*	*			*
LPI(20:3/0:0)_2		*	*					*				*
LPI(20:4/0:0)-	*					*	*		*			*
LPI(22:5/0:0)-	*	*	*	*			*			*	*	*
LPS(18:0/0:0)-	*	*	*	*		*	*	*	*	*	*	*
HDPL-	*	*	*	*		*	*	*	*	*	*	*
IDPL-	*	*					*					
LDPL-	*	*		*					*			
VLPL-					*							*
H1PL-	*	*	*	*	*	*	*	*	*	*	*	*
H2PL-	*	*	*	*	*	*	*	*	*	*	*	*
H3PL-	*	*	*	*	*	*	*	*	*	*	*	*
H4PL-	*	*	*	*	*	*	*	*	*	*	*	*
L1PL-	*	*	*	*	*	*	*	*	*	*	*	*
L2PL-	*	*	*	*	*	*	*	*	*	*	*	*
L3PL-	*	*	*	*	*	*	*	*	*	*	*	*
L4PL-	*	*	*	*	*	*	*	*	*	*	*	*
L5PL-	*	*	*	*	*	*	*	*	*	*	*	*
L6PL-	*	*	*	*	*	*	*	*	*	*	*	*
V1PL-	*	*	*	*	*	*	*	*	*	*	*	*
V2PL-	*	*	*	*	*	*	*	*	*	*	*	*
V3PL-	*	*	*	*	*	*	*	*	*	*	*	*
V4PL-	*	*	*	*	*	*	*	*	*	*	*	*
V5PL-	*	*	*	*	*	*	*	*	*	*	*	*
Sphingosine-1-phosphate	*	*	*	*	*	*	*	*	*	*	*	*
Cer(d18:1/16:0)-	*	*	*	*	*	*	*	*	*	*	*	*
Cer(d18:1/18:0)-	*	*	*	*	*	*	*	*	*	*	*	*
Cer(d18:1/20:0)-	*	*	*	*	*	*	*	*	*	*	*	*
Cer(d18:1/22:0)-	*	*	*	*	*	*	*	*	*	*	*	*
Cer(d18:1/24:0)-	*	*	*	*	*	*	*	*	*	*	*	*
Cer(d18:1/24:1)-	*	*	*	*	*	*	*	*	*	*	*	*
Cer(d18:2/22:0)-	*	*	*	*	*	*	*	*	*	*	*	*
Cer(d18:2/24:0)-	*	*	*	*	*	*	*	*	*	*	*	*
Cer(d34:0); Cer(d18:0/16:0)-	*	*	*	*	*	*	*	*	*	*	*	*
Cer(d41:1); Cer(d18:1/23:0)-	*	*	*	*	*	*	*	*	*	*	*	*
Cer(d42:3); Cer(d18:2/24:1)-	*	*	*	*	*	*	*	*	*	*	*	*
Cer(t18:0/24:1)-	*	*	*	*	*	*	*	*	*	*	*	*
HexCer(d18:1/16:0)-	*	*	*	*	*	*	*	*	*	*	*	*
HexCer(d18:1/22:0)-	*	*	*	*	*	*	*	*	*	*	*	*
HexCer(d18:1/23:0)-	*	*	*	*	*	*	*	*	*	*	*	*
HexCer(d18:1/24:0)-	*	*	*	*	*	*	*	*	*	*	*	*
SulfoHexCer(d18:1/16:0(OH))-	*	*	*	*	*	*	*	*	*	*	*	*
SulfoHexCer(d18:1/24:0(OH))-	*	*	*	*	*	*	*	*	*	*	*	*
SulfoHexCer(d18:1/24:1(OH))-	*	*	*	*	*	*	*	*	*	*	*	*
SulfoHexCer(d18:2/16:0(OH))-	*	*	*	*	*	*	*	*	*	*	*	*
LacCer(d18:1/16:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LacCer(d42:3)-	*	*	*	*	*	*	*	*	*	*	*	*
SM(d16:1/22:0)-	*	*	*	*	*	*	*	*	*	*	*	*
SM(d17:1/16:0)-	*	*	*	*	*	*	*	*	*	*	*	*
SM(d17:1/24:1)-	*	*	*	*	*	*	*	*	*	*	*	*
SM(d18:0/16:0)-	*	*	*	*	*	*	*	*	*	*	*	*
SM(d18:1/16:0)-	*	*	*	*	*	*	*	*	*	*	*	*
SM(d18:1/18:0)-	*	*	*	*	*	*	*	*	*	*	*	*
SM(d18:1/20:0)-	*	*	*	*	*	*	*	*	*	*	*	*
SM(d18:1/20:1)-	*	*	*	*	*	*	*	*	*	*	*	*
SM(d18:1/22:0)-	*	*	*	*	*	*	*	*	*	*	*	*
SM(d18:1/23:0)-	*	*	*	*	*	*	*	*	*	*	*	*
SM(d18:1/24:0)-	*	*	*	*	*	*	*	*	*	*	*	*
SM(d18:1/24:1)-	*	*	*	*	*	*	*	*	*	*	*	*
SM(d18:2/14:0)-	*	*	*	*	*	*	*	*	*	*	*	*
SM(d18:2/16:0)_1	*	*	*	*	*	*	*	*	*	*	*	*
SM(d18:2/18:0)-	*	*	*	*	*	*	*	*	*	*	*	*
Total SOFA Score												
Cardio SOFA Score												
Coagulation SOFA Score												
Renal SOFA Score												
Respiratory SOFA Score												
Liver SOFA Score												
Total SOFA Score												
Cardio SOFA Score												
Coagulation SOFA Score												
Renal SOFA Score												
Respiratory SOFA Score												
Liver SOFA Score												

SOFA Scores

SOFA Scores



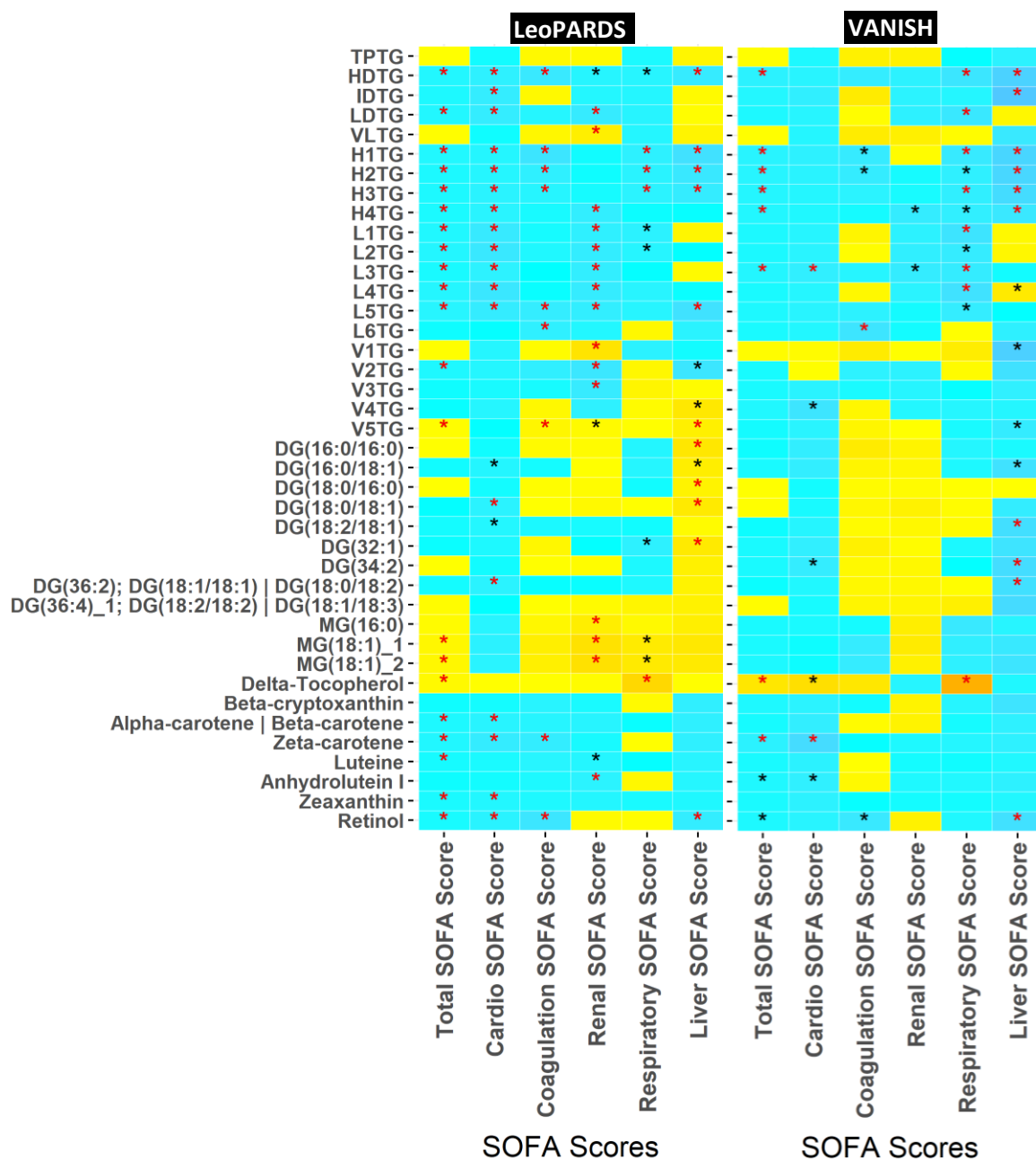


Figure 6.9: The results of LME models based on the SOFA scores for combined BI LISA, LPOS ppR and LNEG ppR log transformed data with age and sex as confounders for LeoPARDS and VANISH. LME model output slope (change in metabolite levels with SOFA scores) is presented for each SOFA score. The scale for slope ranges from positive to negative values, where positive values indicate higher levels of metabolites when the SOFA score is high whilst negative values indicate lower levels of metabolites when the SOFA score is high and stronger the colour the more the levels of metabolites with higher SOFA scores. Metabolites with slopes which are significantly different from 0 are highlighted in black asterisk whilst those that remained significant after correction are shown in red. Abbreviations: CH = Cholesterol; FC = Free Cholesterol; PL = Phospholipid; TG = Triglycerides; A1 = Apo-A1; A2 = Apo-A2, AB = Apo-B; HD = High density lipoprotein (HDL); VL = Very low density lipoprotein (VLDL); ID = Intermediate density lipoprotein (IDL); LD = Low density lipoprotein; TPCH = Total plasma Cholesterol; VLCH = VLDL Cholesterol; V1-V5CH = VLDL Cholesterol subfractions 1-5; IDCH = IDL Cholesterol; LDCH = LDL Cholesterol; L1-6CH = LDL Cholesterol subfractions 1-6; HDCH = HDL Cholesterol; H1-4CH = HDL Cholesterol subfractions 1-4; VLFC = VLDL Free Cholesterol; V1-V5FC = VLDL Free Cholesterol subfractions 1-5; IDFC = IDL Free Cholesterol; LDFC = LDL Free Cholesterol; L1-L6FC =

LDL Free Cholesterol subfractions 1-6; HDLC = HDL Free Cholesterol; H1-H4FC = HDL Free Cholesterol subfractions 1-4; TPA1 = Total Plasma A1; HDA1 = HDL Apo-A1; H1-H4A1 = HDL Apo A1 subfractions 1-4; TPA2 = Total plasma A2; H1-H4A2 = HDL Apo A2 subfractions 1-4; TPAB = Total plasma Apo-B; LDAB = LDL Apo-B; L1-L6AB = LDL Apo-B subfractions 1-6; IDAB = IDL Apo-B; TPBN: Total blood particle number; VLPN = VLDL Particle Number; IDPN = IDL Particle Number; LDPN = LDL Particle Number; L1-L6PN = LDL Particle number subfractions 1-6; TPTG = Total Plasma Triglycerides; VLTG = VLDL Triglycerides; V1-V5TG = VLDL Triglycerides subfractions 1-5; IDTG = IDL Triglycerides; LDTG = LDL Triglycerides; L1-L6TG = LDL Triglycerides subfractions 1-6; HDTG = HDL Triglycerides; H1-H4TG = HDL Triglycerides subfractions 1-4; VLPL = HDL Phospholipids; V1-V5PL = VLDL Phospholipid subfractions 1-5; IDPL = IDL Phospholipids; LDPL = LDL Phospholipids; L1-L6PL = LDL Phospholipids subfractions 1-6; HDPL = HDL Phospholipids; H1-H4PL = HDL Phospholipids subfractions 1-4; PC = Phosphatidylcholine; LPC = Lysophosphatidylcholine; PE = Phosphatidylethanolamine; LPE = Lysophosphatidylethanolamine; PI = Phosphatidylinositol; LPI = Lysophosphatidylinositol; LPG = Lysophosphatidylglycerol; PA = Phosphatidic acid; LPA = Lysophosphatidic acid; LPS = Lysophosphatidylserine; SM = Sphingomyelin; Cer = Ceramide; LacCer = Lactosylceramide; HexCer = Hexosylceramide; SulfoHexCer = Sulfohexsoylceramide; CE = Cholesteryl esters; DG = Diacylglycerol; MG = Monoacylglycerols; TG = Triglycerides; CAR = Carnitine; FA = Fatty acid. Note: Carbon atoms and double bonds in all lipid species are represented as (C atoms:double bond); d indicates 2 hydroxyl groups in the sphenoid base of ceramides and sphigomyelins; DC denotes for dicarboxylic acid; Prefixes "OH", "O" and "P" represents different substituents/linkages in the lipid species: "OH" denotes for hydroxyl group, "O" represents an alkyl ether linkage whilst "P" refers to the plasmalogen link. Lipid species with abbreviations _1 and _2 indicate same m/z value but different retention times for those lipids.

6.5 Discussion

In this study, I have investigated the trajectories of lipids and lipoproteins for septic shock survivors and non-survivors by using NMR and UHPLC-MS methodologies in the two cohorts. Additionally, in this chapter, lipid and lipoprotein changes based on organ failure scores (i.e., SOFA scores) from the two cohorts were also investigated.

Overall, we found that there are no significant differences in the metabolic trajectories of lipids and lipoproteins for survivors and non-survivors from both cohorts. However, the metabolic changes over time occur in the same direction in the two clinical groups.

The findings from the trajectory analysis revealed that fatty acyls such as medium chain acyl carnitines mostly decreased over time in all patients irrespective of whether they survived or died at 28-days. A lack of significant difference between these groups suggests that trajectories were similar no matter what the outcome. However, our results study showed that medium-chain acyl carnitines were significantly higher in both survivors and non-survivors at day 0 than at day 4 in both cohorts and these results suggest increased incomplete fatty acid oxidation at day 0 in both groups due to mitochondrial dysfunction (162, 253).

Additionally, the trajectory analysis also revealed that medium- and long chain acyl carnitines (four fatty acyl carnitines such as hydroxydodecenoylcarnitine CAR(12:1-OH), eicoseneoylcarnitine CAR(20:1), eicosadienoylcarnitine CAR(20:2), arachidyl carnitine CAR(20:0) at day 0 and 2 fatty acyl carnitines (such as octanoylcarnitine CAR(8:0), hydroxydodecenoylcarnitine CAR(12:1-OH) at day 4) were significantly higher in patients who went on to die than those who survived in both cohorts at baseline and day 4. These results suggest that non-survivors might have an enhanced incomplete β -oxidation due to mitochondrial dysfunction (162, 253) in non-surviving patients with septic shock. Our findings were also in accordance with other metabolomic studies (146-149, 163) and the univariate results reported previously in chapters 3 and 5.

Long chain acyl carnitines mostly increased over time in surviving patients whilst decreased over time in non-surviving patients. The trajectories of two long-chain acyl carnitines (palmitoylcarnitine CAR(16:0) and eicoseneoylcarnitine CAR (20:1)), increased over time in survivors from both cohorts whilst these two long-chain acyl carnitines decreased in non-surviving patients from both cohorts. Surprisingly, these two long-chain acyl carnitines had the opposite pattern to medium-chain acyl-carnitines and it was found that these two long chain acyl carnitines were significantly higher in survivors at day 4 than at baseline in both cohorts whilst non-survivors also had significantly higher levels of two long chain acyl carnitines at day 0 in comparison to day 4. . These results suggest increased incomplete fatty acid oxidation (i.e., β -oxidation) due to mitochondrial dysfunction (162, 253) in non-survivors with reduced uptake of long chain fatty acids into the mitochondria. However, increased levels of these long-chain acyl carnitines in survivors over time might suggest increased uptake of fatty acids into the mitochondria where they can be converted into acetyl-CoA via β -oxidation which can then enter TCA cycle and generate more energy for survival.

Our study revealed that, higher organ failure scores (such as total SOFA, renal SOFA, coagulation SOFA and liver SOFA scores) were associated with higher levels of medium and long-chain acylcarnitines indicating that as the organ dysfunction gets worse or as the patients progress towards organ failure they will have high levels of these acyl carnitines probably due to dysfunctional fatty acid oxidation due to mitochondrial dysfunction (162, 253). This is also supported by evidence that people with liver failure have increased

mitochondrial dysfunction and hence enhanced incomplete fatty oxidation (162, 253). Elevated levels of acyl carnitines have also been reported in patients with chronic kidney disease and maintenance haemodialysis as a result of impaired excretory function of the failing kidneys (339).

Our results revealed that most of the fatty acids decreased over time in both survivors and non-survivors, with no difference in trajectory between these groups. However, the results from the trajectory analysis revealed that 11 FAs such as one medium chain SFA (lauric acid FA(12:0)), 4 MUFAs and 6 PUFAs (octadecadienoic acid FA(18:2), eicosadienoic acid FA(20:2), ETA, DPA, DHA, tetracosapentaenoic acid FA(24:5)) were significantly lower in surviving and non-surviving sepsis patients at day 4 than at baseline in both cohorts. Most of these fatty acids have been produced by activated macrophages and have shown to cause a reduction in type I inflammation mediated by macrophages (340) and our results revealed that both survivors and non-survivors had increased levels of these fatty acids at baseline probably to counter act increased inflammation in these two groups at baseline and the levels of these fatty acids decreased over time probably due to the reduced inflammation on day 4. However, survivors had bigger degree of change from baseline indicating that these fatty acids decrease more as the patients get better whilst the non-survivors had lower degree of change from baseline indicating that these patients start off with extremely high levels of these fatty acids which decrease over time but not significant enough hence, smaller change.

PUFAs especially, C20 PUFAs such as ETA, EPA and arachidonic acid play important roles in the formation of eicosanoids, lipid mediators belonging to a subgroup of oxylipins (298) with a role in immunomodulation as well as in other physiological processes (298, 299) such as vasodilation and inflammation. SPMs including resolvins, protectins and maresins are formed from two PUFAs including EPA and DHA (300, 301) and as our results indicated that non-surviving patients had significantly lower levels of four PUFAs including ETA, EPA, DPA (i.e., FA(22:5)₂), tetracosatetraenoic acid FA(24:4)) than survivors on day 0 but only in LeoPARDS and the trend for all these PUFAs was the same in VANISH. These results might indicate that EPA is quickly metabolized to exhibit anti-inflammatory activity via the production of SPMs, which are biologically active during organ damage and tissue

inflammation (300). All of these FAs have not been previously associated with sepsis mortality however, Lin et al. reported that two PUFAs (including EPA and DHA) were decreased in septic patients with respect to healthy controls (302) and as we also found reduced levels in septic shock non-survivors which suggests a disease continuum in our case.

Our study revealed that fatty acids such as SFAs (capric acid FA(10:0) in total SOFA and respiratory SOFA), 2 MUFAs and PUFAs (tetracosenoic acid FA(24:1) and tetracosatetraenoic acid FA(24:4)) in total SOFA, coagulation SOFA and Liver SOFA scores), and 6 MUFAs and PUFAs (Hexadecenoic acid FA(16:1), heptadecenoic acid FA(17:1), octadecenoic acid FA(18:1), eicosenoic acid FA(20:1), eicosadienoic acid FA(20:2), docosatetraenoic only in liver SOFA score) increase as the total SOFA, coagulation, respiratory and liver score increase in both cohorts. These results might suggest increased lipolysis (337) in patients who progress towards organ failure which leads to the accumulation of these FAs in these organs causing lipotoxicity which leads to multiple organ dysfunction/ failure in patients with sepsis (242).

Surprisingly, two very long chain saturated fatty acids (SFAs) (lignoceric acid FA(24:0) and hexacosanoic acid FA(26:0)), derived from the elongation of palmitic or stearic acid or from the diet, were significantly higher in both survivors and non-survivors at day 4 compared to baseline. Long chain fatty acids play a crucial role in macrophage development, polarization and, type 1 inflammation (341, 342) and we found that long chain fatty acid lignoceric acid FA(24:0) significantly increased as the coagulation SOFA score increased in both cohorts suggesting that as the patients with sepsis get worse, the levels of lignoceric acid FA(24:0) increase.

Glycosphingolipids such as LacCers (generated by glycosylation of Cers) and sulfoglycosphingolipids such as SulfoHexCers increased over time in both survivors and non-survivors in both groups but there was no significant difference between the overall trajectory of these glycosphingolipids for two groups from both cohorts however, two LacCers (LacCer(d18:1/16:0) and LacCer(d42:3)), 2SulfoHexCers (SulfoHexCers) (SulfoHexCer(d18:1/24:0(OH) and SulfoHexCer(d18:1/24:1(OH))) were significantly higher in surviving patients at day 4 than at baseline in both cohorts whilst only SulfoHexCer(d18:1/24:1(OH) was significantly elevated in non-survivors at day 4 than at

baseline in both cohorts. It has been reported that LacCer can induce various pro-inflammatory cytokines such as TNF- α and macrophage inflammatory protein 2 by interacting with 1,3- β -glucan in leukocyte membrane which leads to an increase in oxidative burst and anti-microbial function of leukocytes (343). Our results suggest that patients who went on to survive might have increased glycosylation of Cers into LacCers as the levels of Cers mentioned previously were higher in these patients on day 4 and higher levels of LacCer in survivors at day 4 might result in an increased activation of cytosolic phospholipase A2 which might lead to the formation of increased arachidonic acid, a starting material for the synthesis of prostaglandins and eicosanoids (343).

Sphingolipids such as SMs and Cers play crucial roles in the regulation of cellular processes (such as lymphocyte trafficking, mediating pro- and anti-inflammatory effects, and maintaining the integrity of the endothelial barrier (303). Our study revealed that sphingolipids including SMs mostly increased over time in both survivors and non-survivors in both cohorts and this increase was steeper in patients who survived. The trajectories of Cers for survivors and non-survivors fluctuated across the two cohorts and the overall trajectories of none of the Cers were significantly different between survivors and non-survivors. These results were also consistent when the levels of SMs and Cers for both survivors and non-survivors were compared between day 0 and day 4. Cers result from the catabolism of SMs via the action of sphingomyelinases during inflammation (304) and act as second messengers (344). As our results showed that SMs increase over time in both groups therefore there will be more availability of SMs at later timepoint which will lead to the formation of Cers via the breakdown of SMs causing the levels of Cers to increase over time in both survivors and non-survivors, but an increased catabolism of SMs will only occur in patients who went on to survive than those who went on to die as higher levels of SMs will be present in these surviving patients than survivors. This also fits well with our results which showed that sphingolipids including 10 SMs and 4 Cers (Cer(d18:1/18:0), Cer(d18:1/20:0), Cer(t18:0/24:1)), and Cer(d41:1); Cer(d18:1/23:0), Cer(d17:1/24:0)) were significantly lower in non-survivors than survivors at day 4 in both cohorts. As Cers originate from the breakdown of SMs, low levels of Cers in patients who die might suggest a reduction in its precursors i.e., SMs in comparison to those who survived or it might also hint that most of the Cers could be bound to CD14 (306), a specific surface expression marker for monocytes

and macrophages (306, 345, 346). CD14 is a glycerophosphatidylinositol-anchored protein (347) which can bind lipopolysaccharide, but only in the presence of lipopolysaccharide binding protein (a secretory class 1 acute-phase protein produced by hepatocytes (348)). It has various roles including NF- κ B-dependent production of cytokines via the recognition of small doses of lipopolysaccharide, promoting TLR4 endocytosis as well as enhancing the expression of type-I IFN and causing the activation of nuclear factor of activated T-cells (NFAT) transcription factor family members (347). As Cers are structurally similar to lipopolysaccharide, thereby act as ligands for CD14 (306), hence reduced Cers in the serum of patients who went on to die. However, only Cer(d18:1/18:0) was found to be significantly lower in non-survivors at day 4 than at baseline in both cohorts.

Our study revealed that sphingolipids such as SMs and Cers decrease as the organ failure scores such as total SOFA (13 SMs, 2 Cers (Cer(d18:1/24:0) and Cer(d41:1); Cer(d18:1/23:0) | Cer(d17:1/24:0)), cardiac (8 SMs), coagulation (8 SMs), respiratory (SM(d18:1/18:0), and liver (10 SMs) SOFA scores increase in both cohorts. Lower levels of SM have been reported in sepsis patients with acute lung injury in comparison to healthy subjects (251) which also supports our findings. Sphingosine-1-phosphate, a sphingolipid which results from the hydrolysis of Cers followed by phosphorylation via sphingosine kinases 1 and 2 (349), decreased as the coagulation and liver score increased in septic shock patients from both cohorts and these results might suggest there might be increased hydrolysis of Cers in patients who progress towards organ failure.

Hypocholesterolemia and low level of TGs have been linked with a poor prognosis in patients with sepsis (338) and our study showed that there was a lack of significant difference in the overall trajectories of the free triglycerides and lipoprotein bound triglycerides of survivors and non-survivors from the cohorts however, one free triglyceride TG(54:5)₃ had a significant difference between the overall trajectory of survivors and non-survivors but only in LeoPARDS and this lipid species increased over time in both groups and the increase was more steep in patients who went on to survive. The trend for this free triglyceride was also the same in the alternate cohort for both groups. Additionally, free triglycerides (26 TGs in survivors and 7 TGs in non-survivors) and lipoprotein bound triglycerides (HDTG, H3TG, IDTG only in survivors and LDTG, L1-L5TG, H4TG in both groups)

were significantly higher in survivors at day 4 than at baseline in both cohorts. Elevated levels of free triglycerides and lipoprotein bound triglycerides in both groups at day 4 than at day 0 might suggest reduced lipolysis over time in both groups, hence lower hydrolysis of TGs into glycerol and FAs in a two-step process (337, 350) and lipolysis might be lower in patients who went on to survive than those who went on to die. Our results might suggest attenuation of inflammatory response in macrophages (351) over time in both survivors and non-survivors due to the upregulation of hypoxia-inducible lipid droplet-associated (HILPDA) protein which results in down-regulation of adipose triglyceride lipase (ATGL) enzyme, thereby reducing TG hydrolysis via ATGL (351) in both survivors and non-survivors which will in turn result in decreased availability of arachidonic acid, a precursor for pro-inflammatory mediators, thereby suppressing the synthesis of prostaglandin E2 (PGE2) and IL-6 (351). Our findings about TGs, also fit well with our results where we found that DGs (DG(18:2/18:1), and DG(36:2); DG(18:1/18:1) | DG(18:0/18:2)) and MGs (MG(16:0), MG(18:1)_1, MG(18:1)_2), the breakdown products resulting from the first and second step in the hydrolysis of TGs via ATGL (337) and hormone-sensitive lipase (352), were significantly higher in survivors at day 4 than at baseline whilst only MGs (such as MG(18:1)_1, MG(18:1)_2) were significantly higher in non-survivors at day 4 than at baseline in both cohorts. These results suggest that over time as the levels of TGs increase the hydrolysis of TGs might be occurring but not fully hence leading to the accumulation of DGs and MGs on day 4. Additionally, we found that free triglycerides mostly increased as the organ failure SOFA scores (coagulation, respiratory, and total SOFA) increase in both cohorts, which suggests as the patients progress towards organ failure the levels of these circulating free triglycerides rise in the serum reflecting increased formation of triglycerides and decreased activity of lipoprotein lipase (296, 353).

Cholesterol, one of the important lipid molecule present in plasma cell membranes is required for normal cell function (327). It exists in the plasma in two main forms, free cholesterol and CEs, both of which are part of all lipoprotein classes including HDL, LDL, IDL, VLDL and chylomicrons (328). Some studies have shown that there is an important association between innate immunity and cholesterol metabolism (329-331). Our study revealed that cholesterol sulfate_2 was significantly higher in both surviving and non-surviving patients at day 4 than at baseline in both cohorts. These results suggest that both

survivors and non-survivors might be starting off at low levels of cholesterol sulfate₂ which increase from baseline in both groups and this increase was steeper in patients who went on to die than those who survived. These results might also suggest there might be lower metabolism of cholesterol over time in both survivors and non-survivors. Cholesterol metabolism leads to the formation of other essential compounds such as bile acids and adrenal and gonadal steroids hormones (327). However, there were no significant difference between the cholesterol sulfate₂ levels of survivors and non-survivors at both baseline and at day 4 in both cohorts.

Our study also revealed that lipoprotein bound cholesterol and free cholesterol (L1CH, IDFC, L1FC in both groups whilst IDCH, H3CH, V4 & V5CH, VLFC, V4FC only in survivors) were significantly higher in both surviving and non-surviving patients at day 4 than at baseline whilst some lipoprotein bound cholesterol and free cholesterol classes (HDFC, H1FC in both groups whilst L6CH, V1CH, H4FC, L6FC, V5FC only in survivors) were significantly lower in both survivors and non-survivors at day 4 than at baseline in both cohorts. As lipoprotein subfractions from the same classes change either direction it's difficult to draw conclusions and these results suggest that there might be a complex mechanism changing over time which are not yet fully known. Additionally, the results from the analysis of organ failure scores revealed that lipoprotein bound cholesterol (TPCH, HDCH, H1-H3CH in both liver and total SOFA; TPCH, H1 & H2CH in coagulation; L1CH total SOFA and cardio) and free cholesterol (L1FC in cardio SOFA and total SOFA; HDFC in both coagulation and liver SOFA), CEs (CE(20:4) and CE(20:5) in total SOFA, coagulation and liver) decreased as these organ failure scores increased in both cohorts. Hypcholesterolemia is a strong prognostic marker of poor outcomes in patients with sepsis (354) and our results suggest that patients who progress towards organ failure have increased cholesterol metabolism or reduced production as sepsis is known to affect cholesterol transport, synthesis, and metabolism (354).

Phospholipids, the principal component of the biological cell membranes are crucial for the synthesis and stability of lipoproteins (307). Our results from the trajectory analysis revealed that the two most abundant phospholipid classes i.e., PEs and PCs (307) mostly increased over time in both survivors and non-survivors in both cohorts but there was a lack of

significant difference in the overall trajectories of these glycerophospholipids between survivors and non-survivors in the two cohorts. However, PCs and PEs were significantly elevated in both survivors and non-survivors at day 4 in comparison to baseline in both cohorts. Phospholipids including 4PCs (PC(16:0/20:4)₃, PC(16:0/20:5), PC(16:0/22:6), PC(18:0/22:6) and 2 PEs (PE(O-16:1/22:6) and/or PE(P-16:0/22:6), PE(O-18:1/22:6) and/or PE(P-18:0/22:6)) were significantly elevated in patients who went on to die than those who survived in both cohorts at day 4.

The results from the analysis of SOFA scores revealed that glycerophospholipids such as PCs (PC(32:1); PC(14:0/18:1) | PC(16:0/16:1) in coagulation, liver and total SOFA, 5PCs (PC(16:0/16:0), PC(18:0/18:2), PC(33:1); PC(15:0/18:1) | PC(16:0/17:1) in both coagulation and liver SOFA, 2PCs (PC(16:0/18:3), PC(18:0/18:1)) only in coagulation SOFA, 5PCs (PC(34:0); PC(18:0/16:0) | PC(16:0/18:0), PC(O-16:0/14:0), PC(O-16:0/15:0), PC(O-16:0/16:0), PC(O-18:0/16:0)) only in liver SOFA score) and PEs (PE(36:1); PE(18:0/18:1) | PE(18:1/18:0) in total SOFA, coagulation SOFA and respiratory SOFA), PE(16:0/18:1) in both total SOFA and coagulation SOFA, and 2 PEs (PE(16:0/18:2), PE(18:0/18:2) only in coagulation SOFA) mostly increased as the organ failure scores increase whilst PCs including long chain and unsaturated PCs PC(16:0/22:6) in both total SOFA and liver SOFA, PC(O-16:1/18:2) and/or PC(P-16:0/18:2) in cardiac SOFA and PC(18:0/22:6) only in Liver SOFA score) and PE(O-20:1/20:4) and/or PE(P-20:0/20:4) (only in liver SOFA) decreased as the organ failure scores increased in septic patients from both cohorts. These results suggest that as the patients progress towards organ failure have reduced levels of plasmalogen PE, plasmolgen PC or acyl alkyl PCs (with long chain SFAs at C-1 position and very long chain PUFAs at C-2 position of glycerol moiety) and diacyl PCs (with long chain SFAs at C-1 position and very long chain PUFAs at C-2 position of glycerol moiety) whilst acyl alkyl PEs, acyl alkyl PCs with long chain SFAs at C-1 and C-2 positions of glycerol moiety, acyl alkyl PCs with long chain SFAs at C-1 and MUFAs or PUFAs at C-2 positions of glycerol moiety, diacyl PCs (with long chain SFAs at C-1 and C-2, or SFAs at C-1 and long chain MUFAs or PUFAs at C2 positions of glycerol moiety). This shows us that there is a complex mechanism which is taking place in patients who progress towards organ failure and these glycerophospholipid species might provide an overall indication of their disease state, but the full mechanism is still not fully known.

LPCs the catabolic product of PCs which result from the cleavage of PC from HDL via endothelial lipase or from the hydrolysis of PC on the cell membranes by phospholipases A1 or A2 (308) were mostly found to increase over time in survivors and non-survivors from both cohorts, but there was a lack of significant difference in the overall trajectories of the two clinical groups in both cohorts. However, LPCs (including 45 LPCs in survivors and 42 LPCs in non-survivors) were significantly higher in both surviving and non-surviving patients at day 4 than at baseline in both cohorts. The degree of change in patients who went on to survive in both cohorts was bigger from baseline which indicates that patients are starting off at low levels at baseline which increase rapidly over time whilst in non-survivors the degree of change was smaller though the LPCs levels might start off low and increase steadily but not much. LPCs exhibit various different roles ranging from both anti and pro-inflammatory (308) to immuno-modulation (such as regulating the function of monocytes and macrophages (309), inhibiting platelet aggregation and promoting the secretion of NO in endothelial cells (308). LPCs induce IL-1 β secretion and foam cell synthesis through inflammasome-mediated pathway in human monocytes and endothelial cells (355). In this study, we found that LPCs decreased as the cardiac, coagulation, liver and total SOFA scores increased in both cohorts. These results also fit well with our findings from trajectory analysis which revealed that 27 LPCs were significantly lower in patients who went on to die than those who survived at day 4 in both cohorts and these results were also supported by other metabolomic studies (149, 151, 156, 158) and our results from the univariate analysis of lipids and lipoproteins reported previously in chapter 5. Low levels of LPCs in non-surviving patients might suggest either reduced hydrolysis of PCs due to the low levels of PCs in these patients in comparison to survivors or it might suggest an enhanced hydrolysis of LPC into LPA via phospholipase D or autotaxin (303). LPC administration in septic mice and rats has been shown to increase the clearance of bacteria, reducing organ injury, and improving survival (356-359).

Phospholipids such as LPEs are constituents of pulmonary surfactant and they modulate inflammation (360). LPEs which result from the hydrolysis of PEs by phospholipase A2 (308) mostly increased over time in patients who survived and those who went on to die in both cohorts. The degree of change in patients who went on to survive in both cohorts was bigger from baseline which indicates that patients start off at low levels at baseline which

increase rapidly over time whilst in non-survivors the degree of change was smaller though the LPEs levels start of low and increase steadily over time. Additionally, 5 LPEs (5 LPEs (LPE(0:0/18:2), LPE(0:0/22:6), LPE(18:2/0:0), LPE(20:4/0:0), LPE(22:6/0:0))) were significantly reduced in patients who went on to die than those who survived at day 4 in both cohorts. These findings were also consistent with our previous results from the univariate results of lipids and lipoproteins reported previously in chapter 5. Our results suggest that there might be reduced hydrolysis of PEs into LPEs (308) in patients who went on to die than those in comparison to survivors at day 4.

The results from the analysis of SOFA scores revealed that glycerophospholipids such as LPCs and LPEs which are catabolic products of PCs and PEs, respectively (308) decreased as the total SOFA (44 LPCs, 9 LPEs), cardiac SOFA (41 LPCs, 6 LPEs), coagulation SOFA (42 LPCs, 6 LPEs) and liver SOFA scores (8 LPEs) increased in septic shock patients from both cohorts and these results suggest that there might be an increased breakdown of PCs and PEs as the patients progress towards organ failure which might cause the levels of LPCs and LPEs to accumulate in these patients.

LPGs, bioactive lipids formed from the hydrolysis of PGs via phospholipase A2 (308) are an inherent component of lung surfactant with anti-inflammatory roles (310) and these LPGs increased over time in both survivors and non-survivors from both cohorts. Our results also revealed that LPGs (LPG (18:1/0:0) in both survivors and non-survivors and LPG (18:2/0:0) only in survivors) were significantly higher in patients who survived and those who went on to die at day 4 in comparison to baseline. These results might suggest increased hydrolysis of PGs into LPGs as well as increased modulation of endothelial cell activity over time (308) in both survivors and non-survivors though an increased activation of these two process might probably be lower in survivors in comparison to non-survivors as these patients get better. Our results from the analysis of SOFA scores revealed that LPGs (LPG(18:2/0:0) in both total SOFA and LIVER sofa score and LPG(18:0/0:0) in total SOFA) increase as these organ failure scores increase in septic shock patients from both cohorts and these results might suggest enhanced breakdown of PGs into LPGs (308) as the patients progress towards organ failure.

LPIs originate from the hydrolysis of PIs, (phospholipids crucial in intracellular signalling (149)) by phospholipases A1 and A2 (308) and our results showed that LPIs increase over time in both survivors and non-survivors from both cohorts. These results were also consistent when the levels of LPIs for both survivors and non-survivors were compared between day 0 and day 4. It was found that four LPIs were significantly higher in both survivors and non-survivors on day 4 in comparison to baseline. These results suggest that there might be increased hydrolysis of PIs over time in both groups, but the hydrolysis of PIs might be still lower in survivors than non-survivors. LPIs exhibit several roles ranging from regulating fat deposition, glucose homeostasis, stimulating insulin secretion (308). As our results from the trajectory analysis revealed that patients who went on to die had significantly elevated levels of LPI(18:1/0:0)_1 than patients who went on to survive at day 4 in both cohorts which might suggest increased stimulation of insulin release from islets of Langerhans (308) in patients who went on to die than those who survived. LPIs have not been previously linked with mortality associated with sepsis. Our study also revealed that 2LPIs (LPI(18:2/0:0), LPI(22:5/0:0)) decreased as the liver SOFA score increased in septic shock patients from both cohorts and these results might indicate that patients who progress towards liver failure will have high level of these two LPIs.

PA a precursor for DG and LPA plays a role in enhancing thrombosis mediated via the exposure of PS on human erythrocytes (311) and our study revealed that PAs (PA(16:0/18:1) in three SOFA scores (total SOFA, coagulation and liver) and PA(36:2);PA(18:0/18:2) | PA(18:1/18:1) (in total SOFA, coagulation, liver and respiratory) increased as the organ failure scores increased in both cohorts. These results indicate that as the patients progress towards organ failure they have increased thrombosis (311). LPA, a lipid mediator of inflammation takes part in regulating the thrombotic stability by stimulating the release of NETs from neutrophils through lysophosphatidic acid receptors 1-6 (312). Our results showed that LPA(22:6/0:0) resulting from the breakdown of PA, increases as the liver SOFA score increases in septic shock patients from both cohorts and these results might suggest that as the patients progress towards liver failure, they might have increased NET production via LPA receptors which will lead to increased thrombosis in these patients.

Lipoprotein bound phospholipids mostly increased over time in both survivors and non-survivors in both cohorts with few exceptions. Our results indicated that lipoprotein bound phospholipids (including HDPL, IDPL, LDPL, H2 & H3PL, L1 & L3PL, V4PL in survivors and L1PL in non-survivors) were significantly elevated in surviving and non-surviving patients at day 4 than at baseline in both cohorts. These results were also in accordance with all the measured phospholipid classes from UHPLC-MS data. Additionally, our results from the trajectory analysis also revealed that HDL phospholipid and its subfraction 3 were significantly lower in patients who went on to die than those who survived at day 4 in both cohorts. These results were also in accordance with our previous results from UHPLC-MS data as well as findings from de la Llera Moya et al. who revealed that decreased expression of phospholipids and Apo-A1 causes alterations in HDL composition in LPS induced human endotoxemia (320). Our results on SOFA scores revealed that phospholipid bound lipoproteins (HDL phospholipid and its subfractions 1-3 in total SOFA, cardio, coagulation, and liver SOFA, L1PL in both total SOFA and cardio SOFA and L6PL only in liver SOFA score) decrease as these organ failure scores increase in septic shock patients from both cohorts indicating as the patients get worse and progress towards organ failure the levels of these lipoprotein phospholipids decline in the serum also consistent with the findings of glycerophospholipids from UHPLC-MS data from both cohorts.

Apolipoproteins are involved in lipid transport and metabolism (321) and there are several types of apolipoproteins which exhibit different roles. Apo-A1 and Apo-A2 are the two most abundant apolipoproteins (321) and almost 70% of HDL contains Apo-A1 (324) whereas 20% of HDL contains Apo-A2 (322). Apo-A1 is not only involved in the activation of LACT (an enzyme which esterifies free cholesterol into CEs)(324), but it also has an essential role in the HDL anti-inflammatory functions, where it attaches and neutralizes bacterial toxins thereby exhibiting protective effects against sepsis (296). Apo-A2, a cofactor of LCAT (322) (an enzyme involved in the metabolism of lipoproteins with both phospholipase A2 and an acyltransferase action (322)) also regulates the activity of extracellular LPL (322) (an enzyme responsible for degradation of circulating TGs in the bloodstream) located on vascular endothelial surface (323). Apo-B is the main component of three lipoprotein classes (such as LDL, VLDL & IDL) is also a ligand for the LDL receptor (324). This apolipoprotein is crucial for the assembly and secretion of VLDL (322) and has roles in the development of

atherosclerosis (325). The findings from our study revealed that none of the apolipoproteins were common between the two cohorts based on the significant difference between the overall trajectories of survivors and non-survivors. However, the results from the trajectory analysis also revealed that apolipoproteins such as Apo-A1 (H2A1 only in survivors), Apo-A2 (HDA2, H3A2 in both groups and TPA2, H2A2 only in survivors) and Apo-B (TPAB, IDAB, L1 & L3AB in both groups and LDAB, L2 & L6AB, VLAB only in survivors) were significantly higher in survivors and non-survivors at day 4 than at baseline in both cohorts. Our results about lipoprotein classes with Apo-A might suggest that there might be an increased inactivation of bacterial endotoxins via protein-protein interactions (361) in survivors at day 4. It has been reported that lps-binding protein interacts with Apo-B of LDL and VLDL in patients with sepsis thus increasing the lps-LDL/VLDL interaction (326). As our results indicated increased levels of apolipoproteins (especially lipoprotein classes with Apo-A2 and Apo-B) over time which might indicate that there might be increased activity of LPL, as well as increased scavenging capacity of LDL, VLDL and IDL for the bacterial toxin (i.e., lps) at day 4 in both survivors and non-survivors, but these processes might be more enhanced in patients who went on to survive. Surprisingly there were some conflicting findings on apolipoproteins, and we found that higher density LDL Apo-B subfraction 6 (L6AB) in both survivors and non-survivors and TPA1 only in non-survivors were significantly lower at day 4 than at baseline in both cohorts. This type of lipoprotein detail has not been previously reported in the literature in patient with septic shock focusing on mortality and these types of conflicting results suggest that there might be a complex mechanism occurring in these patients at a molecular and a biological level which still needs to be explored. However, when we compared survivors and non-survivors at baseline and at day 4 we found that only H2A1 was significantly reduced in patients who went on to die than those who went on to survive at day 4, also consistent with our previous results from the univariate analysis of lipoproteins and our result might suggest a lowered inactivation of bacterial endotoxins via protein-protein interactions (361) in patients who went on to die than those who survived.

Our study revealed that apolipoproteins such as Apo-A1 (TPA2 in both liver and total SOFA, H1A1 in coagulation and liver, H2 & H3A1, H1 & H2A2 in both coagulation, liver, and total SOFA, H3A2 only in total SOFA, H2A1 in renal SOFA) and Apo-B (L1AB in total SOFA and L6AB

in liver SOFA) decreased as the these organ failure SOFA scores increased in septic shock patients from both cohorts and these result suggest that the patients who progress towards organ failure might have reduced clearance of bacterial toxins probably due to the lowered inactivation of these endotoxins by HDL and they might also have reduced LPL activity as well as decreased of scavenging capacity of other lipoprotein classes such as LDL, VLDL and IDL for the bacterial toxin.

6.6 Conclusion

The targeted data analysis of serum samples from sepsis patients revealed that the metabolic trajectories of lipids and lipoproteins for patients who went on to die and those who survived in both cohorts were similar and there was a lack of significant difference in the overall trajectories between these two clinical groups. Pathways related to energy and lipid and phospholipid metabolism were disturbed over time in both clinical groups. The validation models for mortality did not perform well probably due to the less commonality of metabolites in the predictive models built in two cohorts or due to the metabolic trajectories being very similar in survivors and non-survivors. This study also revealed that lipids and lipoprotein profiles vary with organ failure and mostly sepsis patients with higher levels of lipids and lipoproteins were associated with higher organ dysfunction. The knowledge of these metabolic changes will allow us to understand the pathological processes in sepsis patients and in future it might help us in monitoring the clinical course of these patients.

Chapter 7 – Final Discussion and Conclusion

7.1 Discussion

This study focused on the metabolic differences and the trajectories of patients with septic shock using the concentration of metabolites in serum. NMR and UHPLC-MS were used to obtain these concentrations. The predominant focus was on the differences between survivors and non-survivors. The work was performed independently in two different cohorts belonging to the, VANISH and LeoPARDS clinical trials. Additionally, this study also investigated how metabolic profiles vary with organ failure and treatment. To our knowledge this is the largest metabolomic study of septic shock. The use of two independent cohorts allowed us to validate our findings.

As shown in the results, some of the metabolites were statistically significant after correction only in LeoPARDS, but these metabolites had the same pattern in VANISH (see tables 3.5, 3.6, 5.5, 5.6, 5.9, 5.10). This reflects higher statistical power of LeoPARDS due to larger sample size in comparison to VANISH.

The main goal of this study was to understand what happens metabolically over time in survivors and non-survivors and to learn what happens at baseline. There is much known about the metabolic differences between survivors and non-survivors at baseline, summarised in chapter 1 (table 1.4). We found that at baseline, septic shock patients who went on to die had accumulated levels of: i) amino acids and derivatives (tyrosine, phenylalanine, alanine, glutamine, glutamate, glycine, histidine, lysine, valine, creatinine, creatine); ii) oxidative stress related metabolites (N6, N6, N6-trimethyllysine, proline, N,N-dimethylglycine, betaine, N1-acetylspermidine, symmetric/asymmetric dimethylarginine (SDAMA/ADMA)); iii) energy related metabolites (lactic acid, pyruvate, citrate, formic acid, sucrose, acylcarnitines (short-medium- and long-chain), 1-methylnicotinamide and its metabolites (N1-methyl-2-pyridone-5-carboxamide (2-Py) and N1-methyl-4-pyridone-3-carboxamide (4-Py)); iv) phospholipids (lysophosphatidylglycerols (LPGs), lysophosphatidylinositols (LPIs), and phosphatidic acid (PA)(36:2);PA(18:0/18:2) | PA(18:1/18:1)), and choline) than those who survived. On the other hand, metabolites including glucose, arginine, fatty acids (FAs), triglycerides (TGs), phospholipids (such as phosphatidylethanolamines (PEs), phosphatidylcholines (PCs), and lysophosphatidylcholines (LPCs)) and sphingolipids (such as sphingomyelins (SMs), ceramides (Cers)) were lower in those who went on to die than those who survived. All these findings were consistent across

the two cohorts and most of these metabolites followed a similar pattern even after 24 h of ICU admission. However, at 24h not all metabolites were significantly different between the two clinical groups after correction for multiple comparisons and only aromatic amino acids (tyrosine, phenylalanine) and 2-PY remained significantly increased in non-survivors compared to survivors in both cohorts. Besides these, only observed in LeoPARDS cohort there were some additional metabolites including BCAAs (leucine and valine), acetate, cortisol, sucrose, and bilirubin which were significantly elevated in patients who went on to die and not for those who survived whilst hypoxanthine and arginine were reduced in septic shock non-survivors when compared to survivors after 24 h of ICU admission.

Our findings from the trajectory analysis revealed that in general there was a lack of significant difference in the overall trajectories of survivors and non-survivors and both groups had similar trend over time. A summary of the main findings and the metabolic pathways altered in sepsis survivors and non-survivors is shown in figure 7.1.

As mentioned previously, amino acid concentrations appear increased in patients with sepsis. This could result from increased catabolism of proteins, carbohydrates, and lipids as well as DNA damage (158, 233). Fluctuations in aromatic amino acids such as tyrosine and phenylalanine, which are precursors of catecholamines (231), are related to oxidative stress which lead to the overproduction of hydroxyl radicals converting L-phenylalanine enzymatically in the kidneys to para-tyrosine (234). Our study revealed that patients who died had accumulated levels of these two amino acids at the first two timepoints in accordance with other studies (66, 147, 149, 151, 152, 154, 164-166, 229). Accumulation of these aromatic amino acids in non-survivors might suggest increased muscle proteolysis (235) and these two metabolites could be converted into fumarate and enter the tricarboxylic acid (TCA) cycle (66) (figure 7.1) to compensate for the increased energy demand in patients who went on to die. The findings for phenylalanine were also consistent in the results from the trajectory analysis which revealed that the phenylalanine was significantly higher in non-survivors with respect to survivors at baseline as supported by other metabolomic studies (66, 147, 151, 152, 164-166). In both survivors and non-survivors, levels of these amino acids increased over time to a similar degree, meaning that non-survivors still had higher levels than survivors on day 4 and this might suggest increased muscle breakdown over time in both groups.

Red - Metabolites higher in non-survivors in comparison to survivors at baseline
 Blue - Metabolites lower in non survivors in comparison to survivors at baseline
 Bold - Metabolites common and significant in both cohorts
 Underlined - Metabolites only significant in LeoPARDS (Same pattern in VANISH)
 Not bold & Not underlined - Significant in LeoPARDS but not after B-H corection
 ♠ Decreases over time in both survivors & non-survivors
 ♣ Increases over time in both survivors & non-survivors
 ♥ Increases over time in survivors & decreases in non-survivors
 ♦ Decreases over time in survivors & increases in non-survivors
 – Trajectory opposite in VANISH but only in non-survivors
 + Trajectory opposite in VANISH but only in survivors
 × Trajectory opposite in VANISH for both survivors & non-survivors

Figure 7.1: A summary of the main findings and the metabolic pathways involved in the pathology of sepsis survivors and non-survivors identified using the Kyoto encyclopedia of genes and genomes (KEGG). This figure was adapted from (144). Highlighted pathways in yellow correspond to metabolic derangements in sepsis and are linked to: energy metabolism; amino acid metabolism; purine and pyrimidine metabolism; lipid metabolism; cholesterol metabolism. The seven pathophysiological processes involved in the metabolic response to sepsis are highlighted in turquoise: renal dysfunction; vasodilation; oxidative stress; mitochondrial dysfunction; lactic acidosis; tissue hypoxia; reduced stroke volume and cardiac output and organ dysfunction. Abbreviations: TG = triglyceride; PS = phosphatidylserine; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PG = phosphatidylglycerol; PI = phosphatidylinositol; PA = phosphatidic acid; LPC = lysophosphatidylcholine; LPE = lysophosphatidylethanolamine; LPG = lysophosphatidylglycerol; DAG = diacylglycerol; LPI = lysophosphatidylinositol; TAG = triacylglycerol; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; DiHETE = dihydroxyeicosatetraenoic acid; EET = epoxyeicosatrienoic acid; HpETE = hydroperoxy-eicosatetraenoic acid; HETE = hydroxyeicosatetraenoic acid; HDHA = hydroperoxy-docosahexaenoic acid; HEPE = hydroxypentaenoic acid; TX = thromboxanes; LT = leukotrienes; PGG2 = prostaglandin G2, PGH2 = prostaglandin H2 ; PGE2 = prostaglandin E2 ; PGI2 = prostaglandin I2 ; PGF2 α = prostaglandin F2 α ; keto-PGF1 α = keto-prostaglandin F1 α ; Rv = resolvin; CoA = coenzyme A; HDL= High density lipoprotein; LDL = Low density lipoprotein; Apo-A1 – Apolipoprotein A-1; TCA = tricarboxylic acid; DMG = dimethylglycine; DMA = dimethylamine; ADMA = asymmetric dimethylarginine; SDMA = symmetric dimethylarginine; GABA = γ -aminobutyric acid; NAD⁺ = nicotinamide adenine dinucleotide; 2-Py = N1-methyl-2-pyridone-5-carboxamide; 4-Py = N1-methyl-4-pyridone-5-carboxamide; L-DOPA = levodopa; NH₃ = ammonia; CO₂ = carbon dioxide; ROS = reactive oxygen species; CH₃ = methyl; NO = nitric oxide; iNOS = inducible nitric oxide synthase; SV = stroke volume.

Our study revealed that amino acids such as BCAAs, glycine, glutamine and alanine were elevated in non-survivors, indicative of increased muscle breakdown (figure 7.1). Some of these amino acids are substrates for generating energy via gluconeogenesis and other processes to meet the increased energy demand seen in patients with more severe sepsis (238, 239). Our results were also in accordance with other metabolomic studies (147, 152, 158, 166) and imply a greater degree of catabolism in non-survivors. All these amino acids except for BCAAs decreased over time in patients who went on to die whilst these amino acids even BCAAs increased over time in patients who survived. The metabolic trajectories of essential BCAAs suggests increased proteolysis over time in both survivors and non-survivors (figure 7.1) and these amino acids have been shown to have various effects such as promoting wound healing, preventing hepatic encephalopathy, and stimulating insulin secretion (362). Glutamine is involved in immune function, synthesis of glutathione and purines and pyrimidines (363). A decline in the levels of amino acids including glycine, glutamine, and alanine over time in non-survivors might suggest rapid consumption of these amino acids to generate energy or other metabolites for various other processes whilst survivors showed an accumulation of these metabolites over time indicative of reduced consumption of these amino acids as the patients get better over time. Glycine, an amino

acid with a cytoprotective role (240) is also involved in attenuating the immune response (241). It has been reported that glycine administration reduces liver injury in endotoxic shock (240) and our study revealed that high levels of glycine were associated with higher liver dysfunction in patients with sepsis and this might reflect that high levels of glycine are present because of liver damage to exhibit protective effects. Despite benefits, there is a lack of clinical data available hence the mechanisms related to its protective effects and the safety dose still need to be determined (364).

The amino acid lysine takes part in various process such as production of cytokines, regulation of nitric oxide (NO) synthesis (236), proliferation of lymphocytes, antiviral and anti-inflammation (365). Lysine was significantly increased in non-survivors with respect to survivors at baseline and after 24h of admission (figure 7.1) also supported by findings from the Liu et al. study (66). This amino acid increased over time in both survivors and non-survivors with a steeper increase in patients who died reflecting an increased regulation of NO synthesis pathway over time. Lysine supplementation in mice has shown to lower inflammation as well as to reduce the number of hypotensive episodes with respect to those who received placebo (366) and our results might suggest that lysine is probably present in higher concentrations to reduce the inflammation in sicker patients.

Markers of oxidative stress such as SDMA/ADMA were found to be accumulated at baseline in non-survivors compared to those who survived (figure 7.1). This is also consistent with our results from the trajectory analysis as well as supported by other metabolomic studies (154, 155). As SDMA/ADMA are competitive inhibitors of NO synthase (246) and therefore increased levels of these markers are probably present to counteract increased NO in the serum of patients who die. NO has various roles ranging from vasodilation of blood vessels as seen in sepsis (figure 7.1), to acting as a crucial mediator in the immune response to infection (where it inhibits cell proliferation and migration, reduces platelet adhesion and aggregation and also lowers the monocyte stickiness) (155, 246). Additionally, our study revealed that there was a lack of significant difference in the overall trajectory of SDMA/ADMA between survivors and non-survivors. However, the levels of this oxidative marker were higher in both survivors and non-survivors on day 4 in comparison to day 0, an increase over time (figure 7.1). The post-translationally modified forms of arginine, SDMA/ADMA (283) are also uremic toxins as well as risk indicators for cardiovascular

disease and mortality (283, 367). It has been reported that administering ADMA in humans will lower cardiac output and renal plasma flow and will increase vascular resistance (368). Our results revealed that patients with higher organ failure SOFA scores (total SOFA, coagulation, cardiac, renal, and liver) were associated with higher levels of SDMA/ADMA, supporting these previous findings.

Arginine, the urea cycle metabolite, one of the crucial amino acids involved in the production of NO, SDMA/ADMA, creatine, and polyamines (155, 247) was reduced in septic shock non-survivors in relation to survivors (figure 7.1). Our results were consistent with other metabolomic studies (159, 165) and suggest downregulation of urea cycle in non-survivors or increased conversion of this amino acid to SDMA/ADMA to counteract increased NO in these patients as described previously (figure 7.1). Arginine has been shown to reduce the heart rate as well as improve haemodynamics in congestive heart failure (369) via the NO arginine pathway and we found that there is an inverse relationship between arginine and organ failure scores (including cardiac and total SOFA score) (369) which supports the cardioprotective aspect of arginine.

The polyamine, N1-acetylspermidine was significantly elevated in non-survivors with respect to survivors at the first three timepoints. The accumulation of this polyamine in non-surviving patients suggests increased apoptosis of alveolar macrophages in the lungs due to the increased formation of reactive oxygen species (ROS) during polyamine metabolism (257). This finding was also supported by our results from organ failure where we found that higher levels of N1-acetylspermidine were associated with greater degrees of respiratory, failure as well as high renal, liver, and total SOFA scores. Additionally, this polyamine decreased over time in all patients no matter the outcome, indicative of decreased polyamine metabolism over time in all patients with septic shock.

The modified purine nucleoside, N2,N2-dimethylguanosine accumulated in patients who went on to die compared to those who survived reflecting increased oxidative stress (figure 7.1) which leads to enhanced metabolism of transfer ribonucleic acid (tRNA) resulting in the release of this nucleoside (145) in the cytoplasm. These results were also in accordance with our findings from the trajectory analysis and other metabolomic studies (145, 146). Additionally, higher levels of N2,N2-dimethylguanosine in sepsis patients were associated

with higher organ failure scores (including cardiac, renal, liver, and total SOFA) reflecting the relationship between oxidative stress and organ dysfunction. Hypoxanthine, a purine base also associated with ROS which upon oxidation by xanthine oxidase leads to uric acid, an end product of purine metabolism (248) was reduced in non-survivors with respect to survivors and this might be due to the increased conversion of hypoxanthine into uric acid (figure 7.1). However, hypoxanthine has not been previously linked with sepsis associated mortality as this metabolite has not been measured in most of the metabolomic studies. The trajectories of these two purine metabolites were significantly different between survivors and non-survivors in LeoPARDS. These two metabolites increased over time in non-survivors and decreased over time in survivors indicating purine metabolism increases over time in patients who die but decreases in patients who survive (figure 7.1). Additionally, our study also revealed that reduced levels of hypoxanthine are associated with worse coagulation, and total SOFA and these results also relate with our findings from the mortality analysis.

Accumulation of proline, a substrate for the synthesis of superoxide radicals (249) in non-survivors suggests that patients who die have increased oxidative damage due to aggravated stress during inflammation (249). This amino acid increased over time in survivors and non-survivor (figure 7.1). These findings were also in accordance with the results from other studies (147, 149, 154). Increased oxidative stress can lead to damage to the kidneys and liver (160), consistent with our results from organ failure analysis which showed that higher levels of proline were associated with higher organ failure scores including renal and liver scores.

We have found differences in the energy related metabolites between survivors and non-survivors. This is due to the increased energy demands in patients with sepsis which are fulfilled by increased cellular respiration (239). In sepsis, glycolysis and TCA cycle metabolites can accumulate due to mitochondrial dysfunction (158) and we found that lactate (a clinical biomarker for prognosis of sepsis patients (233)), pyruvate and citric acid were accumulated in non-survivors at baseline (figure 7.1). These results indicate an upregulation of TCA cycle to cope with increased formation of pyruvate which results from the increased stimulation of glycolysis by endogenous adrenaline leading to lactate formation (259) and lactic acidosis (260) (figure 7.1). Our trajectory analysis also

demonstrated elevated levels of lactate at days 0 and 4 in non-survivors with respect to survivors. The findings from the baseline differences and the trajectory analysis were also supported by other metabolomic studies (66, 146-149, 157, 158). Our study revealed that lactic acid and citric acid, decreased over time in both survivors and non-survivors reflecting decreased glycolysis and TCA activity over time (figure 7.1). High levels of lactic acid levels were also linked with higher organ failures scores (including coagulation, renal, respiratory, cardiac, and liver failure).

Fluctuations in the glucose levels are generally observed in critically ill patients and both high and low levels are associated with poor outcomes (261). We found significantly reduced levels of glucose in patients who went on to die compared to those who survived both at baseline and in the trajectory analysis (figure 7.1). This suggests that patients who die might have increased inhibition of gluconeogenesis in the liver by cytokines leading to depletion of glycogen stores (263, 264) or it might also reflect enhanced metabolism of glucose into pyruvate to cope with the increased demand for energy fitting our previous findings about pyruvate and lactate (figure 7.1). Our results were not in accordance with other metabolomic studies (152, 159), which are relatively small, but were supported by the findings from Kushimoto and co-workers, who reported that hypoglycaemia (glucose <70mg/dL) in septic shock was linked with higher mortality (262). Our study also revealed that there is a lack of significant difference in the overall trajectories of glucose between survivors and non-survivors with no significant difference between day 0 and day 4 levels in both clinical groups and this could be due to the treatment with insulin to actively reduce glucose levels. Interestingly, accumulated levels of sucrose were found in patients who went on to die compared to those who survived at baseline, also consistent with the results from the trajectory analysis where sucrose was found to be significantly higher in non-survivors than survivors on day 0. These results were also in accordance with other studies (152, 159) and might suggest increased consumption of glucose by the cells for the generation of energy, but not much of the sucrose in the serum is being metabolised to glucose to replace the glucose levels in non-survivors.

Our study revealed that non-survivors had high levels of 1-methylnicotinamide (MNA) and its metabolites (2-Py and 4-Py) at the first three timepoints and these findings were also supported by Seymour and co-workers who reported higher levels of 2-Py in non-survivors

(145). These results suggest increased metabolism of oxidised nicotinamide adenine dinucleotide (NAD^+) (255) via poly(ADP-ribose) polymerase-1 (PARP-1) activation which is triggered by deoxyribonucleic acid (DNA) damage from oxidative stress (294) in non-survivors which leads to the build-up of nicotinamide and other catabolic products (MNA, 2-Py and 4-Py) (256) (figure 7.1). Our results suggest that non-survivors might exhibit increased activity of the nicotinamide N-methyltransferase (NNMT) enzyme which methylates excess nicotinamide (one of the end-products of NAD^+ degradation) to MNA which gets further oxidised to (2-Py and 4-Py) via aldehyde oxidase (370). It has been reported that, exogenous MNA exhibits vasoprotective effects by releasing prostaglandin (PG) I₂, a vasodilator and a potent inhibitor of platelet aggregation (371) and our results showed higher levels of MNA in sepsis patients with higher cardiac SOFA and coagulation score which suggests that increased MNA is probably present to exert its vasoprotective effects in these patients. Our results indicated that higher levels of MNA and its metabolites (2-Py and 4-Py) are associated with worse organ failure this reflects that sepsis patients with increased organ failure have increased NAD^+ degradation as well as increased activation of the NNMT-MNA pathway perhaps in a compensatory role to protect against further organ damage. All these metabolites (MNA, 2-Py and 4-Py) increased over time in survivors and non-survivors suggesting increased NAD^+ degradation and activation of the NNMT-MNA pathway over time to protect against endothelial damage via the release of PGI₂ (371). Elevated levels of 2-Py and 4-Py could lead to toxicity in patients with renal failure and has been reported to be uremic toxins (372, 373). We found that higher levels of these two metabolites are associated with higher renal SOFA score which indicates increased toxicity due to accumulation of these two metabolites in patients with renal dysfunction.

L-carnitine takes part in a shuttle system where it is involved in importing long-chain fatty acids into the mitochondria for fatty acid oxidation (161, 374, 375) and conversion into acetyl-coenzyme A (acetyl-CoA), for the TCA cycle and reduced nicotinamide dinucleotide (NADH) and flavin adenine dinucleotide (FADH_2) for oxidative phosphorylation (297) (figure 7.1). N6,N6,N6-trimethyllysine, a precursor in the biosynthesis of carnitine (268) was significantly higher in non-survivors than survivors at baseline, also consistent with our trajectory analysis where this metabolite was elevated in non-surviving patients on both days 0 and 4. This small molecules metabolite increased over time in both survivors and

non-survivors from the two cohorts and this increase was steeper in patients who went on to die. Higher levels of N6, N6, N6-trimethyllysine were also associated with higher organ failure scores (total SOFA, coagulation and renal) indicating patients with increased organ dysfunction have altered carnitine biosynthesis. N6,N6,N6-trimethyllysine is a precursor for the atherogenic metabolite, trimethylamine N-oxide (TMAO) and has been reported to be a risk predictor of incident cardiovascular disease (269). TMAO activates platelet hyper-reactivity, enhances formation of foam cells, reduces reverse cholesterol transport and stimulates inflammatory responses thereby leading to the progression of atherosclerosis, heart failure and chronic kidney disease (CKD) (376). Accumulation of N6,N6,N6-trimethyllysine in patients with renal failure might reflect increased formation of TMAO by the gut microbiota and higher levels of TMAO have been associated with decreased glomerular filtration rate in patients with CKD (270).

Fatty acid oxidation is one of the main fuel source for cells (297) and there seems to be a disruption in this process in patients with sepsis. Our study showed that fatty acyls such as acyl carnitines accumulated at baseline in non-surviving patients with respect to survivors reflecting enhanced incomplete fatty acid oxidation (i.e., β -oxidation) due to mitochondrial dysfunction (162, 253) (figure 7.1). These results were also consistent with other metabolomic studies (146-149, 163) and our trajectory results where acylcarnitines were significantly elevated in non-survivors with respect to survivors at day 0. Our study revealed that acyl carnitines mostly decreased over time in all patients irrespective of whether they survived or died at 28-days. Long chain acylcarnitines (especially hexadecenoylcarnitine, stearoylcarnitine, eicoseneoylcarnitine, and palmitoylcarnitine) had a different trajectory for survivors in comparison to other carnitine sub-classes (i.e., short- and medium chain acylcarnitines). The overall trajectory of these fatty acyls was significantly different between survivors and non-survivors and all these carnitines decreased over time in patients who went on to die whilst increased over time in patients who went on to survive (figure 7.1). These results suggest increased incomplete fatty acid oxidation (i.e., β -oxidation) due to mitochondrial dysfunction (162, 253) in non-survivors with reduced uptake of long chain fatty acids into the mitochondria. However, increased levels over time of these long-chain acyl carnitines in survivors might suggest increased uptake of fatty acids into the mitochondria where they can be converted into acetyl-CoA via β -oxidation which can then

enter TCA cycle and generate more energy for survival (figure 7.1). Higher levels of medium- and long-chain acyl carnitines were associated with higher organ failure scores (total SOFA, renal SOFA, and liver SOFA, coagulation SOFA scores) in patients with sepsis. This is supported by evidence that people with liver failure have increased mitochondrial dysfunction and hence enhanced incomplete fatty oxidation (162, 253). Our results were also in accordance with other studies which revealed that higher levels of short- and medium-chain acyl carnitines are linked to liver and renal dysfunction as well as infection/inflammation (175). Elevated levels of acylcarnitines have also been reported in patients with chronic kidney disease and maintenance hemodialysis as a result of impaired excretory function of the failing kidneys (339).

During sepsis, the levels of the peroxisome proliferator-activated receptor (PPAR- α) (a transcription factor which induces genes involved β -oxidation of fatty acids) are reduced and fatty acid breakdown via β -oxidation is compromised, resulting in the accumulation of FAs in the liver, heart, and kidney as well as in the blood (242). Generally, reduced breakdown of FAs in patients with sepsis results in energy deficiency and lipotoxicity as well as mitochondrial damage due to the accumulation of FAs and their metabolites such as fatty acyl carnitines (242). We found reduced levels of FAs (such as polyunsaturated fatty acids (PUFAs) including eicosapentaenoic acid (EPA), eicosatrienoic acid (ETA), arachidonic acid, docosapentaenoic acid (DPA) and eicosadienoic acid) in patients who went on to die than those who survived at baseline and after 24 h of ICU admission which suggests elevated carnitine-independent uptake of FAs into the mitochondria (377) in these patients also supporting our previous findings on acylcarnitines (figure 7.1). Additionally, reduced levels of these FAs especially EPA and docosahexanoic acid (DHA), both of which are involved in the formation of specialized pro-resolving mediators (SPMs) including resolvins, protectins and maresins (300, 301), might also reflect rapid metabolism of PUFAs in patients who die into SPMs, which are biologically active during organ damage and tissue inflammation (300). These results are also consistent with our findings from the trajectory analysis which revealed that ETA, EPA, DPA, tetracosatetraenoic acid were significantly lower in non-survivors than survivors at day 0 in LeoPARDS. All of these FAs have not been previously associated with sepsis mortality however, one study reported reduced levels of EPA and DHA in patients with sepsis when compared to healthy controls (302) and in our case we

had reduced levels in patients who went on to die therefore it might suggest the continuum of the disease.

Our study also revealed that FAs including saturated fatty acids (SFAs), mono-unsaturated fatty acids (MUFAs) and PUFAs (including ETA, EPA, DPA, DHA) decreased over time in both survivors and non-survivors, with no difference in trajectory between these groups (figure 7.1). Elevated levels of these FAs in both survivors and non-survivors at day 0 with respect to day 4 might result from increased type I inflammation mediated by macrophages (340) at baseline which reduces over time. High levels of these FAs may counteract inflammation in the tissues via the increased formation of SPMs from EPA and DHA (300) at day 0 and a decline as inflammation improves. Accumulation of FAs might also suggest increased lipolysis at baseline in comparison to day 4 as reflected by lower TGs at this time point in both survivors and non-survivors which suggests increased hydrolysis of TGs into FAs (figure 7.1). Additionally, we found that higher levels of fatty acids such as SFAs, MUFAs and PUFAs are associated with higher SOFA scores (including respiratory, coagulation, liver and total SOFA scores). These results suggest increased lipolysis (337) in patients who progress towards organ failure which leads to the accumulation of these FAs in these organs causing lipotoxicity which leads to multiple organ dysfunction in patients with sepsis (242).

Lower levels of FAs in non-survivors with respect to survivors at baseline might also indicate reduced lipolysis at baseline which also fits well with our findings about TGs which were significantly reduced in non-survivors when compared to survivors (figure 7.1). These results might suggest decreased hydrolysis of TGs into FAs and glycerol (337, 350) or it might indicate that the energy stores are more depleted in patients who die hence, not able to meet the increased energy requirements. Our study revealed that TGs, one of the main sources of energy, mostly increased over time in both survivors and non-survivors with levels of TGs significantly higher at day 4 in comparison to day 0 in both survivors and non-survivors which suggests reduced lipolysis over time in both groups (figure 7.1), hence decreased hydrolysis of TGs into glycerol and FAs (337, 350). These results might suggest upregulation of the hypoxia-inducible lipid droplet-associated (HILPDA) protein over time which results in down-regulation of the adipose triglyceride lipase (ATGL), thereby leading to reduced hydrolysis of TGs via ATGL (351). This will in turn result in decreased availability of arachidonic acid, a precursor for pro-inflammatory mediators thereby suppressing the

synthesis of prostaglandin E₂ (PGE₂) which will overall lead to attenuation of the inflammatory response (351) over time.

Our findings about TG also fit well with the metabolic trajectories of diacylglycerols (DGs) and monoacylglycerols (MGs), the breakdown products of TGs resulting from the first and second step in the hydrolysis of TGs via adipose triglyceride lipase (337) and hormone-sensitive lipase (352). These lipids were significantly higher in both survivors and non-survivors at day 4 in comparison to day 0 indicating as the levels of TGs increase over time, the hydrolysis of TGs also increases. Increased levels of free triglycerides were associated with higher organ failure scores (such as total SOFA, coagulation SOFA and respiratory SOFA) in patients with sepsis, which suggests as the patients progress towards organ failure the levels of these circulating free triglycerides rise in the serum reflecting increased formation of triglycerides and decreased activity of lipoprotein lipase (296, 353).

In general, fasting-induced lipolysis could primarily be activated by catecholamines such as noradrenaline and other hormones (including cortisol, glucagon, growth hormone and adrenocorticotrophic hormone) (337). Our study revealed that cortisol was significantly higher in non-survivors at baseline and after 24 h of admission but not across both cohorts (figure 7.1). These results were also consistent with the findings from the trajectory analysis which revealed that cortisol was higher in patients who went on to die with respect to those who survive on both days (0 and 4) in accordance with other metabolomic studies (145, 146). Our results suggest that patients who went on to die had increased production of cortisol by the adrenal cortex via enhanced stimulation of adrenocorticotrophic hormone (ACTH) due to the stress response. Cortisol exerts its effects by indirectly suppressing the expression of inflammatory cytokines, such as IL-1 β , TNF, and IL-6, as well as attenuating the release of ACTH and corticotropin-releasing hormone (CRH) by the anterior pituitary gland (90, 254). Accumulation of cortisol in non-survivors might also enhance the activity of catecholamines (254). Our study also revealed that cortisol decreases over time in both survivors and non-survivors (figure 7.1) probably due to reduction in the stress response (90, 254). In septic shock, patients can be treated with corticosteroids as an adjunctive therapy, and they have been consistently proven to lower the shock duration (91-93). We observed that patients with sepsis who had received hydrocortisone had significantly higher

levels of cortisol in comparison to those who received placebo. This suggests conversion of hydrocortisone into cortisol in the liver which can bind to glucocorticoid receptors and will exert many different effects such as causing reduced vasodilation, vascular permeability and leukocyte migration as well as inhibition of phospholipase A2 resulting in reduced production of arachidonic acid (89). Patients with higher organ dysfunction had higher cortisol levels.

Lipid metabolism is altered in patients with sepsis and the activation of several lipid pathways are a part of underlying sepsis pathology (303). Our study revealed that phospholipids, including PCs, PEs, LPCs and LPEs were reduced in patients who went on to die compared to those who survived whilst PAs and LPGs were consistently elevated in non-survivors at baseline (figure 7.1) and after 24 h of admission. These findings were also supported by other metabolomic studies (149, 151, 156, 158). All these phospholipids (PCs, PEs, LPCs, LPEs and LPGs) increased over time in both survivors and non-survivors from and this increase was steeper in patients who went on to survive in comparison to those who went on to die (figure 7.1). Reduced levels of LPCs in non-survivors might be due to enhanced cleavage of PC from HDL via endothelial lipase or increased hydrolysis of PCs on the cell membranes by phospholipases A1 or A2 (308) generating LPCs which are rapidly hydrolysed into LPA via phospholipase D or autotaxin (303). LPCs are crucial regulators of both the innate and the adaptive immune system and have many roles ranging from anti and pro-inflammatory (308) effects to immuno-modulation (309), inhibition of platelet aggregation and promotion of the secretion of NO in endothelial cells (308). As our study revealed that LPCs increase over time in both survivors and non-survivors it suggests increased conversion of PCs into LPCs over time. The physiological significance of LPCs is still not fully known (378) however, LPC administration in septic mice and rats has shown to increase the clearance of bacteria, reducing organ injury, and improving survival (356-359) and in our case low levels might suggest decreased clearance of bacteria in patients who die.

Phosphatidic acid (PA), a precursor for DG and lysophosphatidic acid (LPA), enhances thrombosis via exposure of phosphatidylserine (PS) on human erythrocytes (311). Accumulation of PAs in non-survivors might suggest increased thrombosis compared to

those who survived. LPA, a catabolic product of PA and a bioactive lipid mediator of inflammation can regulate thrombotic stability by stimulating the release of NETs from neutrophils through lysophosphatidic acid receptors 1-6 (312). In sepsis, increased release of neutrophil extracellular traps (NETs) have been shown to contribute towards the pathogenesis of this heterogeneous condition (312) by causing increased intravascular thrombosis and disseminated intravascular coagulation (DIC) which eventually leads to organ damage (312). As non-survivors had higher concentrations of LPA (22:6/0:0) with respect to survivors at ICU admission (figure 7.1) it suggests increased NET production. This is supported by higher levels of PA and LPA being associated with higher organ failure liver SOFA score suggesting that liver failure could be driven by micro-thromboses.

PGs, which are inherent components of lung surfactant, exhibit anti-inflammatory roles (310). Non-survivors had accumulated levels of LPGs resulting from the hydrolysis of PGs (figure 7.1) via phospholipase A2 (308) suggesting increased modulation of endothelial cell activity as well as enhanced activation of natural killer cell trafficking (308). As LPGs increased over time in both groups it indicates increased hydrolysis of PGs into LPGs as well as increased modulation of endothelial cell activity over time (308). Additionally, higher levels of LPG(18:2/0:0) were associated with higher total SOFA and liver sofa score in patients with sepsis.

Sphingolipids such as SMs and Cers have important roles in the regulation of cellular processes, such as lymphocyte trafficking, mediating pro- and anti-inflammatory effects, and maintaining the integrity of the endothelial barrier (303). These lipid species were reduced in non-survivors compared to survivors at all timepoints, but these sphingolipids mostly increased over time in both (figure 7.1). Our results for SMs were in accordance with literature (151) and suggest that non-survivors have a reduction in the precursor SMs and hence lower production of Cers via sphingomyelinase during inflammation (304) which act as second messengers (344) as well as receptor agonists (305) for the lps CD14 receptor (306) as they are structurally similar to bacterial toxin (lps) which has a role in sepsis pathology. Low levels of sphingolipids were associated with higher total, coagulation, respiratory and liver SAOFA scores. Lower levels of SM have been reported in sepsis patients with acute lung injury in comparison to healthy subjects (251) which also supports our findings.

Sphingolipid, sphingosine-1-phosphate (S1P), a potent regulator of endothelial integrity and immune response, which results from the hydrolysis of Cers followed by phosphorylation via sphingosine kinases 1 and 2 (98), decreased as the coagulation and liver score increased in septic shock patients. S1P is important for wound healing processes upon platelet activation, and it is also involved in maintaining the vascular barrier integrity via S1P₁ (a G-protein coupled receptor) and prevents lethal responses to leak-inducing mediators (379). Hence, Lower levels of S1P in patients with higher organ dysfunction might reflect reduced healing as well as increased endothelial dysfunction (379). It has been reported that low levels of S1P decline significantly in patients with advanced stages of chronic liver disease and were also a predictor of an early mortality (380).

The validation of the results from models of mortality from VANISH and LeoPARDS revealed that no matter which way the validation was carried, the predictive models performed the same for both cohorts, but the models were not good, probably due to the less commonality in the metabolites incorporated into the models. Additionally, the predictive models for trajectories did not work as there were no significant differences between survivors and non-survivors or probably due to the use of log fold change data from the individual timepoint samples in the prediction models.

Our study has some strengths as well as some limitations. The major strengths of this study were that the two cohorts involved in this study had large sample sizes as well as samples available from four different timepoints allowing longitudinal data analysis to determine the metabolic trajectories. Consistent results were found across the two cohorts despite different sample sizes, which is also a major strength of our study. Targeted data from two different analytical platforms (NMR and UHPLC-MS) was used in our study which provides higher number of metabolites for investigation. In our study some metabolites such as creatine could be measured using both analytical platforms (NMR and UHPLC-MS) contributing towards the strength of our study and the results obtained for the common metabolites were consistent across the two analytical platforms in both cohorts also confirming our findings.

There were some limitations of this study. Firstly, the sample size of one of the cohorts (i.e., VANISH) was almost 1/3 of the sample size of the other cohort (i.e., LeoPARDS) so, VANISH

had less statistical power than LeoPARDS and due to this reason, some of the results from the statistical analyses were not significant after correction in VANISH. Secondly, there are fewer samples at later timepoints, and this subset of samples is a biased selection of patients, who have either not recovered fast enough to be discharged from ICU or who have not already died so the metabolic trajectories only reflect those patients who remain on ICU. The methods to correct for this are quite complicated and would need building further models, where LME model is combined with a cox regression model to factor in the time to ICU discharge for these patients to account for the early discharges, but this approach could be considered as a further work, but it was outside the scope of this project. Thirdly, the results for metabolites obtained from the two analytical platforms might not match sometimes probably due to the ion suppression of these metabolites during the UHPLC-MS profiling and in those cases the results from NMR were mainly used for the interpretation of the findings as it is more reliable than UHPLC-MS. Another limitation of this study is that different drugs were used for treatment in the two clinical trials so the findings from the drug analysis on VANISH could not be validated in LeoPARDS. The LeoPARDS dataset contains samples from almost the whole population but VANISH is a subset of the trial population with only half of the population being sampled, as not all the recruiting sites could take samples. This could be a biased selection, which might not be representative of the entire trial. This could partly explain why some findings in LeoPARDS were not also seen in VANISH. Finally, although we tried using LeoPARDS and VANISH to validate each other they are not drawn from the same population and so we cannot be sure that the metabolic trajectories in both are the same. For example, because of the differences in the trial designs patients in LeoPARDS tended to be sicker, with more severe shock than those in VANISH. Additionally, the timings for sample collection were not the same in VANISH and LeoPARDS, especially for third and fourth timepoint. However, despite the difference in the sample timings, most of the observed metabolic changes were in the same direction in the two cohorts.

7.2 Conclusion

Our study revealed that there were baseline differences between survivors and non-survivors, suggesting that patients with sepsis have perturbations in the metabolic pathways associated with energy, lipid, protein, nucleotide, purine, and phospholipid metabolism.

These pathways were found to be more altered in patients who went on to die than those who went on to survive. Overall, non-survivors had inadequate resolution of inflammation and energy imbalance in comparison to survivors and these mechanisms are known to be altered during sepsis leading to dysfunctional lipid metabolism. The overall metabolic trajectories of small molecules, lipids and lipoproteins for survivors and non-survivors were similar. However, there were some metabolites which had significantly different trajectories for survivors and non-survivors, and those metabolites include acyl carnitines, fatty acids, and some small molecules. The knowledge of metabolites which differentiate survivors from non-survivors at baseline is useful, but knowing how these metabolites change over time in the two clinical groups is clinically beneficial as it could allow novel ways to track clinical progress.

8. References

1. Perner A, Gordon AC, Angus DC, Lamontagne F, Machado F, Russell JA, et al. The intensive care medicine research agenda on septic shock. *Intensive Care Med.* 2017;43(9):1294-305.
2. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA.* 2016;315(8):801-10.
3. Vincent JL. The Clinical Challenge of Sepsis Identification and Monitoring. *PLoS Med.* 2016;13(5):e1002022.
4. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, et al. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest.* 1992;101(6):1644-55.
5. Perner A, Gordon AC, De Backer D, Dimopoulos G, Russell JA, Lipman J, et al. Sepsis: frontiers in diagnosis, resuscitation and antibiotic therapy. *Intensive Care Med.* 2016;42(12):1958-69.
6. Levy MM, Fink MP, Marshall JC, Abraham E, Angus D, Cook D, et al. 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. *Crit Care Med.* 2003;31(4):1250-6.
7. Sprung CL, Reinhart K. Definitions for Sepsis and Septic Shock. *JAMA.* 2016;316(4):456-7.
8. Kleinpell RM, Schorr CA, Balk RA. The New Sepsis Definitions: Implications for Critical Care Practitioners. *Am J Crit Care.* 2016;25(5):457-64.
9. Abraham E. New Definitions for Sepsis and Septic Shock: Continuing Evolution but With Much Still to Be Done. *JAMA.* 2016;315(8):757-9.
10. Gul F, Arslantas MK, Cinel I, Kumar A. Changing Definitions of Sepsis. *Turk J Anaesthesiol Reanim.* 2017;45(3):129-38.
11. Chiu C, Legrand M. Epidemiology of sepsis and septic shock. *Curr Opin Anaesthesiol.* 2021;34(2):71-6.
12. Rudd KE, Johnson SC, Agesa KM, Shackelford KA, Tsoi D, Kievlan DR, et al. Global, regional, and national sepsis incidence and mortality, 1990–2017: analysis for the Global Burden of Disease Study. *The Lancet.* 2020;395(10219):200-11.
13. Liu V, Escobar GJ, Greene JD, Soule J, Whippy A, Angus DC, et al. Hospital deaths in patients with sepsis from 2 independent cohorts. *JAMA.* 2014;312(1):90-2.

14. Sharma NK, Ferreira BL, Tashima AK, Brunialti MKC, Torquato RJS, Bafi A, et al. Lipid metabolism impairment in patients with sepsis secondary to hospital acquired pneumonia, a proteomic analysis. *Clin Proteomics*. 2019;16:29.
15. Levy MM, Artigas A, Phillips GS, Rhodes A, Beale R, Osborn T, et al. Outcomes of the Surviving Sepsis Campaign in intensive care units in the USA and Europe: a prospective cohort study. *The Lancet Infectious Diseases*. 2012;12(12):919-24.
16. Bray A, Kampouraki E, Winter A, Jesuthasan A, Messer B, Graziadio S. High Variability in Sepsis Guidelines in UK: Why Does It Matter? *Int J Environ Res Public Health*. 2020;17(6).
17. Gaieski DF, Edwards JM, Kallan MJ, Carr BG. Benchmarking the incidence and mortality of severe sepsis in the United States. *Crit Care Med*. 2013;41(5):1167-74.
18. Kaukonen KM, Bailey M, Suzuki S, Pilcher D, Bellomo R. Mortality related to severe sepsis and septic shock among critically ill patients in Australia and New Zealand, 2000-2012. *JAMA*. 2014;311(13):1308-16.
19. Paoli CJ, Reynolds MA, Sinha M, Gitlin M, Crouser E. Epidemiology and Costs of Sepsis in the United States-An Analysis Based on Timing of Diagnosis and Severity Level. *Crit Care Med*. 2018;46(12):1889-97.
20. Hex N, editor *The Cost of Sepsis Care in the UK Final Report* 2017.
21. Machon C, Sheikh F, Fox-Robichaud A. Social determinants of health associated with the development of sepsis in adults: a scoping review protocol. *BMJ Open*. 2020;10(10):e039146.
22. Cecconi M, Evans L, Levy M, Rhodes A. Sepsis and septic shock. *The Lancet*. 2018;392(10141):75-87.
23. Hecker A, Reichert M, Reuss CJ, Schmoch T, Riedel JG, Schneck E, et al. Intra-abdominal sepsis: new definitions and current clinical standards. *Langenbecks Arch Surg*. 2019;404(3):257-71.
24. Chen Y, Hu Y, Zhang J, Shen Y, Huang J, Yin J, et al. Clinical characteristics, risk factors, immune status and prognosis of secondary infection of sepsis: a retrospective observational study. *BMC Anesthesiol*. 2019;19(1):185.
25. Lindstrom AC, Eriksson M, Martensson J, Oldner A, Larsson E. Nationwide case-control study of risk factors and outcomes for community-acquired sepsis. *Sci Rep*. 2021;11(1):15118.
26. O'Keefe GE, Hybki DL, Munford RS. The G→A Single Nucleotide Polymorphism at the -308 Position in the Tumor Necrosis Factor- α Promoter Increases the Risk for Severe Sepsis after Trauma. *Journal of Trauma and Acute Care Surgery*. 2002;52(5).
27. Tang GJ, Huang SL, Yien HW, Chen WS, Chi CW, Wu CW, et al. Tumor necrosis factor gene polymorphism and septic shock in surgical infection. *Crit Care Med*. 2000;28(8):2733-6.

28. Thompson CM, Holden TD, Rona G, Laxmanan B, Black RA, O'Keefe GE, et al. Toll-like receptor 1 polymorphisms and associated outcomes in sepsis after traumatic injury: a candidate gene association study. *Ann Surg.* 2014;259(1):179-85.
29. Waterer GW, Quasney MW, Cantor RM, Wunderink RG. Septic shock and respiratory failure in community-acquired pneumonia have different TNF polymorphism associations. *Am J Respir Crit Care Med.* 2001;163(7):1599-604.
30. Boyd JH, Russell JA, Fjell CD. The meta-genome of sepsis: host genetics, pathogens and the acute immune response. *J Innate Immun.* 2014;6(3):272-83.
31. Akin A, Alp E, Altindiş M, Azak E, Batirel A, ÇAĞ Y, et al. Current Diagnosis and Treatment Approach to Sepsis. *Mediterranean Journal of Infection Microbes and Antimicrobials.* 2018.
32. Vincent JL, Rello J, Marshall J, Silva E, Anzueto A, Martin CD, et al. International study of the prevalence and outcomes of infection in intensive care units. *Jama.* 2009;302(21):2323-9.
33. Leligdowicz A, Dodek PM, Norena M, Wong H, Kumar A, Kumar A. Association between source of infection and hospital mortality in patients who have septic shock. *Am J Respir Crit Care Med.* 2014;189(10):1204-13.
34. Beale R, Reinhart K, Brunkhorst FM, Dobb G, Levy M, Martin G, et al. Promoting Global Research Excellence in Severe Sepsis (PROGRESS): lessons from an international sepsis registry. *Infection.* 2009;37(3):222-32.
35. Tolsma V, Schwebel C, Azoulay E, Darmon M, Souweine B, Vesin A, et al. Sepsis severe or septic shock: outcome according to immune status and immunodeficiency profile. *Chest.* 2014;146(5):1205-13.
36. Tanriover MD, Guven GS, Sen D, Unal S, Uzun O. Epidemiology and outcome of sepsis in a tertiary-care hospital in a developing country. *Epidemiol Infect.* 2006;134(2):315-22.
37. Vincent J-L, Marshall JC, Namendys-Silva SA, François B, Martin-Loeches I, Lipman J, et al. Assessment of the worldwide burden of critical illness: the Intensive Care Over Nations (ICON) audit. *The Lancet Respiratory Medicine.* 2014;2(5):380-6.
38. Cohen J, Cristofaro P, Carlet J, Opal S. New method of classifying infections in critically ill patients. *Crit Care Med.* 2004;32(7):1510-26.
39. Morgan MP, Szakmany T, Power SG, Olaniyi P, Hall JE, Rowan K, et al. Sepsis Patients with First and Second-Hit Infections Show Different Outcomes Depending on the Causative Organism. *Front Microbiol.* 2016;7:207.
40. Ani C, Farshidpanah S, Bellinghausen Stewart A, Nguyen HB. Variations in organism-specific severe sepsis mortality in the United States: 1999-2008. *Crit Care Med.* 2015;43(1):65-77.

41. Cohen J. The immunopathogenesis of sepsis. *Nature*. 2002;420(6917):885-91.
42. Gotts JE, Matthay MA. Sepsis: pathophysiology and clinical management. *BMJ*. 2016;353:i1585.
43. Cohen J. The immunopathogenesis of sepsis. *Nature*. 2002;420(6917):885-91.
44. Gyawali B, Ramakrishna K, Dhamoon AS. Sepsis: The evolution in definition, pathophysiology, and management. *SAGE Open Med*. 2019;7:2050312119835043.
45. Cinel I, Dellinger RP. Advances in pathogenesis and management of sepsis. *Current Opinion in Infectious Diseases*. 2007;20(4).
46. Jacobi J. Pathophysiology of sepsis. *Am J Health Syst Pharm*. 2002;59 Suppl 1:S3-8.
47. Chen GY, Nunez G. Sterile inflammation: sensing and reacting to damage. *Nat Rev Immunol*. 2010;10(12):826-37.
48. Mollnes TE, Huber-Lang M. Complement in sepsis-when science meets clinics. *FEBS Lett*. 2020;594(16):2621-32.
49. Jain S, Gautam V, Naseem S. Acute-phase proteins: As diagnostic tool. *J Pharm Bioallied Sci*. 2011;3(1):118-27.
50. van der Poll T, van de Veerdonk FL, Scicluna BP, Netea MG. The immunopathology of sepsis and potential therapeutic targets. *Nat Rev Immunol*. 2017;17(7):407-20.
51. Charchafli J, Wei J, Labaze G, Hou YJ, Babarsh B, Stutz H, et al. The role of complement system in septic shock. *Clin Dev Immunol*. 2012;2012:407324.
52. Zetoune FS, Ward PA. Role of Complement and Histones in Sepsis. *Front Med (Lausanne)*. 2020;7:616957.
53. Evans T. Diagnosis and management of sepsis. *Clin Med (Lond)*. 2018;18(2):146-9.
54. Morin EE, Guo L, Schwendeman A, Li XA. HDL in sepsis - risk factor and therapeutic approach. *Front Pharmacol*. 2015;6:244.
55. Cambiaghi A, Pinto BB, Brunelli L, Falcetta F, Aletti F, Bendjelid K, et al. Characterization of a metabolomic profile associated with responsiveness to therapy in the acute phase of septic shock. *Sci Rep*. 2017;7(1):9748.
56. Lorente L, Martin MM, Gonzalez-Rivero AF, Ferreres J, Sole-Violan J, Labarta L, et al. Serum levels of caspase-cleaved cytokeratin-18 and mortality are associated in severe septic patients: pilot study. *PLoS One*. 2014;9(10):e109618.
57. Roth GA, Krenn C, Brunner M, Moser B, Ploder M, Spittler A, et al. Elevated serum levels of epithelial cell apoptosis-specific cytokeratin 18 neoepitope m30 in critically ill patients. *Shock*. 2004;22(3):218-20.

58. Moore DJ, Greystoke A, Butt F, Wurthner J, Growcott J, Hughes A, et al. A Pilot Study Assessing the Prognostic Value of CK18 and nDNA Biomarkers in Severe Sepsis Patients. *Clinical Drug Investigation*. 2012;32(3):179-87.
59. Cobb JP, Danner RL. Nitric Oxide and Septic Shock. *JAMA*. 1996;275(15):1192-6.
60. Zeerleder S, Hack CE, Willemin WA. Disseminated intravascular coagulation in sepsis. *Chest*. 2005;128(4):2864-75.
61. Riewald M, Petrovan RJ, Donner A, Mueller BM, Ruf W. Activation of endothelial cell protease activated receptor 1 by the protein C pathway. *Science*. 2002;296(5574):1880-2.
62. Badrinath K, Shekhar M, Sreelakshmi M, Srinivasan M, Thunga G, Nair S, et al. Comparison of Various Severity Assessment Scoring Systems in Patients with Sepsis in a Tertiary Care Teaching Hospital. *Indian J Crit Care Med*. 2018;22(12):842-5.
63. Knaus WA, Draper EA, Wagner DP, Zimmerman JE. APACHE II: a severity of disease classification system. *Crit Care Med*. 1985;13(10):818-29.
64. Vincent JL, Moreno R, Takala J, Willatts S, De Mendonça A, Bruining H, et al. The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure. On behalf of the Working Group on Sepsis-Related Problems of the European Society of Intensive Care Medicine. *Intensive Care Med*. 1996;22(7):707-10.
65. Vincent JL, de Mendonça A, Cantraine F, Moreno R, Takala J, Suter PM, et al. Use of the SOFA score to assess the incidence of organ dysfunction/failure in intensive care units: results of a multicenter, prospective study. Working group on "sepsis-related problems" of the European Society of Intensive Care Medicine. *Crit Care Med*. 1998;26(11):1793-800.
66. Liu Z, Triba MN, Amathieu R, Lin X, Bouchemal N, Hantz E, et al. Nuclear magnetic resonance-based serum metabolomic analysis reveals different disease evolution profiles between septic shock survivors and non-survivors. *Crit Care*. 2019;23(1):169.
67. Evans L, Rhodes A, Alhazzani W, Antonelli M, Coopersmith CM, French C, et al. Surviving sepsis campaign: international guidelines for management of sepsis and septic shock 2021. *Intensive Care Med*. 2021;47(11):1181-247.
68. Antcliffe DB, Burnham KL, Al-Beidh F, Santhakumaran S, Brett SJ, Hinds CJ, et al. Transcriptomic Signatures in Sepsis and a Differential Response to Steroids. From the VANISH Randomized Trial. *Am J Respir Crit Care Med*. 2019;199(8):980-6.
69. Rello J, Valenzuela-Sanchez F, Ruiz-Rodriguez M, Moyano S. Sepsis: A Review of Advances in Management. *Adv Ther*. 2017;34(11):2393-411.
70. Kumar A, Roberts D, Wood KE, Light B, Parrillo JE, Sharma S, et al. Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock. *Crit Care Med*. 2006;34(6):1589-96.

71. Harbarth S, Garbino J, Pugin J, Romand JA, Lew D, Pittet D. Inappropriate initial antimicrobial therapy and its effect on survival in a clinical trial of immunomodulating therapy for severe sepsis. *Am J Med.* 2003;115(7):529-35.
 72. Garnacho-Montero J, Garcia-Garmendia JL, Barrero-Almodovar A, Jimenez-Jimenez FJ, Perez-Paredes C, Ortiz-Leyba C. Impact of adequate empirical antibiotic therapy on the outcome of patients admitted to the intensive care unit with sepsis. *Crit Care Med.* 2003;31(12):2742-51.
 73. Armand-Lefèvre L, Angebault C, Barbier F, Hamelet E, Defrance G, Ruppé E, et al. Emergence of imipenem-resistant gram-negative bacilli in intestinal flora of intensive care patients. *Antimicrob Agents Chemother.* 2013;57(3):1488-95.
 74. Rubinstein E, Keynan Y. Short-course therapy for severe infections. *International Journal of Antimicrobial Agents.* 2013;42:S22-S4.
 75. Howell MD, Davis AM. Management of Sepsis and Septic Shock. *Jama.* 2017;317(8):847-8.
 76. Levy MM, Artigas A, Phillips GS, Rhodes A, Beale R, Osborn T, et al. Outcomes of the Surviving Sepsis Campaign in intensive care units in the USA and Europe: a prospective cohort study. *Lancet Infect Dis.* 2012;12(12):919-24.
 77. Rhodes A, Evans LE, Alhazzani W, Levy MM, Antonelli M, Ferrer R, et al. Surviving Sepsis Campaign: International Guidelines for Management of Sepsis and Septic Shock: 2016. *Crit Care Med.* 2017;45(3):486-552.
 78. Avila AA, Kinberg EC, Sherwin NK, Taylor RD. The Use of Fluids in Sepsis. *Cureus.* 2016;8(3):e528.
 79. Smith MD, Maani CV. Norepinephrine. StatPearls. Treasure Island (FL): StatPearls Publishing
- Copyright © 2022, StatPearls Publishing LLC.; 2022.
80. Barrett LK, Singer M, Clapp LH. Vasopressin: mechanisms of action on the vasculature in health and in septic shock. *Crit Care Med.* 2007;35(1):33-40.
 81. Kakihana Y, Ito T, Nakahara M, Yamaguchi K, Yasuda T. Sepsis-induced myocardial dysfunction: pathophysiology and management. *J Intensive Care.* 2016;4:22.
 82. Vieillard-Baron A, Cecconi M. Understanding cardiac failure in sepsis. *Intensive Care Med.* 2014;40(10):1560-3.
 83. Parker MM, Shelhamer JH, Bacharach SL, Green MV, Natanson C, Frederick TM, et al. Profound but reversible myocardial depression in patients with septic shock. *Ann Intern Med.* 1984;100(4):483-90.

84. Sanfilippo F, Corredor C, Fletcher N, Landesberg G, Benedetto U, Foex P, et al. Diastolic dysfunction and mortality in septic patients: a systematic review and meta-analysis. *Intensive Care Med.* 2015;41(6):1004-13.
 85. Vieillard-Baron A, Caille V, Charron C, Belliard G, Page B, Jardin F. Actual incidence of global left ventricular hypokinesia in adult septic shock. *Crit Care Med.* 2008;36(6):1701-6.
 86. Schmittinger CA, Torgersen C, Luckner G, Schröder DC, Lorenz I, Dünser MW. Adverse cardiac events during catecholamine vasopressor therapy: a prospective observational study. *Intensive Care Med.* 2012;38(6):950-8.
 87. Wang Q, Yokoo H, Takashina M, Sakata K, Ohashi W, Abedelzaher LA, et al. Anti-Inflammatory Profile of Levosimendan in Cecal Ligation-Induced Septic Mice and in Lipopolysaccharide-Stimulated Macrophages. *Crit Care Med.* 2015;43(11):e508-20.
 88. Gordon AC, Perkins GD, Singer M, McAuley DF, Orme RM, Santhakumaran S, et al. Levosimendan for the Prevention of Acute Organ Dysfunction in Sepsis. *N Engl J Med.* 2016;375(17):1638-48.
 89. Yasir M, Goyal A, Sonthalia S. Corticosteroid Adverse Effects. StatPearls. Treasure Island (FL): StatPearls Publishing
- Copyright © 2022, StatPearls Publishing LLC.; 2022.
90. Vandewalle J, Libert C. Glucocorticoids in Sepsis: To Be or Not to Be. *Front Immunol.* 2020;11:1318.
 91. Annane D, Renault A, Brun-Buisson C, Megarbane B, Quenot JP, Siami S, et al. Hydrocortisone plus Fludrocortisone for Adults with Septic Shock. *N Engl J Med.* 2018;378(9):809-18.
 92. Gordon AC, Mason AJ, Thirunavukkarasu N, Perkins GD, Cecconi M, Cepkova M, et al. Effect of Early Vasopressin vs Norepinephrine on Kidney Failure in Patients With Septic Shock: The VANISH Randomized Clinical Trial. *JAMA.* 2016;316(5):509-18.
 93. Venkatesh B, Finfer S, Cohen J, Rajbhandari D, Arabi Y, Bellomo R, et al. Adjunctive Glucocorticoid Therapy in Patients with Septic Shock. *N Engl J Med.* 2018;378(9):797-808.
 94. Liang H, Song H, Zhai R, Song G, Li H, Ding X, et al. Corticosteroids for Treating Sepsis in Adult Patients: A Systematic Review and Meta-Analysis. *Front Immunol.* 2021;12:709155.
 95. Lamontagne F, Rochwerg B, Lytvyn L, Guyatt GH, Moller MH, Annane D, et al. Corticosteroid therapy for sepsis: a clinical practice guideline. *BMJ.* 2018;362:k3284.
 96. Nicholson JK, Lindon JC, Holmes E. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica.* 1999;29(11):1181-9.

97. Schlotterbeck G, Ross A, Dieterle F, Senn H. Metabolic profiling technologies for biomarker discovery in biomedicine and drug development. *Pharmacogenomics*. 2006;7(7):1055-75.
98. Beckonert O, Keun HC, Ebbels TM, Bundy J, Holmes E, Lindon JC, et al. Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nat Protoc*. 2007;2(11):2692-703.
99. Holmes E, Wilson ID, Lindon JC. Chapter 1 - An Overview of Metabolic Phenotyping and Its Role in Systems Biology. In: Lindon JC, Nicholson JK, Holmes E, editors. *The Handbook of Metabolic Phenotyping*: Elsevier; 2019. p. 1-51.
100. Clarke CJ, Haselden JN. Metabolic profiling as a tool for understanding mechanisms of toxicity. *Toxicol Pathol*. 2008;36(1):140-7.
101. Parkinson JA. Chapter 2 - NMR Spectroscopy Methods in Metabolic Phenotyping. In: Lindon JC, Nicholson JK, Holmes E, editors. *The Handbook of Metabolic Phenotyping*: Elsevier; 2019. p. 53-96.
102. Gerothanassis IP, Troganis A, Exarchou V, Barbarossou K. Nuclear Magnetic Resonance (Nmr) Spectroscopy: Basic Principles and Phenomena, and Their Applications to Chemistry, Biology and Medicine. *Chem Educ Res Pract*. 2002;3(2):229-52.
103. Keeler J. *Understanding NMR Spectroscopy*. 2013.
104. Hosur RV, Kakita VMR. Basic Concepts. In: Hosur RV, Kakita VMR, editors. *A Graduate Course in NMR Spectroscopy*. Cham: Springer International Publishing; 2022. p. 1-40.
105. Darbeau RW. Nuclear Magnetic Resonance (NMR) Spectroscopy: A Review and a Look at Its Use as a Probative Tool in Deamination Chemistry. *Applied Spectroscopy Reviews*. 2006;41(4):401-25.
106. Ross A, Schlotterbeck G, Dieterle F, Senn H. Chapter 3 - NMR Spectroscopy Techniques for Application to Metabonomics. In: Lindon JC, Nicholson JK, Holmes E, editors. *The Handbook of Metabonomics and Metabolomics*. Amsterdam: Elsevier Science B.V.; 2007. p. 55-112.
107. Alonso A, Marsal S, Julia A. Analytical methods in untargeted metabolomics: state of the art in 2015. *Front Bioeng Biotechnol*. 2015;3:23.
108. Tran TD, Caruthers SD, Hughes M, Marsh JN, Cyrus T, Winter PM, et al. Clinical applications of perfluorocarbon nanoparticles for molecular imaging and targeted therapeutics. *Int J Nanomedicine*. 2007;2(4):515-26.
109. Kubala E, Munoz-Alvarez KA, Topping G, Hundshammer C, Feuerecker B, Gomez PA, et al. Hyperpolarized ¹³C Metabolic Magnetic Resonance Spectroscopy and Imaging. *J Vis Exp*. 2016(118).

110. Krivdin LB. Tritium NMR: A compilation of data and a practical guide. *Magn Reson Chem.* 2023;61(4):195-247.
111. Herb K. Konstantin's gyromagnetic ratio table [Online]. 2023 [Accessed on 22 Oct 2023]. Available from: https://www.kherb.io/docs/nmr_table.html.
112. Jimenez B, Holmes E, Heude C, Tolson RF, Harvey N, Lodge SL, et al. Quantitative Lipoprotein Subclass and Low Molecular Weight Metabolite Analysis in Human Serum and Plasma by $(1)H$ NMR Spectroscopy in a Multilaboratory Trial. *Anal Chem.* 2018;90(20):11962-71.
113. Dona AC, Jimenez B, Schafer H, Humpfer E, Spraul M, Lewis MR, et al. Precision high-throughput proton NMR spectroscopy of human urine, serum, and plasma for large-scale metabolic phenotyping. *Anal Chem.* 2014;86(19):9887-94.
114. Zhang X, Zhu X, Wang C, Zhang H, Cai Z. Non-targeted and targeted metabolomics approaches to diagnosing lung cancer and predicting patient prognosis. *Oncotarget.* 2016;7(39):63437-48.
115. Pitt JJ. Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry. *Clin Biochem Rev.* 2009;30(1):19-34.
116. Kaklamanos G, Aprea E, Theodoridis G. 11 - Mass spectrometry: principles and instrumentation. In: Pico Y, editor. *Chemical Analysis of Food (Second Edition)*: Academic Press; 2020. p. 525-52.
117. Rockwood AL, Kushnir MM, Clarke NJ. *Mass Spectrometry. Principles and Applications of Clinical Mass Spectrometry* 2018. p. 33-65.
118. Fjeldsted JC. Chapter 2 - Advances in Time-of-Flight Mass Spectrometry. In: Pérez S, Eichhorn P, Barceló D, editors. *Comprehensive Analytical Chemistry.* 71: Elsevier; 2016. p. 19-49.
119. Stewart D, Dhungana S, Clark R, Pathmasiri W, McRitchie S, Sumner S. Chapter 4 - Omics Technologies Used in Systems Biology. In: Fry RC, editor. *Systems Biology in Toxicology and Environmental Health.* Boston: Academic Press; 2015. p. 57-83.
120. Allen DR, McWhinney BC. Quadrupole Time-of-Flight Mass Spectrometry: A Paradigm Shift in Toxicology Screening Applications. *Clin Biochem Rev.* 2019;40(3):135-46.
121. On the Accurate Understanding of Mass Measurement Accuracy in Q-TOF MS 2019.
122. Brenton AG, Godfrey AR. Accurate mass measurement: terminology and treatment of data. *J Am Soc Mass Spectrom.* 2010;21(11):1821-35.
123. Dunn WB, Lewis MR. Chapter 3 - The Role of Ultra Performance Liquid Chromatography-Mass Spectrometry in Metabolic Phenotyping. In: Lindon JC, Nicholson JK, Holmes E, editors. *The Handbook of Metabolic Phenotyping*: Elsevier; 2019. p. 97-136.

124. Worley B, Powers R. Multivariate Analysis in Metabolomics. *Curr Metabolomics*. 2013;1(1):92-107.
125. The problem of multicollinearity. In: Allen MP, editor. *Understanding Regression Analysis*. Boston, MA: Springer US; 1997. p. 176-80.
126. Dormann CF, Elith J, Bacher S, Buchmann C, Carl G, Carré G, et al. Collinearity: a review of methods to deal with it and a simulation study evaluating their performance. *Ecography*. 2013;36(1):27-46.
127. Debik J, Sangermani M, Wang F, Madssen TS, Giskeodegard GF. Multivariate analysis of NMR-based metabolomic data. *NMR Biomed*. 2022;35(2):e4638.
128. Sathya R, Abraham A. Comparison of Supervised and Unsupervised Learning Algorithms for Pattern Classification. *International Journal of Advanced Research in Artificial Intelligence*. 2013;2.
129. Posma JM. Chapter 9 - Multivariate Statistical Methods for Metabolic Phenotyping. In: Lindon JC, Nicholson JK, Holmes E, editors. *The Handbook of Metabolic Phenotyping*: Elsevier; 2019. p. 261-308.
130. Blaise BJ, Correia GDS, Haggart GA, Surowiec I, Sands C, Lewis MR, et al. Statistical analysis in metabolic phenotyping. *Nat Protoc*. 2021;16(9):4299-326.
131. Liu C, Zhang X, Nguyen TT, Liu J, Wu T, Lee E, et al. Partial least squares regression and principal component analysis: similarity and differences between two popular variable reduction approaches. *Gen Psychiatr*. 2022;35(1):e100662.
132. Gromski PS, Muhamadali H, Ellis DI, Xu Y, Correa E, Turner ML, et al. A tutorial review: Metabolomics and partial least squares-discriminant analysis--a marriage of convenience or a shotgun wedding. *Anal Chim Acta*. 2015;879:10-23.
133. Triba MN, Le Moyec L, Amathieu R, Goossens C, Bouchemal N, Nahon P, et al. PLS/OPLS models in metabolomics: the impact of permutation of dataset rows on the K-fold cross-validation quality parameters. *Molecular BioSystems*. 2015;11(1):13-9.
134. Montesinos López OA, Montesinos López A, Crossa J. Overfitting, Model Tuning, and Evaluation of Prediction Performance. *Multivariate Statistical Machine Learning Methods for Genomic Prediction* 2022. p. 109-39.
135. Szymanska E, Saccenti E, Smilde AK, Westerhuis JA. Double-check: validation of diagnostic statistics for PLS-DA models in metabolomics studies. *Metabolomics*. 2012;8(Suppl 1):3-16.
136. Gramatica P. On the development and validation of QSAR models. *Methods Mol Biol*. 2013;930:499-526.
137. Bono R, Alarcon R, Blanca MJ. Report Quality of Generalized Linear Mixed Models in Psychology: A Systematic Review. *Front Psychol*. 2021;12:666182.

138. Zhao Y. Chapter 5 - Regression. In: Zhao Y, editor. *R and Data Mining*: Academic Press; 2013. p. 41-50.
139. van Oijen M. Linear Modelling: LM, GLM, GAM and Mixed Models. *Bayesian Compendium* 2020. p. 137-40.
140. Peng H, Lu Y. Model selection in linear mixed effect models. *Journal of Multivariate Analysis*. 2012;109:109-29.
141. Harrison XA, Donaldson L, Correa-Cano ME, Evans J, Fisher DN, Goodwin CED, et al. A brief introduction to mixed effects modelling and multi-model inference in ecology. *PeerJ*. 2018;6:e4794.
142. Magezi DA. Linear mixed-effects models for within-participant psychology experiments: an introductory tutorial and free, graphical user interface (LMMgui). *Front Psychol*. 2015;6:2.
143. Cloarec O, Dumas M-E, Craig A, Barton RH, Trygg J, Hudson J, et al. Statistical Total Correlation Spectroscopy: An Exploratory Approach for Latent Biomarker Identification from Metabolic ¹H NMR Data Sets. *Analytical Chemistry*. 2005;77(5):1282-9.
144. Hussain H, Vutipongsatorn K, Jiménez B, Antcliffe DB. Patient Stratification in Sepsis: Using Metabolomics to Detect Clinical Phenotypes, Sub-Phenotypes and Therapeutic Response. *Metabolites*. 2022;12(5).
145. Seymour CW, Yende S, Scott MJ, Pribis J, Mohnney RP, Bell LN, et al. Metabolomics in pneumonia and sepsis: an analysis of the GenIMS cohort study. *Intensive Care Med*. 2013;39(8):1423-34.
146. Rogers AJ, McGeachie M, Baron RM, Gazourian L, Haspel JA, Nakahira K, et al. Metabolomic derangements are associated with mortality in critically ill adult patients. *PLoS One*. 2014;9(1):e87538.
147. Liu Z, Yin P, Amathieu R, Savarin P, Xu G. Application of LC-MS-based metabolomics method in differentiating septic survivors from non-survivors. *Anal Bioanal Chem*. 2016;408(27):7641-9.
148. Langley RJ, Tsalik EL, van Velkinburgh JC, Glickman SW, Rice BJ, Wang C, et al. An integrated clinico-metabolomic model improves prediction of death in sepsis. *Sci Transl Med*. 2013;5(195):195ra95.
149. Li GB, Hu HR, Pan WF, Li B, Ou ZY, Liang HY, et al. Plasma Metabolic Profiling of Pediatric Sepsis in a Chinese Cohort. *Front Cell Dev Biol*. 2021;9:643979.
150. Su L, Huang Y, Zhu Y, Xia L, Wang R, Xiao K, et al. Discrimination of sepsis stage metabolic profiles with an LC/MS-MS-based metabolomics approach. *BMJ Open Respir Res*. 2014;1(1):e000056.

151. Khaliq W, Grossmann P, Neugebauer S, Kleyman A, Domizi R, Calcinaro S, et al. Lipid metabolic signatures deviate in sepsis survivors compared to non-survivors. *Comput Struct Biotechnol J*. 2020;18:3678-91.
152. Roth E, Funovics J, Mühlbacher F, Schemper M, Mauritz W, Sporn P, et al. Metabolic disorders in severe abdominal sepsis: Glutamine deficiency in skeletal muscle. *Clinical Nutrition*. 1982;1(1):25-41.
153. Mierzchala-Pasierb M, Lipinska-Gediga M, Fleszar MG, Lewandowski L, Serek P, Placzowska S, et al. An analysis of urine and serum amino acids in critically ill patients upon admission by means of targeted LC-MS/MS: a preliminary study. *Sci Rep*. 2021;11(1):19977.
154. Cambiaghi A, Diaz R, Martinez JB, Odena A, Brunelli L, Caironi P, et al. An Innovative Approach for The Integration of Proteomics and Metabolomics Data In Severe Septic Shock Patients Stratified for Mortality. *Sci Rep*. 2018;8(1):6681.
155. Winkler MS, Nierhaus A, Rosler G, Lezius S, Harlandt O, Schwedhelm E, et al. Symmetrical (SDMA) and asymmetrical dimethylarginine (ADMA) in sepsis: high plasma levels as combined risk markers for sepsis survival. *Crit Care*. 2018;22(1):216.
156. Ferrario M, Cambiaghi A, Brunelli L, Giordano S, Caironi P, Guatteri L, et al. Mortality prediction in patients with severe septic shock: a pilot study using a target metabolomics approach. *Sci Rep*. 2016;6:20391.
157. Jaurila H, Koivukangas V, Koskela M, Gaddnas F, Myllymaa S, Kullaa A, et al. (1)H NMR Based Metabolomics in Human Sepsis and Healthy Serum. *Metabolites*. 2020;10(2).
158. Wang J, Sun Y, Teng S, Li K. Prediction of sepsis mortality using metabolite biomarkers in the blood: a meta-analysis of death-related pathways and prospective validation. *BMC Med*. 2020;18(1):83.
159. Garcia-Simon M, Morales JM, Modesto-Alapont V, Gonzalez-Marrachelli V, Vento-Rehues R, Jorda-Minana A, et al. Prognosis Biomarkers of Severe Sepsis and Septic Shock by 1H NMR Urine Metabolomics in the Intensive Care Unit. *PLoS One*. 2015;10(11):e0140993.
160. Mickiewicz B, Tam P, Jenne CN, Leger C, Wong J, Winston BW, et al. Integration of metabolic and inflammatory mediator profiles as a potential prognostic approach for septic shock in the intensive care unit. *Crit Care*. 2015;19:11.
161. Reuter SE, Evans AM. Carnitine and Acylcarnitines. *Clinical Pharmacokinetics*. 2012;51(9):553-72.
162. Otsubo C, Bharathi S, Uppala R, Ilkayeva OR, Wang D, McHugh K, et al. Long-chain Acylcarnitines Reduce Lung Function by Inhibiting Pulmonary Surfactant. *J Biol Chem*. 2015;290(39):23897-904.
163. Kamisoglu K, Haimovich B, Calvano SE, Coyle SM, Corbett SA, Langley RJ, et al. Human metabolic response to systemic inflammation: assessment of the concordance between experimental endotoxemia and clinical cases of sepsis/SIRS. *Crit Care*. 2015;19:71.

164. Sprung CL, Cerra FB, Freund HR, Schein RMH, Konstantinides FN, Marcial EH, et al. Amino acid alterations and encephalopathy in the sepsis syndrome. *Critical Care Medicine*. 1991;19(6).
165. Su L, Li H, Xie A, Liu D, Rao W, Lan L, et al. Dynamic changes in amino acid concentration profiles in patients with sepsis. *PLoS One*. 2015;10(4):e0121933.
166. Huang SS, Lin JY, Chen WS, Liu MH, Cheng CW, Cheng ML, et al. Phenylalanine- and leucine-defined metabolic types identify high mortality risk in patients with severe infection. *Int J Infect Dis*. 2019;85:143-9.
167. Drobnik W, Liebisch G, Audebert FX, Frohlich D, Gluck T, Vogel P, et al. Plasma ceramide and lysophosphatidylcholine inversely correlate with mortality in sepsis patients. *J Lipid Res*. 2003;44(4):754-61.
168. Dalli J, Colas RA, Quintana C, Barragan-Bradford D, Hurwitz S, Levy BD, et al. Human Sepsis Eicosanoid and Proresolving Lipid Mediator Temporal Profiles: Correlations With Survival and Clinical Outcomes. *Crit Care Med*. 2017;45(1):58-68.
169. Jones TN, Janani L, Gordon AC, Al-Beidh F, Antcliffe DB. A Novel Role for Cytochrome P450 Epoxygenase Metabolites in Septic Shock. *Crit Care Explor*. 2022;4(1):e0622.
170. Lee SH, Park MS, Park BH, Jung WJ, Lee IS, Kim SY, et al. Prognostic Implications of Serum Lipid Metabolism over Time during Sepsis. *Biomed Res Int*. 2015;2015:789298.
171. Cirstea M, Walley KR, Russell JA, Brunham LR, Genga KR, Boyd JH. Decreased high-density lipoprotein cholesterol level is an early prognostic marker for organ dysfunction and death in patients with suspected sepsis. *J Crit Care*. 2017;38:289-94.
172. Chien JY, Jerng JS, Yu CJ, Yang PC. Low serum level of high-density lipoprotein cholesterol is a poor prognostic factor for severe sepsis. *Crit Care Med*. 2005;33(8):1688-93.
173. Reisinger AC, Posch F, Hackl G, Marsche G, Sourij H, Bourgeois B, et al. Branched-Chain Amino Acids Can Predict Mortality in ICU Sepsis Patients. *Nutrients*. 2021;13(9).
174. Evans CR, Karnovsky A, Puskarich MA, Michailidis G, Jones AE, Stringer KA. Untargeted Metabolomics Differentiates L-Carnitine Treated Septic Shock 1-Year Survivors and Nonsurvivors. *J Proteome Res*. 2019;18(5):2004-11.
175. Chung KP, Chen GY, Chuang TY, Huang YT, Chang HT, Chen YF, et al. Increased Plasma Acetylcarnitine in Sepsis Is Associated With Multiple Organ Dysfunction and Mortality: A Multicenter Cohort Study. *Crit Care Med*. 2019;47(2):210-8.
176. Wang L, Ko ER, Gilchrist JJ, Pittman KJ, Rautanen A, Pirinen M, et al. Human genetic and metabolite variation reveals that methylthioadenosine is a prognostic biomarker and an inflammatory regulator in sepsis. *Sci Adv*. 2017;3(3):e1602096-e.

177. Sharma NK, Tashima AK, Brunialti MKC, Ferreira ER, Torquato RJS, Mortara RA, et al. Proteomic study revealed cellular assembly and lipid metabolism dysregulation in sepsis secondary to community-acquired pneumonia. *Sci Rep*. 2017;7(1):15606.
178. Mickiewicz B, Duggan GE, Winston BW, Doig C, Kubes P, Vogel HJ, et al. Metabolic profiling of serum samples by ¹H nuclear magnetic resonance spectroscopy as a potential diagnostic approach for septic shock. *Crit Care Med*. 2014;42(5):1140-9.
179. Mickiewicz B, Vogel HJ, Wong HR, Winston BW. Metabolomics as a novel approach for early diagnosis of pediatric septic shock and its mortality. *Am J Respir Crit Care Med*. 2013;187(9):967-76.
180. Gaddnas F, Koskela M, Koivukangas V, Risteli J, Oikarinen A, Laurila J, et al. Markers of collagen synthesis and degradation are increased in serum in severe sepsis: a longitudinal study of 44 patients. *Crit Care*. 2009;13(2):R53.
181. Vermont CL, den Brinker M, Kakeci N, de Kleijn ED, de Rijke YB, Joosten KF, et al. Serum lipids and disease severity in children with severe meningococcal sepsis. *Crit Care Med*. 2005;33(7):1610-5.
182. van Leeuwen HJ, Heezius EC, Dallinga GM, van Strijp JA, Verhoef J, van Kessel KP. Lipoprotein metabolism in patients with severe sepsis. *Crit Care Med*. 2003;31(5):1359-66.
183. Fanos V, Caboni P, Corsello G, Stronati M, Gazzolo D, Noto A, et al. Urinary ¹H-NMR and GC-MS metabolomics predicts early and late onset neonatal sepsis. *Early Human Development*. 2014;90:S78-S83.
184. Gordon AC, Mason AJ, Perkins GD, Ashby D, Brett SJ. Protocol for a randomised controlled trial of VAsopressin versus Noradrenaline as Initial therapy in Septic sHock (VANISH). *BMJ Open*. 2014;4(7):e005866.
185. Orme RM, Perkins GD, McAuley DF, Liu KD, Mason AJ, Morelli A, et al. An efficacy and mechanism evaluation study of Levosimendan for the Prevention of Acute oRgan Dysfunction in Sepsis (LeoPARDS): protocol for a randomized controlled trial. *Trials*. 2014;15:199.
186. Nieminen MS, Fruhwald S, Heunks LM, Suominen PK, Gordon AC, Kivikko M, et al. Levosimendan: current data, clinical use and future development. *Heart Lung Vessel*. 2013;5(4):227-45.
187. Antcliffe DB, Santhakumaran S, Orme RML, Ward JK, Al-Beidh F, O'Dea K, et al. Levosimendan in septic shock in patients with biochemical evidence of cardiac dysfunction: a subgroup analysis of the LeoPARDS randomised trial. *Intensive Care Med*. 2019;45(10):1392-400.
188. Takis PG, Jiménez B, Al-Saffar NMS, Harvey N, Chekmeneva E, Misra S, et al. A Computationally Lightweight Algorithm for Deriving Reliable Metabolite Panel Measurements from 1D ¹H NMR. *Analytical Chemistry*. 2021;93(12):4995-5000.

189. Sands CJ, Wolfer AM, Correia GD, Sadawi N, Ahmed A, Jimenez B, et al. The nPYc-Toolbox, a Python module for the pre-processing, quality-control, and analysis of metabolic profiling datasets. *Bioinformatics*. 2019.
190. Izzi-Engbeaya C, Comninou AN, Clarke SA, Jomard A, Yang L, Jones S, et al. The effects of kisspeptin on beta-cell function, serum metabolites and appetite in humans. *Diabetes Obes Metab*. 2018;20(12):2800-10.
191. Lewis MR, Pearce JT, Spagou K, Green M, Dona AC, Yuen AH, et al. Development and Application of Ultra-Performance Liquid Chromatography-TOF MS for Precision Large Scale Urinary Metabolic Phenotyping. *Anal Chem*. 2016;88(18):9004-13.
192. Lewis MR, Chekmeneva E, Camuzeaux S, Sands CJ, Yuen AHY, David M, et al. An Open Platform for Large Scale LC-MS-Based Metabolomics. *ChemRxiv*. 2022.
193. Wolfer AM, Correia GDS, Sands CJ, Camuzeaux S, Yuen AHY, Chekmeneva E, et al. peakPanther, an R package for large-scale targeted extraction and integration of annotated metabolic features in LC-MS profiling datasets. *Bioinformatics*. 2021;37(24):4886-8.
194. Martens L, Chambers M, Sturm M, Kessner D, Levander F, Shofstahl J, et al. mzML—a Community Standard for Mass Spectrometry Data*. *Molecular & Cellular Proteomics*. 2011;10(1):R110.000133.
195. R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. 2021.
196. Wickham H. *ggplot2: Elegant Graphics for Data Analysis*: Springer-Verlag New York; 2016.
197. The MathWorks Inc. MATLAB version: 9.8 (R2020a). 2020.
198. Fay MP, Proschan MA. Wilcoxon-Mann-Whitney or t-test? On assumptions for hypothesis tests and multiple interpretations of decision rules. *Stat Surv*. 2010;4:1-39.
199. Campbell I. Chi-squared and Fisher-Irwin tests of two-by-two tables with small sample recommendations. *Statistics in medicine*. 2007;26(19):3661-75.
200. Yates F. Contingency Tables Involving Small Numbers and the χ^2 Test. Supplement to the *Journal of the Royal Statistical Society*. 1934;1(2):217-35.
201. Jafari M, Ansari-Pour N. Why, When and How to Adjust Your P Values? *Cell J*. 2019;20(4):604-7.
202. Nicholson JK, Foxall PJD, Spraul M, Farrant RD, Lindon JC. 750 MHz ¹H and ¹H-¹³C NMR Spectroscopy of Human Blood Plasma. *Analytical Chemistry*. 1995;67(5):793-811.
203. Nagana Gowda GA, Gowda YN, Raftery D. Expanding the Limits of Human Blood Metabolite Quantitation Using NMR Spectroscopy. *Analytical Chemistry*. 2014;87(1):706-15.

204. Royston JP. Algorithm AS 181: The W Test for Normality. *Journal of the Royal Statistical Society*. 1982.
205. Wickham H. Reshaping Data with the reshape Package. *Journal of Statistical Software*. 2007;21(12):1 - 20.
206. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez J-C, et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics*. 2011;12(1):77.
207. Kuhn M. Building Predictive Models in R Using the caret Package. *Journal of Statistical Software*. 2008;28(5):1 - 26.
208. Broadhurst DI, Kell DB. Statistical strategies for avoiding false discoveries in metabolomics and related experiments. *Metabolomics*. 2006;2(4):171-96.
209. Nahm FS. Receiver operating characteristic curve: overview and practical use for clinicians. *Korean J Anesthesiol*. 2022;75(1):25-36.
210. Monaghan TF, Rahman SN, Agudelo CW, Wein AJ, Lazar JM, Everaert K, et al. Foundational Statistical Principles in Medical Research: Sensitivity, Specificity, Positive Predictive Value, and Negative Predictive Value. *Medicina (Kaunas)*. 2021;57(5).
211. Fawcett T. An introduction to ROC analysis. *Pattern Recognition Letters*. 2006;27(8):861-74.
212. Halligan S, Altman DG, Mallett S. Disadvantages of using the area under the receiver operating characteristic curve to assess imaging tests: a discussion and proposal for an alternative approach. *Eur Radiol*. 2015;25(4):932-9.
213. gscoreia89, jaketmp, carolinesands. Gscoreia89/pyChemometrics osotest [Online]. Zenodo; 2021 [Accessed on 2 Oct 2021]. Available from: <https://doi.org/10.5281/zenodo.4441074>.
214. DeBruine LM, Barr DJ. Understanding Mixed-Effects Models Through Data Simulation. *Advances in Methods and Practices in Psychological Science*. 2021;4(1).
215. Tanaka E, Hui FKC, editors. Symbolic Formulae for Linear Mixed Models. *Statistics and Data Science*; 2019 2019//; Singapore: Springer Singapore.
216. Liu X. Chapter 4 - Restricted maximum likelihood and inference of random effects in linear mixed models. In: Liu X, editor. *Methods and Applications of Longitudinal Data Analysis*. Oxford: Academic Press; 2016. p. 95-131.
217. Heise MJ, Mon SK, Bowman LC. Utility of linear mixed effects models for event-related potential research with infants and children. *Dev Cogn Neurosci*. 2022;54:101070.
218. Bates D, Mächler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*. 2015;67(1):1 - 48.

219. Lenth RV. emmeans: Estimated Marginal Means, aka Least-Squares Means. 2021.
220. Satterthwaite FE. An Approximate Distribution of Estimates of Variance Components. *Biometrics Bulletin*. 1946;2(6):110-4.
221. Luke SG. Evaluating significance in linear mixed-effects models in R. *Behav Res Methods*. 2017;49(4):1494-502.
222. Halekoh U, Højsgaard S. A Kenward-Roger Approximation and Parametric Bootstrap Methods for Tests in Linear Mixed Models – The R Package pbkrtest. *Journal of Statistical Software*. 2014;59(9):1 - 32.
223. Wishart DS, Guo A, Oler E, Wang F, Anjum A, Peters H, et al. HMDB 5.0: the Human Metabolome Database for 2022. *Nucleic Acids Res*. 2022;50(D1):D622-D31.
224. Pepper DJ, Demirkale CY, Sun J, Rhee C, Fram D, Eichacker P, et al. Does Obesity Protect Against Death in Sepsis? A Retrospective Cohort Study of 55,038 Adult Patients. *Crit Care Med*. 2019;47(5):643-50.
225. Oami T, Karasawa S, Shimada T, Nakada TA, Abe T, Ogura H, et al. Association between low body mass index and increased 28-day mortality of severe sepsis in Japanese cohorts. *Sci Rep*. 2021;11(1):1615.
226. Pepper DJ, Sun J, Welsh J, Cui X, Suffredini AF, Eichacker PQ. Increased body mass index and adjusted mortality in ICU patients with sepsis or septic shock: a systematic review and meta-analysis. *Crit Care*. 2016;20(1):181.
227. Wang S, Liu X, Chen Q, Liu C, Huang C, Fang X. The role of increased body mass index in outcomes of sepsis: a systematic review and meta-analysis. *BMC Anesthesiol*. 2017;17(1):118.
228. Bates D, Mächler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*. 2015;67(1).
229. Naveen Kumar MS, Gupta G, Kumar V, Jagannathan NR, Sinha S, Mewar S, et al. Differentiation between sepsis survivors and sepsis non-survivors through blood serum metabolomics: A proton nuclear magnetic resonance spectroscopy (NMR) study. *Magn Reson Imaging*. 2022;89:49-57.
230. García V, Mollineda RA, Sánchez JS. Index of Balanced Accuracy: A Performance Measure for Skewed Class Distributions. *Pattern Recognition and Image Analysis. Lecture Notes in Computer Science* 2009. p. 441-8.
231. Fernstrom JD, Fernstrom MH. Tyrosine, phenylalanine, and catecholamine synthesis and function in the brain. *J Nutr*. 2007;137(6 Suppl 1):1539S-47S; discussion 48S.
232. Nicholson JK, Gartland KP. ¹H NMR studies on protein binding of histidine, tyrosine and phenylalanine in blood plasma. *NMR Biomed*. 1989;2(2):77-82.

233. Englert JA, Rogers AJ. Metabolism, Metabolomics, and Nutritional Support of Patients with Sepsis. *Clin Chest Med*. 2016;37(2):321-31.
234. Szelig L, Kun S, Woth G, Molnar GA, Zrinyi Z, Katai E, et al. Time courses of changes of para-, meta-, and ortho-tyrosine in septic patients: A pilot study. *Redox Rep*. 2016;21(4):180-9.
235. Cooney RN, Kimball SR, Vary TC. REGULATION OF SKELETAL MUSCLE PROTEIN TURNOVER DURING SEPSIS: MECHANISMS AND MEDIATORS. *Shock*. 1997;7(1).
236. Li P, Yin YL, Li D, Kim SW, Wu G. Amino acids and immune function. *Br J Nutr*. 2007;98(2):237-52.
237. Hohn A, Iovino I, Cirillo F, Drinhaus H, Kleinbrahm K, Boehm L, et al. Bioinformatical Analysis of Organ-Related (Heart, Brain, Liver, and Kidney) and Serum Proteomic Data to Identify Protein Regulation Patterns and Potential Sepsis Biomarkers. *BioMed research international*. 2018;2018:3576157.
238. Ludwig KR, Hummon AB. Mass spectrometry for the discovery of biomarkers of sepsis. *Mol Biosyst*. 2017;13(4):648-64.
239. Wasyluk W, Zwolak A. Metabolic Alterations in Sepsis. *J Clin Med*. 2021;10(11).
240. Zhong Z, Wheeler MD, Li X, Froh M, Schemmer P, Yin M, et al. L-Glycine: a novel antiinflammatory, immunomodulatory, and cytoprotective agent. *Current Opinion in Clinical Nutrition & Metabolic Care*. 2003;6(2).
241. Mauriz JL, Matilla B, Culebras JM, González P, González-Gallego J. Dietary glycine inhibits activation of nuclear factor kappa B and prevents liver injury in hemorrhagic shock in the rat. *Free Radical Biology and Medicine*. 2001;31(10):1236-44.
242. Van Wyngene L, Vandewalle J, Libert C. Reprogramming of basic metabolic pathways in microbial sepsis: therapeutic targets at last? *EMBO Mol Med*. 2018;10(8).
243. Stringer KA, Younger JG, McHugh C, Yeomans L, Finkel MA, Puskarich MA, et al. Whole Blood Reveals More Metabolic Detail of the Human Metabolome than Serum as Measured by ¹H-NMR Spectroscopy: Implications for Sepsis Metabolomics. *Shock*. 2015;44(3):200-8.
244. Patel SS, Molnar MZ, Tayek JA, Ix JH, Noori N, Benner D, et al. Serum creatinine as a marker of muscle mass in chronic kidney disease: results of a cross-sectional study and review of literature. *J Cachexia Sarcopenia Muscle*. 2013;4(1):19-29.
245. Chiarla C, Giovannini I, Siegel JH. High phosphoserine in sepsis: panel of clinical and plasma amino acid correlations. *Springerplus*. 2014;3:279-.
246. Arlouskaya Y, Sawicka A, Glowala M, Giebultowicz J, Korytowska N, Talalaj M, et al. Asymmetric Dimethylarginine (ADMA) and Symmetric Dimethylarginine (SDMA)

Concentrations in Patients with Obesity and the Risk of Obstructive Sleep Apnea (OSA). *J Clin Med*. 2019;8(6).

247. Luiking YC, Poeze M, Ramsay G, Deutz NE. Reduced citrulline production in sepsis is related to diminished de novo arginine and nitric oxide production. *Am J Clin Nutr*. 2009;89(1):142-52.

248. Maiuolo J, Oppedisano F, Gratteri S, Muscoli C, Mollace V. Regulation of uric acid metabolism and excretion. *Int J Cardiol*. 2016;213:8-14.

249. Phang JM, Pandhare J, Liu Y. The metabolism of proline as microenvironmental stress substrate. *J Nutr*. 2008;138(10):2008s-15s.

250. Beloborodova N, Pautova A, Sergeev A, Fedotcheva N. Serum Levels of Mitochondrial and Microbial Metabolites Reflect Mitochondrial Dysfunction in Different Stages of Sepsis. *Metabolites*. 2019;9(10).

251. Stringer KA, Serkova NJ, Karnovsky A, Guire K, Paine R, 3rd, Standiford TJ. Metabolic consequences of sepsis-induced acute lung injury revealed by plasma (1)H-nuclear magnetic resonance quantitative metabolomics and computational analysis. *Am J Physiol Lung Cell Mol Physiol*. 2011;300(1):L4-L11.

252. Zhao G, He F, Wu C, Li P, Li N, Deng J, et al. Betaine in Inflammation: Mechanistic Aspects and Applications. *Front Immunol*. 2018;9:1070.

253. Reuter SE, Evans AM. Carnitine and acylcarnitines: pharmacokinetic, pharmacological and clinical aspects. *Clin Pharmacokinet*. 2012;51(9):553-72.

254. Bendel S, Karlsson S, Pettila V, Loisa P, Varpula M, Ruokonen E, et al. Free cortisol in sepsis and septic shock. *Anesth Analg*. 2008;106(6):1813-9.

255. Deen CPJ, Veen AV, Gomes-Neto AW, Geleijnse JM, Berg K, Heiner-Fokkema MR, et al. Urinary Excretion of N(1)-Methylnicotinamide and N(1)-Methyl-2-Pyridone-5-Carboxamide and Mortality in Kidney Transplant Recipients. *Nutrients*. 2020;12(7).

256. Slominska EM, Smolenski RT, Szolkiewicz M, Leaver N, Rutkowski B, Simmonds HA, et al. Accumulation of plasma N-methyl-2-pyridone-5-carboxamide in patients with chronic renal failure. *Mol Cell Biochem*. 2002;231(1-2):83-8.

257. Lasbury ME, Merali S, Durant PJ, Tschang D, Ray CA, Lee CH. Polyamine-mediated apoptosis of alveolar macrophages during *Pneumocystis pneumonia*. *J Biol Chem*. 2007;282(15):11009-20.

258. Poole RC, Halestrap AP. Transport of lactate and other monocarboxylates across mammalian plasma membranes. *Am J Physiol*. 1993;264(4 Pt 1):C761-82.

259. James JH, Luchette FA, McCarter FD, Fischer JE. Lactate is an unreliable indicator of tissue hypoxia in injury or sepsis. *The Lancet*. 1999;354(9177):505-8.

260. Pravda J. Metabolic theory of septic shock. *World J Crit Care Med.* 2014;3(2):45-54.
261. Cryer PE, Axelrod L, Grossman AB, Heller SR, Montori VM, Seaquist ER, et al. Evaluation and management of adult hypoglycemic disorders: an Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab.* 2009;94(3):709-28.
262. Kushimoto S, Abe T, Ogura H, Shiraishi A, Saitoh D, Fujishima S, et al. Impact of blood glucose abnormalities on outcomes and disease severity in patients with severe sepsis: An analysis from a multicenter, prospective survey of severe sepsis. *PLoS One.* 2020;15(3):e0229919.
263. Maitra SR, Wojnar MM, Lang CH. Alterations in tissue glucose uptake during the hyperglycemic and hypoglycemic phases of sepsis. *Shock.* 2000;13(5):379-85.
264. Metzger S, Nusair S, Planer D, Barash V, Pappo O, Shilyansky J, et al. Inhibition of hepatic gluconeogenesis and enhanced glucose uptake contribute to the development of hypoglycemia in mice bearing interleukin-1 β -secreting tumor. *Endocrinology.* 2004;145(11):5150-6.
265. Zeisel SH, da Costa K-A. Choline: an essential nutrient for public health. *Nutrition reviews.* 2009;67(11):615-23.
266. Al-Humadi A, Al-Humadi H, Liapi C. Novel insight on the impact of choline-deficiency in sepsis. *Annals of Research Hospitals.* 2019;3:12-.
267. McCann MR, George De la Rosa MV, Rosania GR, Stringer KA. L-Carnitine and Acylcarnitines: Mitochondrial Biomarkers for Precision Medicine. *Metabolites.* 2021;11(1).
268. Maas MN, Hintzen JCJ, Porzberg MRB, Mecinovic J. Trimethyllysine: From Carnitine Biosynthesis to Epigenetics. *Int J Mol Sci.* 2020;21(24).
269. Li XS, Wang Z, Cajka T, Buffa JA, Nemet I, Hurd AG, et al. Untargeted metabolomics identifies trimethyllysine, a TMAO-producing nutrient precursor, as a predictor of incident cardiovascular disease risk. *JCI Insight.* 2018;3(6).
270. Pelletier CC, Croyal M, Ene L, Aguesse A, Billon-Crossouard S, Krempf M, et al. Elevation of Trimethylamine-N-Oxide in Chronic Kidney Disease: Contribution of Decreased Glomerular Filtration Rate. *Toxins (Basel).* 2019;11(11).
271. Ploder M, Neurauter G, Spittler A, Schroecksadel K, Roth E, Fuchs D. Serum phenylalanine in patients post trauma and with sepsis correlate to neopterin concentrations. *Amino Acids.* 2008;35(2):303-7.
272. Peterson JW, Boldogh I, Popov VL, Saini SS, Chopra AK. Anti-inflammatory and antisecretory potential of histidine in *Salmonella*-challenged mouse small intestine. *Lab Invest.* 1998;78(5):523-34.
273. Long CL, Birkhahn RH, Geiger JW, Betts JE, Schiller WR, Blakemore WS. Urinary excretion of 3-methylhistidine: an assessment of muscle protein catabolism in adult normal

subjects and during malnutrition, sepsis, and skeletal trauma. *Metabolism*. 1981;30(8):765-76.

274. Kovarik M, Muthny T, Sispera L, Holecek M. Effects of beta-hydroxy-beta-methylbutyrate treatment in different types of skeletal muscle of intact and septic rats. *J Physiol Biochem*. 2010;66(4):311-9.

275. Goldman R. The clinical evaluation of renal function. *Calif Med*. 1956;85(6):376-80.

276. Pandey S, Adnan Siddiqui M, Azim A, Trigun SK, Sinha N. Serum metabolic profiles of septic shock patients based upon co-morbidities and other underlying conditions. *Mol Omics*. 2021.

277. Connelly MA, Otvos JD, Shalaurova I, Playford MP, Mehta NN. GlycA, a novel biomarker of systemic inflammation and cardiovascular disease risk. *J Transl Med*. 2017;15(1):219.

278. Otvos JD, Shalaurova I, Wolak-Dinsmore J, Connelly MA, Mackey RH, Stein JH, et al. GlycA: A Composite Nuclear Magnetic Resonance Biomarker of Systemic Inflammation. *Clin Chem*. 2015;61(5):714-23.

279. Ritchie SC, Kettunen J, Brozynska M, Nath AP, Havulinna AS, Mannisto S, et al. Elevated serum alpha-1 antitrypsin is a major component of GlycA-associated risk for future morbidity and mortality. *PLoS One*. 2019;14(10):e0223692.

280. Niwa T, Takeda N, Yoshizumi H. RNA metabolism in uremic patients: accumulation of modified ribonucleosides in uremic serum. Technical note. *Kidney Int*. 1998;53(6):1801-6.

281. Kirsanov K, Fetisov T, Antoshina E, Trukhanova L, Gor'kova T, Vlasova O, et al. Toxicological Properties of 7-Methylguanine, and Preliminary Data on its Anticancer Activity. *Front Pharmacol*. 2022;13:842316.

282. Dzúrik R, Lajdová I, Spustová V, Opatrný K, Jr. Pseudouridine excretion in healthy subjects and its accumulation in renal failure. *Nephron*. 1992;61(1):64-7.

283. Oliva-Damaso E, Oliva-Damaso N, Rodriguez-Esparragon F, Payan J, Baamonde-Laborda E, Gonzalez-Cabrera F, et al. Asymmetric (ADMA) and Symmetric (SDMA) Dimethylarginines in Chronic Kidney Disease: A Clinical Approach. *Int J Mol Sci*. 2019;20(15).

284. Baylis C. Arginine, arginine analogs and nitric oxide production in chronic kidney disease. *Nat Clin Pract Nephrol*. 2006;2(4):209-20.

285. Ramos CO, Campos KKD, Costa GP, Cangussú SD, Talvani A, Bezerra FS. Taurine treatment decreases inflammation and oxidative stress in lungs of adult mice exposed to cigarette smoke. *Regul Toxicol Pharmacol*. 2018;98:50-7.

286. Tsunekawa M, Wang S, Kato T, Yamashita T, Ma N. Taurine Administration Mitigates Cisplatin Induced Acute Nephrotoxicity by Decreasing DNA Damage and Inflammation: An Immunocytochemical Study. *Adv Exp Med Biol*. 2017;975 Pt 2:703-16.

287. Qaradakh T, Gadanec LK, McSweeney KR, Abraham JR, Apostolopoulos V, Zulli A. The Anti-Inflammatory Effect of Taurine on Cardiovascular Disease. *Nutrients*. 2020;12(9).
288. Ma Y, Zhang Y, Li R, Deng S, Qin Q, Ran C, et al. Mechanism of taurine reducing inflammation and organ injury in sepsis mice. *Cell Immunol*. 2022;375:104503.
289. Xu Y-J, Arneja A, Tappia P, Dhalla N. The potential health benefits of Taurine in cardiovascular disease. *Experimental and clinical cardiology*. 2008;13:57-65.
290. Supinski GS, Wang L, Schroder EA, Callahan LAP. Taurine administration ablates sepsis induced diaphragm weakness. *Respir Physiol Neurobiol*. 2020;271:103289.
291. Ping F, Guo Y, Cao Y, Shang J, Yao S, Zhang J, et al. Metabolomics Analysis of the Renal Cortex in Rats With Acute Kidney Injury Induced by Sepsis. *Front Mol Biosci*. 2019;6:152.
292. Chen J, Xue X, Cai J, Jia L, Sun B, Zhao W. Protective effect of taurine on sepsis-induced lung injury via inhibiting the p38/MAPK signaling pathway. *Mol Med Rep*. 2021;24(3).
293. Cheng F, Zhou Y, Wang M, Guo C, Cao Z, Zhang R, et al. A review of pharmacological and pharmacokinetic properties of stachydrine. *Pharmacol Res*. 2020;155:104755.
294. Amjad S, Nisar S, Bhat AA, Shah AR, Frenneaux MP, Fakhro K, et al. Role of NAD(+) in regulating cellular and metabolic signaling pathways. *Mol Metab*. 2021;49:101195.
295. Gallazzini M, Burg MB. What's new about osmotic regulation of glycerophosphocholine. *Physiology (Bethesda)*. 2009;24:245-9.
296. Pirillo A, Catapano AL, Norata GD. HDL in infectious diseases and sepsis. *Handb Exp Pharmacol*. 2015;224:483-508.
297. Fitzpatrick SF. Immunometabolism and Sepsis: A Role for HIF? *Front Mol Biosci*. 2019;6:85.
298. Noverr MC, Erb-Downward JR, Huffnagle GB. Production of eicosanoids and other oxylipins by pathogenic eukaryotic microbes. *Clin Microbiol Rev*. 2003;16(3):517-33.
299. Yoshikai Y. Roles of prostaglandins and leukotrienes in acute inflammation caused by bacterial infection. *Current Opinion in Infectious Diseases*. 2001;14(3).
300. Duvall MG, Levy BD. DHA- and EPA-derived resolvins, protectins, and maresins in airway inflammation. *Eur J Pharmacol*. 2016;785:144-55.
301. Lavy M, Gauttier V, Poirier N, Barille-Nion S, Blanquart C. Specialized Pro-Resolving Mediators Mitigate Cancer-Related Inflammation: Role of Tumor-Associated Macrophages and Therapeutic Opportunities. *Front Immunol*. 2021;12:702785.

302. Lin SH, Fan J, Zhu J, Zhao YS, Wang CJ, Zhang M, et al. Exploring plasma metabolomic changes in sepsis: a clinical matching study based on gas chromatography-mass spectrometry. *Ann Transl Med.* 2020;8(23):1568.
303. Amunugama K, Pike DP, Ford DA. The lipid biology of sepsis. *J Lipid Res.* 2021;62:100090.
304. Goñi FM, Alonso A. Sphingomyelinases: enzymology and membrane activity. *FEBS Lett.* 2002;531(1):38-46.
305. Opal SM. Endotoxins and other sepsis triggers. *Contrib Nephrol.* 2010;167:14-24.
306. Pfeiffer A, Böttcher A, Orsó E, Kapinsky M, Nagy P, Bodnár A, et al. Lipopolysaccharide and ceramide docking to CD14 provokes ligand-specific receptor clustering in rafts. *Eur J Immunol.* 2001;31(11):3153-64.
307. van der Veen JN, Kennelly JP, Wan S, Vance JE, Vance DE, Jacobs RL. The critical role of phosphatidylcholine and phosphatidylethanolamine metabolism in health and disease. *Biochim Biophys Acta Biomembr.* 2017;1859(9 Pt B):1558-72.
308. Tan ST, Ramesh T, Toh XR, Nguyen LN. Emerging roles of lysophospholipids in health and disease. *Prog Lipid Res.* 2020;80:101068.
309. Ahn WG, Jung JS, Kwon HY, Song DK. Alteration of Lysophosphatidylcholine-Related Metabolic Parameters in the Plasma of Mice with Experimental Sepsis. *Inflammation.* 2017;40(2):537-45.
310. Klein ME, Rieckmann M, Sedding D, Hause G, Meister A, Mader K, et al. Towards the Development of Long Circulating Phosphatidylserine (PS)- and Phosphatidylglycerol (PG)-Enriched Anti-Inflammatory Liposomes: Is PEGylation Effective? *Pharmaceutics.* 2021;13(2).
311. Noh J-Y, Lim K-M, Chung J-H. Phosphatidic Acid Enhances Procoagulant Activity and Thrombosis through Phosphatidylserine Exposure in Human Erythrocytes. *Blood.* 2008;112(11):3846.
312. Pei S, Xu C, Pei J, Bai R, Peng R, Li T, et al. Lysophosphatidic Acid Receptor 3 Suppress Neutrophil Extracellular Traps Production and Thrombosis During Sepsis. *Front Immunol.* 2022;13:844781.
313. Nikitopoulou I, Katsifa A, Kanellopoulou P, Jahaj E, Vassiliou AG, Mastora Z, et al. Autotaxin Has a Negative Role in Systemic Inflammation. *Int J Mol Sci.* 2022;23(14).
314. Sexton T, Chalhoub G, Ye S, Morris W, Annabathula R, Dugan A, et al. Autotaxin Activity Predicts 30-Day Mortality in Sepsis Patients and Correlates With Platelet Count and Vascular Dysfunction. *Shock.* 2020;54(6):738-43.
315. Barbayianni E, Kaffe E, Aidinis V, Kokotos G. Autotaxin, a secreted lysophospholipase D, as a promising therapeutic target in chronic inflammation and cancer. *Progress in Lipid Research.* 2015;58:76-96.

316. Ninou I, Magkrioti C, Aidinis V. Autotaxin in Pathophysiology and Pulmonary Fibrosis. *Frontiers in Medicine*. 2018;5.
 317. Geraldo LHM, Spohr T, Amaral RFD, Fonseca A, Garcia C, Mendes FA, et al. Role of lysophosphatidic acid and its receptors in health and disease: novel therapeutic strategies. *Signal Transduct Target Ther*. 2021;6(1):45.
 318. Vadas P, Scott K, Smith G, Rajkovic I, Stefanski E, Schouten BD, et al. Serum phospholipase A2 enzyme activity and immunoreactivity in a prospective analysis of patients with septic shock. *Life Sciences*. 1992;50(11):807-11.
 319. Vadas P. Elevated plasma phospholipase A2 levels: correlation with the hemodynamic and pulmonary changes in gram-negative septic shock. *J Lab Clin Med*. 1984;104(6):873-81.
 320. de la Llera Moya M, McGillicuddy FC, Hinkle CC, Byrne M, Joshi MR, Nguyen V, et al. Inflammation modulates human HDL composition and function in vivo. *Atherosclerosis*. 2012;222(2):390-4.
 321. Marques LR, Diniz TA, Antunes BM, Rossi FE, Caperuto EC, Lira FS, et al. Reverse Cholesterol Transport: Molecular Mechanisms and the Non-medical Approach to Enhance HDL Cholesterol. *Front Physiol*. 2018;9:526.
 322. Bayly GR. CHAPTER 37 - Lipids and disorders of lipoprotein metabolism. In: Marshall WJ, Lapsley M, Day AP, Ayling RM, editors. *Clinical Biochemistry: Metabolic and Clinical Aspects (Third Edition)*: Churchill Livingstone; 2014. p. 702-36.
 323. Pirahanchi Y, Anoruo M, Sharma S. *Biochemistry, Lipoprotein Lipase*. StatPearls. Treasure Island (FL): StatPearls Publishing
- Copyright © 2022, StatPearls Publishing LLC.; 2022.
324. Feingold KR. Introduction to Lipids and Lipoproteins. In: Feingold KR, Anawalt B, Boyce A, Chrousos G, de Herder WW, Dhatariya K, et al., editors. *Endotext*. South Dartmouth (MA): MDText.com, Inc.
- Copyright © 2000-2023, MDText.com, Inc.; 2000.
325. Olofsson SO, Boren J. Apolipoprotein B: a clinically important apolipoprotein which assembles atherogenic lipoproteins and promotes the development of atherosclerosis. *J Intern Med*. 2005;258(5):395-410.
 326. Vreugdenhil AC, Snoek AM, van 't Veer C, Greve JW, Buurman WA. LPS-binding protein circulates in association with apoB-containing lipoproteins and enhances endotoxin-LDL/VLDL interaction. *J Clin Invest*. 2001;107(2):225-34.
 327. Cox RA, García-Palmieri MR. Cholesterol, Triglycerides, and Associated Lipoproteins. In: Walker HK, Hall WD, Hurst JW, editors. *Clinical Methods: The History, Physical, and Laboratory Examinations*. Boston: Butterworths

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328. Gerl MJ, Vaz WLC, Domingues N, Klose C, Surma MA, Sampaio JL, et al. Cholesterol is Inefficiently Converted to Cholesteryl Esters in the Blood of Cardiovascular Disease Patients. *Scientific Reports*. 2018;8(1).
329. Blanc M, Hsieh WY, Robertson KA, Watterson S, Shui G, Lacaze P, et al. Host defense against viral infection involves interferon mediated down-regulation of sterol biosynthesis. *PLoS Biol*. 2011;9(3):e1000598.
330. Mackenzie JM, Khromykh AA, Parton RG. Cholesterol manipulation by West Nile virus perturbs the cellular immune response. *Cell Host Microbe*. 2007;2(4):229-39.
331. Petersen J, Drake MJ, Bruce EA, Riblett AM, Didigu CA, Wilen CB, et al. The major cellular sterol regulatory pathway is required for Andes virus infection. *PLoS Pathog*. 2014;10(2):e1003911.
332. Nessler N, Launey Y, Aninat C, Morel F, Mallédant Y, Seguin P. Clinical review: The liver in sepsis. *Critical care (London, England)*. 2012;16(5):235-.
333. Trinder M, Genga KR, Kong HJ, Blauw LL, Lo C, Li X, et al. Cholesteryl Ester Transfer Protein Influences High-Density Lipoprotein Levels and Survival in Sepsis. *Am J Respir Crit Care Med*. 2019;199(7):854-62.
334. Oliveira HC, de Faria EC. Cholesteryl ester transfer protein: the controversial relation to atherosclerosis and emerging new biological roles. *IUBMB Life*. 2011;63(4):248-57.
335. Venancio TM, Machado RM, Castoldi A, Amano MT, Nunes VS, Quintao EC, et al. CETP Lowers TLR4 Expression Which Attenuates the Inflammatory Response Induced by LPS and Polymicrobial Sepsis. *Mediators Inflamm*. 2016;2016:1784014.
336. Chang TY, Li BL, Chang CC, Urano Y. Acyl-coenzyme A:cholesterol acyltransferases. *Am J Physiol Endocrinol Metab*. 2009;297(1):E1-9.
337. Edwards M, Mohiuddin SS. Biochemistry, Lipolysis. *StatPearls*. Treasure Island (FL)2022.
338. Chiarla C, Giovannini I, Giuliani F, Zadak Z, Vellone M, Ardito F, et al. Severe hypocholesterolemia in surgical patients, sepsis, and critical illness. *J Crit Care*. 2010;25(2):361 e7- e12.
339. Fouque D, Holt S, Guebre-Egziabher F, Nakamura K, Vianey-Saban C, Hadj-Aïssa A, et al. Relationship between serum carnitine, acylcarnitines, and renal function in patients with chronic renal disease. *J Ren Nutr*. 2006;16(2):125-31.
340. Balestrieri B, Di Costanzo D, Dwyer DF. Macrophage-Mediated Immune Responses: From Fatty Acids to Oxylipins. *Molecules (Basel, Switzerland)*. 2021;27(1):152.

341. Jha AK, Huang SC, Sergushichev A, Lampropoulou V, Ivanova Y, Loginicheva E, et al. Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization. *Immunity*. 2015;42(3):419-30.
342. Okuno T, Gijón MA, Zarini S, Martin SA, Barkley RM, Johnson CA, et al. Altered eicosanoid production and phospholipid remodeling during cell culture. *J Lipid Res*. 2018;59(3):542-9.
343. Chatterjee S, Balram A, Li W. Convergence: Lactosylceramide-Centric Signaling Pathways Induce Inflammation, Oxidative Stress, and Other Phenotypic Outcomes. *Int J Mol Sci*. 2021;22(4).
344. Andrieu-Abadie N, Levade T. Sphingomyelin hydrolysis during apoptosis. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*. 2002;1585(2):126-34.
345. Goyert SM, Ferrero EM, Seremetis SV, Winchester RJ, Silver J, Mattison AC. Biochemistry and expression of myelomonocytic antigens. *J Immunol*. 1986;137(12):3909-14.
346. Mahnke K, Becher E, Ricciardi-Castagnoli P, Luger TA, Schwarz T, Grabbe S. CD14 is expressed by subsets of murine dendritic cells and upregulated by lipopolysaccharide. *Adv Exp Med Biol*. 1997;417:145-59.
347. Zanoni I, Granucci F. Role of CD14 in host protection against infections and in metabolism regulation. *Front Cell Infect Microbiol*. 2013;3:32.
348. Schumann RR, Kirschning CJ, Unbehauen A, Aberle HP, Knope HP, Lamping N, et al. The lipopolysaccharide-binding protein is a secretory class 1 acute-phase protein whose gene is transcriptionally activated by APRF/STAT/3 and other cytokine-inducible nuclear proteins. *Mol Cell Biol*. 1996;16(7):3490-503.
349. Grassi S, Mauri L, Prioni S, Cabitta L, Sonnino S, Prinetti A, et al. Sphingosine 1-Phosphate Receptors and Metabolic Enzymes as Druggable Targets for Brain Diseases. *Front Pharmacol*. 2019;10:807.
350. Schweiger M, Eichmann TO, Taschler U, Zimmermann R, Zechner R, Lass A. Measurement of lipolysis. *Methods Enzymol*. 2014;538:171-93.
351. van Dierendonck X, Vrieling F, Smeehuijzen L, Deng L, Boogaard JP, Croes CA, et al. Triglyceride breakdown from lipid droplets regulates the inflammatory response in macrophages. *Proc Natl Acad Sci U S A*. 2022;119(12):e2114739119.
352. Vaughan M, Berger JE, Steinberg D. HORMONE-SENSITIVE LIPASE AND MONOGLYCERIDE LIPASE ACTIVITIES IN ADIPOSE TISSUE. *J Biol Chem*. 1964;239:401-9.
353. Berbee JF, Havekes LM, Rensen PC. Apolipoproteins modulate the inflammatory response to lipopolysaccharide. *J Endotoxin Res*. 2005;11(2):97-103.

354. Hofmaenner DA, Kleyman A, Press A, Bauer M, Singer M. The Many Roles of Cholesterol in Sepsis: A Review. *American journal of respiratory and critical care medicine*. 2022;205(4):388-96.
355. Correa R, Silva LFF, Ribeiro DJS, Almeida RDN, Santos IO, Correa LH, et al. Lysophosphatidylcholine Induces NLRP3 Inflammasome-Mediated Foam Cell Formation and Pyroptosis in Human Monocytes and Endothelial Cells. *Front Immunol*. 2019;10:2927.
356. Yan JJ, Jung JS, Lee JE, Lee J, Huh SO, Kim HS, et al. Therapeutic effects of lysophosphatidylcholine in experimental sepsis. *Nature Medicine*. 2004;10(2):161-7.
357. Smani Y, Domínguez-Herrera J, Ibáñez-Martínez J, Pachóna J. Therapeutic efficacy of lysophosphatidylcholine in severe infections caused by *Acinetobacter baumannii*. *Antimicrobial Agents and Chemotherapy*. 2015;59(7):3920-4.
358. Murch O, Collin M, Sepodes B, Foster SJ, Mota-Filipe H, Thiernemann C. Lysophosphatidylcholine reduces the organ injury and dysfunction in rodent models of Gram-negative and Gram-positive shock. *British Journal of Pharmacology*. 2006;148(6):769-77.
359. Parra Millán R, Jiménez Mejías ME, Sánchez Encinales V, Ayerbe Algaba R, Gutiérrez Valencia A, Pachón Ibáñez ME, et al. Efficacy of lysophosphatidylcholine in combination with antimicrobial agents against *Acinetobacter baumannii* in experimental murine peritoneal sepsis and pneumonia models. *Antimicrobial Agents and Chemotherapy*. 2016;60(8):4464-70.
360. Makide K, Kitamura H, Sato Y, Okutani M, Aoki J. Emerging lysophospholipid mediators, lysophosphatidylserine, lysophosphatidylthreonine, lysophosphatidylethanolamine and lysophosphatidylglycerol. *Prostaglandins Other Lipid Mediat*. 2009;89(3-4):135-9.
361. Emancipator K, Csako G, Elin RJ. In vitro inactivation of bacterial endotoxin by human lipoproteins and apolipoproteins. *Infection and Immunity*. 1992;60(2):596-601.
362. Holeček M. Branched-chain amino acids in health and disease: metabolism, alterations in blood plasma, and as supplements. *Nutr Metab (Lond)*. 2018;15:33.
363. Tapiero H, Mathé G, Couvreur P, Tew KD. II. Glutamine and glutamate. *Biomedicine & Pharmacotherapy*. 2002;56(9):446-57.
364. Razak MA, Begum PS, Viswanath B, Rajagopal S. Multifarious Beneficial Effect of Nonessential Amino Acid, Glycine: A Review. *Oxidative Medicine and Cellular Longevity*. 2017;2017:1716701.
365. Trongtrakul K, Thonusin C, Pothirat C, Chattipakorn SC, Chattipakorn N. Past Experiences for Future Applications of Metabolomics in Critically Ill Patients with Sepsis and Septic Shocks. *Metabolites*. 2021;12(1).

366. Zhang Y, Yu W, Han D, Meng J, Wang H, Cao G. L-lysine ameliorates sepsis-induced acute lung injury in a lipopolysaccharide-induced mouse model. *Biomed Pharmacother.* 2019;118:109307.
 367. Schlesinger S, Sonntag SR, Lieb W, Maas R. Asymmetric and Symmetric Dimethylarginine as Risk Markers for Total Mortality and Cardiovascular Outcomes: A Systematic Review and Meta-Analysis of Prospective Studies. *PLoS One.* 2016;11(11):e0165811.
 368. Kielstein JT, Impraim B, Simmel S, Bode-Böger SM, Tsikas D, Frölich JC, et al. Cardiovascular effects of systemic nitric oxide synthase inhibition with asymmetrical dimethylarginine in humans. *Circulation.* 2004;109(2):172-7.
 369. Bocchi EA, Vilella de Moraes AV, Esteves-Filho A, Bacal F, Auler JO, Carmona MJ, et al. L-arginine reduces heart rate and improves hemodynamics in severe congestive heart failure. *Clin Cardiol.* 2000;23(3):205-10.
 370. Fedorowicz A, Mateuszuk L, Kopec G, Skorka T, Kutryb-Zajac B, Zakrzewska A, et al. Activation of the nicotinamide N-methyltransferase (NNMT)-1-methylnicotinamide (MNA) pathway in pulmonary hypertension. *Respir Res.* 2016;17(1):108.
 371. Lau KE, Lui F. *Physiology, Prostaglandin I2*. StatPearls. Treasure Island (FL): StatPearls Publishing
- Copyright © 2023, StatPearls Publishing LLC.; 2023.
372. Rutkowski B, Slominska E, Szolkiewicz M, Smolenski RT, Striley C, Rutkowski P, et al. N-methyl-2-pyridone-5-carboxamide: a novel uremic toxin? *Kidney Int Suppl.* 2003(84):S19-21.
 373. Rutkowski B, Rutkowski P, Słomińska E, Smolenski RT, Swierczyński J. Cellular toxicity of nicotinamide metabolites. *J Ren Nutr.* 2012;22(1):95-7.
 374. Knottnerus SJG, Bleeker JC, Wüst RCI, Ferdinandusse S, Ijlst L, Wijburg FA, et al. Disorders of mitochondrial long-chain fatty acid oxidation and the carnitine shuttle. *Reviews in Endocrine and Metabolic Disorders.* 2018;19(1):93-106.
 375. El-Gharbawy A, Vockley J. Inborn Errors of Metabolism with Myopathy: Defects of Fatty Acid Oxidation and the Carnitine Shuttle System. *Pediatr Clin North Am.* 2018;65(2):317-35.
 376. Zhang Y, Wang Y, Ke B, Du J. TMAO: how gut microbiota contributes to heart failure. *Transl Res.* 2021;228:109-25.
 377. Schonfeld P, Wojtczak L. Short- and medium-chain fatty acids in energy metabolism: the cellular perspective. *J Lipid Res.* 2016;57(6):943-54.
 378. Tvrzicka E, Kremmyda LS, Stankova B, Zak A. Fatty acids as biocompounds: their role in human metabolism, health and disease--a review. Part 1: classification, dietary sources

and biological functions. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2011;155(2):117-30.

379. Obinata H, Hla T. Sphingosine 1-phosphate in coagulation and inflammation. Semin Immunopathol. 2012;34(1):73-91.

380. Mücke VT, Maria Schwarzkopf K, Thomas D, Mücke MM, Rüschbaum S, Trebicka J, et al. Serum Sphingosine-1-Phosphate Is Decreased in Patients With Acute-on-Chronic Liver Failure and Predicts Early Mortality. Hepatology Communications. 2020;4(10):1477-86.

9. Appendices

Appendix 1.1 List of all the metabolites from NMR and UHPLC-MS targeted dataset

Metabolites (Small molecules, lipids and lipoproteins)		
Ethanol	H3FC	IDPN
Alanine	H4FC	LDPN
Symmetric Asymmetric Dimethylarginine	L1FC	TBPN
Arginine	L2FC	VLPN
Phenylalanine	L3FC	L1PN
Tyrosine	L4FC	L2PN
Isoleucine	L5FC	L3PN
Leucine	L6FC	L4PN
Valine	V1FC	L5PN
NMR_Creatine	V2FC	L6PN
HPOS_Creatine	V3FC	H1A1
NMR_Creatinine	V4FC	H2A1
HPOS_Creatinine	V5FC	H3A1
Glutamine	CE(20:4)	H4A1
Glycine	CE(20:5)	HDA1
N,N-Dimethylglycine	Capric acid FA(10:0)	TPA1
HPOS_Lysine	Lauric acid FA(12:0)	H1A2
NMR_Lysine	Hexadecenoic acid FA(16:1)	H2A2
N6,N6,N6-Trimethyl-L-lysine	Heptadecenoic acid FA(17:1)	H3A2
N-methyl pipecolic acid	octadecenoic acid FA(18:1)	H4A2
Pipecolic acid N-methyl proline	Octadecadienoic acid FA(18:2)	HDA2
Proline	Octadecatrienoic acid FA(18:3)_1	TPA2
Proline Betaine	Octadecatrienoic acid FA(18:3)_2	IDAB
Betaine	Octadecatetraenoic acid FA(18:4)	LDAB
Histidine	Eicosenoic acid FA(20:1)	TPAB
Urocanic acid	Eicosadienoic acid FA(20:2)	VLAB
N1-Acetylspermidine	Eicosatrienoic acid FA(20:3)	L1AB
Acetic acid	Eicosatetraenoic acid FA(20:4)	L2AB
Citric acid	Eicosapentaenoic acid FA(20:5)	L3AB
Formic acid	Docosatetraenoic acid FA(22:4)	L4AB
Lactic acid	Docosapentaenoic acid FA(22:5)_1	L5AB
Acetaminophen	Docosapentaenoic acid FA(22:5)_2	L6AB
Cortisol	Docosahexaenoic FA(22:6)	Bilirubin
Tetradecenoylcarnitine CAR(14:1)	Lignoceric acid FA(24:0)	Cholesterol
HPOS_Decanoylcarnitine CAR (C10:0)	Tetracosenoic acid FA(24:1)	Cholesterol Sulfate_2
HPOS_Palmitoylcarnitine CAR (C16:0)	Tetracosatetraenoic acid FA(24:4)	HDCH
Stearoylcarnitine CAR(18:0)	Tetracosapentaenoic acid FA(24:5)	IDCH
Oleoylcarnitine CAR(18:1)	Hexacosanoic acid FA(26:0)	LDCH
Hexadecenoylcarnitine CAR(16:1)	Octanoylcarnitine CAR(8:0)	TPCH
3-Hydroxybutyrylcarnitine CAR (C4:0-OH)	Decanoylcarnitine CAR(10:0)	VLCH
a-glycerophosphocholine	Hydroxydodecanoylcarnitine CAR(12:0-OH)	H1CH
3-Hydroxybutyric acid	Hydroxydodecenoylcarnitine CAR(12:1-OH)	H2CH
Acetone	Hydroxytetradecanoylcarnitine	H3CH

	CAR(14:0-OH)	
Trimethylaminoacetone	Tetradecadienoylcarnitine CAR(14:2)	H4CH
Pseudouridine	Palmitoylcarnitine CAR(16:0)	L1CH
N2,N2-Dimethylguanosine	Hydroxypalmitoleoylcarnitine CAR(16:1-OH)	L2CH
1-Methyladenosine	Hexadecadienoylcarnitine CAR(16:2)	L3CH
Hypoxanthine	Carboxyheptadecanoylcarnitine CAR(18:0-DC)	L4CH
7-Methylguanine	Carboxyheptadecenoylcarnitine CAR(18:1-DC)	L5CH
Carnitine	Arachidyl carnitine CAR(20:0)	L6CH
Choline	Eicoseneoylcarnitine CAR(20:1)	V1CH
Glucose	Eicosadienoylcarnitine CAR(20:2)	V2CH
Sucrose	Eicosatrienoylcarnitine CAR(20:3)	V3CH
Dimethyl sulfone	Arachidonoylcarnitine CAR(20:4)	V4CH
Taurine	Tetracosanoylcarnitine CAR(24:0)	V5CH
Pantothenic acid	Hexacosanoylcarnitine CAR(26:0)	HDFC
Niacinamide	Hexacosenoylcarnitine CAR(26:1)	IDFC
N1-methyl-2-pyridone-5-carboxamide	PA(16:0/18:1)	LDFC
N1-methyl-4-pyridone-3-carboxamide	PA(36:2);PA(18:0/18:2) PA(18:1/18:1)	VLFC
1-Methylnicotinamide	PA(O-18:1/20:4) and/or PA(P-18:0/20:4)	H1FC
Trigonelline	LPA(22:6/0:0)	H2FC
PC(14:0/18:2)	LPC(0:0/22:6)	LPE(22:6/0:0)
PC(14:0/20:4)	LPC(14:0/0:0)	LPE(P-18:0/0:0)
PC(14:0/22:6)	LPC(15:0/0:0)	LPG(18:0/0:0)
PC(15:0/18:2)	LPC(16:0/0:0)	LPG(18:1/0:0)
PC(16:0/16:0)	LPC(16:1/0:0)	LPG(18:2/0:0)
PC(16:0/17:0)	LPC(17:0/0:0)	LPI(16:1/0:0)
PC(16:0/18:1)_2	LPC(17:1/0:0)	LPI(18:0/0:0)
PC(16:0/18:2)	LPC(18:0/0:0)	LPI(18:1/0:0)_1
PC(16:0/18:3)	LPC(18:1/0:0)_1	LPI(18:2/0:0)
PC(16:0/20:4)_1	LPC(18:2/0:0)	LPI(20:3/0:0)_1
PC(16:0/20:4)_3	LPC(18:3/0:0)_1	LPI(20:3/0:0)_2
PC(16:0/20:5)	LPC(18:3/0:0)_2	LPI(20:4/0:0)
PC(16:0/22:4)	LPC(19:0/0:0)	LPI(22:5/0:0)
PC(16:0/22:6)	LPC(20:0/0:0)	LPS(18:0/0:0)
PC(18:0/18:0)	LPC(20:1/0:0)	HDPL
PC(18:0/18:1)	LPC(20:2/0:0)	IDPL
PC(18:0/18:2)	LPC(20:3/0:0)_1	LDPL
PC(18:0/20:3)	LPC(20:3/0:0)_2	VLPL
PC(18:0/20:4)	LPC(20:4/0:0)	H1PL
PC(18:0/22:4)	LPC(20:5/0:0)	H2PL
PC(18:0/22:5)	LPC(22:4/0:0)	H3PL
PC(18:0/22:6)	LPC(22:5/0:0)_1	H4PL
PC(18:1/20:4)	LPC(22:5/0:0)_2	L1PL
PC(32:1); PC(14:0/18:1) PC(16:0/16:1)	LPC(22:6/0:0)	L2PL
PC(33:1); PC(15:0/18:1) PC(16:0/17:1)	LPC(24:0/0:0)	L3PL
PC(34:0); PC(18:0/16:0) PC(16:0/18:0)	LPC(O-16:0/0:0)	L4PL
PC(40:3)	LPC(O-18:0/0:0)	L5PL
PC(O-16:0/14:0)	LPC(O-18:1/0:0)_1	L6PL
PC(O-16:0/15:0)	LPC(O-24:0/0:0)	V1PL
PC(O-16:0/16:0)	LPC(O-24:1/0:0)	V2PL

PC(O-16:0/18:2)	LPC(P-16:0/0:0)	V3PL
PC(O-16:1/18:2) and/or PC(P-16:0/18:2)	LPC(P-18:0/0:0)	V4PL
PC(O-16:1/22:6) and/or PC(P-16:0/22:6)	PE(16:0/18:1)	V5PL
PC(O-18:0/16:0)	PE(16:0/18:2)	Sphingosine-1-phosphate
PC(O-18:0/20:3)	PE(16:0/20:4)	Cer(d18:1/16:0)
PC(O-18:0/20:4)	PE(18:0/18:2)	Cer(d18:1/18:0)
PC(O-18:0/22:4)	PE(18:0/20:3)	Cer(d18:1/20:0)
PC(O-18:1/18:1) and/or PC(P-18:0/18:1)	PE(18:0/20:4)	Cer(d18:1/22:0)
PC(O-18:1/22:6) and/or PC(P-18:0/22:6)	PE(18:0/22:4)	Cer(d18:1/24:0)
PC(O-22:0/18:2)	PE(18:0/22:5)	Cer(d18:1/24:1)
PC(O-22:0/20:3)	PE(18:0/22:6)	Cer(d18:2/22:0)
PC(O-24:0/18:2)	PE(36:1); PE(18:0/18:1) PE(18:1/18:0)	Cer(d18:2/24:0)
PC(O-24:0/20:4)	PE(O-16:1/18:2) and/or PE(P-16:0/18:2)	Cer(d34:0); Cer(d18:0/16:0) Cer(d16:0/18:0)
PC(O-24:1/20:3) and/or PC(P-24:0/20:3)	PE(O-16:1/20:4) and/or PE(P-16:0/20:4)	Cer(d41:1); Cer(d18:1/23:0) Cer(d17:1/24:0)
PC(O-24:1/20:4) and/or PC(P-24:0/20:4)	PE(O-16:1/22:5) and/or PE(P-16:0/22:5)	Cer(d42:3); Cer(d18:2/24:1) Cer(d18:1/24:2)
PC(O-24:1/22:4) and/or PC(P-24:0/22:4)	PE(O-16:1/22:6) and/or PE(P-16:0/22:6)	Cer(t18:0/24:1)
PC(O-24:2/20:4) and/or PC(P-24:1/20:4)	PE(O-18:1/18:1) and/or PE(P-18:0/18:1)	HexCer(d18:1/16:0)
PC(O-26:1/20:4) and/or PC(P-26:0/20:4)	PE(O-18:1/18:2) and/or PE(P-18:0/18:2)	HexCer(d18:1/22:0)
LPC(0:0/14:0)	PE(O-18:1/20:4) and/or PE(P-18:0/20:4)	HexCer(d18:1/23:0)
LPC(0:0/15:0)	PE(O-18:1/22:6) and/or PE(P-18:0/22:6)	HexCer(d18:1/24:0)
LPC(0:0/16:0)	PE(O-20:1/20:4) and/or PE(P-20:0/20:4)	SulfoHexCer(d18:1/16:0(OH))
LPC(0:0/16:1)	LPE(0:0/18:0)	SulfoHexCer(d18:1/24:0(OH))
LPC(0:0/18:0)	LPE(0:0/18:1)	SulfoHexCer(d18:1/24:1(OH))
LPC(0:0/18:1)	LPE(0:0/18:2)	SulfoHexCer(d18:2/16:0(OH))
LPC(0:0/18:2)	LPE(0:0/20:4)	LacCer(d18:1/16:0)
LPC(0:0/18:3)	LPE(0:0/22:6)	LacCer(d42:3)
LPC(0:0/20:2)	LPE(16:0/0:0)	SM(d16:1/22:0)
LPC(0:0/20:3)	LPE(18:0/0:0)	SM(d17:1/16:0)
LPC(0:0/20:4)	LPE(18:1/0:0)_1	SM(d17:1/24:1)
LPC(0:0/20:5)	LPE(18:2/0:0)	SM(d18:0/16:0)
LPC(0:0/22:5)_1	LPE(20:4/0:0)	SM(d18:1/16:0)
SM(d18:1/18:0)	TG(51:2)	IDTG
SM(d18:1/20:0)	TG(51:3)	LDTG
SM(d18:1/20:1)	TG(51:4)	VLTG
SM(d18:1/22:0)	TG(52:1)	H1TG
SM(d18:1/23:0)	TG(52:2)	H2TG
SM(d18:1/24:0)	TG(52:3)	H3TG
SM(d18:1/24:1)	TG(52:4)_1	H4TG
SM(d18:2/14:0)	TG(52:5)_1	L1TG
SM(d18:2/16:0)_1	TG(53:1)	L2TG
SM(d18:2/18:0)	TG(53:2)	L3TG
SM(d18:2/22:0)	TG(53:3)	L4TG
SM(d18:2/23:0)	TG(53:4)	L5TG
SM(d18:2/24:0)	TG(53:5)	L6TG

SM(d18:2/24:1)	TG(54:1)	V1TG
SM(d19:1/18:0)	TG(54:2)	V2TG
SM(d30:1); SM(d16:1/14:0) SM(d18:1/12:0)	TG(54:3)	V3TG
SM(d32:1); SM(d16:1/16:0) SM(d18:1/14:0)	TG(54:4)	V4TG
SM(d35:1); SM(d17:1/18:0) SM(d18:1/17:0)	TG(54:5)_1	V5TG
TG(42:1)	TG(54:5)_2	DG(16:0/16:0)
TG(44:1)	TG(54:6)_1	DG(16:0/18:1)
TG(44:2)	TG(54:7)_1	DG(18:0/16:0)
TG(44:3)	TG(55:2)	DG(18:0/18:1)
TG(46:0)	TG(55:4)	DG(18:2/18:1)
TG(46:1)	TG(56:1)	DG(32:1)
TG(46:2)	TG(56:2)	DG(34:2)
TG(46:3)	TG(56:3)	DG(36:2); DG(18:1/18:1) DG(18:0/18:2)
TG(48:0)	TG(56:4)	DG(36:4)_1; DG(18:2/18:2) DG(18:1/18:3)
TG(48:1)	TG(56:5)_2	MG(16:0)
TG(48:2)	TG(56:6)_1	MG(18:1)_1
TG(48:3)	TG(58:1)	MG(18:1)_2
TG(48:4)_1	TG(58:6)_2	Delta-Tocopherol
TG(49:1)	TG(58:7)_2	Beta-cryptoxanthin
TG(49:2)	TG(58:8)	Alpha-carotene Beta-carotene
TG(49:3)	TG(58:9)_1	Zeta-carotene
TG(50:1)	TG(60:8)	Luteine
TG(50:2)	TG(60:9)	Anhydrolutein I
TG(50:3)	TPTG	Zeaxanthin
TG(50:4)	HDTG	Retinol

Appendix 1.2 Script for generating LME models of targeted data.

1) Mortality model

```
Outcome_model_withoutBMI <- lmer(log(Metabolite + 1) ~ 1 + as.numeric(`Time post TP0 (days)`)*`Mortality at Day 29` + `Age` + `Sex` + (as.numeric(`Time post TP0 (days)`)|`Sample Number`) + (1|`Sitename`), data=combinedData, REML=TRUE) # Model without BMI
```

```
Outcome_model_withBMI <- lmer(log(Metabolite + 1) ~ 1 + as.numeric(`Time post TP0 (days)`)*`Mortality at Day 29` + `Age` + `Sex` + `BMI` + (as.numeric(`Time post TP0 (days)`)|`Sample Number`) + (1|`Sitename`), data=combinedData, REML=TRUE) # Model with BMI
```

```
time_trend <- emtrends(Mortality_model_withoutBMI, pairwise ~ `Mortality at Day 29`, var="Time post TP0 (days)", adjust='none')
```

```
time_means <- emmeans(Mortality_model_withoutBMI, pairwise ~ `Time post TP0 (days)`*`Mortality at Day 29`, at=list(`Time post TP0 (days)`=c(0, 4)), adjust='none')
```

2) Noradrenaline vs Vasopressin

```
Drug1_model <- lmer(log(`Metabolite` + 1) ~ 1 + as.numeric(`Time post TP0 (days)`)*`Mortality at Day 29` + `Drug1_Administered` + as.numeric(`Time post TP0 (days)`)*`Drug1` + `Hydrocortisone` + `Placebo` + `Age` + `Sex` + (as.numeric(`Time post TP0 (days)`)|`Sample Number`) + (1|`Sitename`), data=combinedData, REML=TRUE)
```

```
Drug1_time_trend <- emtrends(Drug1_model, pairwise ~ `Drug1`, var="Time post TP0 (days)", adjust='none')
```

```
Drug1_time_means <- emmeans(Drug1_model, pairwise ~ `Time post TP0 (days)`*`Drug1`, at=list(`Time post TP0 (days)`=c(0, 1, 4)), adjust='none')
```

3) Drug2 model (Hydrocortisone vs Placebo)

Metabolic changes with drug 2

```
Drug2_model1 <- lmer(log(`Metabolite` + 1) ~ 1 + as.numeric(`Time post TP0 (days)`)*`Mortality at Day 29` + `Drug2` + `Age` + `Sex` + (as.numeric(`Time post TP0 (days)`)|`Sample Number`) + (1|`Sitename`), data=combinedData, REML=TRUE)
```

```
Drug2_emmeans_model1 <- emmeans(Drug2_model1, pairwise ~ `Drug2`, at=list(`Drug 2` = c("Pre", "Placebo", "Hydrocortisone")), adjust='none')
```

Metabolic trajectories for drug 2

```
Drug2_model2 <- lmer(log(`Metabolite` + 1) ~ 1 + as.numeric(`Time post TP0 (days)`)*`Mortality at Day 29` + as.numeric(`Time post TP0 (days)`)*`Drug2` + `Age` + `Sex` + (as.numeric(`Time post TP0 (days)`)|`Sample Number`) + (1|`Sitename`), data=combinedData, REML=TRUE)
```

```
Drug2_trends_model2 <- emtrends(Drug2_model2 , pairwise ~ `Drug2`, var = "Time post TP0 (days)", adjust='none')
```

```
Drug2_emmeans_model2 <- emmeans(Drug2_model2 , pairwise ~ `Drug2.PP`, at=list(`Drug2` = c("Pre", "Placebo", "Hydrocortisone")), adjust='none')
```

4) Drug2 model with Day29 mortality

```
Drug2 _model_withmortality <- lmer(log(`Metabolite` + 1) ~ 1 + as.numeric(`Time post TP0 (days)`)*`Mortality at Day 29` + `Drug2` + as.numeric(`Time post TP0 (days)`):`Drug2` + as.numeric(`Time post TP0 (days)`):`Mortality at Day 29` + `Drug2` + `Age` + `Sex` + (as.numeric(`Time post TP0 (days)`)|`Sample Number`) + (1|`SITENAME`), data=combinedData, REML=TRUE)
```

```
Day29mortality_Drug2_trends_method2 <- emtrends(Drug2 _model_withmortality , pairwise ~ `Mortality at Day 29` + `Drug2.PP`, var="Time post TP0 (days)", adjust='none')
```

```
Day29mortality_Drug2_emmeans_method2 <- emmeans(Drug2 _model_withmortality , pairwise ~ `Drug2.PP` + `Mortality at Day 29`, at=list(`Time post TP0 (days)`=c(2)), adjust='none')
```

5) SOFA score model

```
SOFA_model <- lmer(log(`Metabolite` + 1) ~ 1 + (as.numeric(`Time post TP0 (days)`)) + `Total SOFA Score` + `Age` + `Sex` + (as.numeric(`Time post TP0 (days)`)|`Sample Number`) + (1|`Sitename`), data = combinedData, REML=TRUE)
```

```
SOFA_trend <- emtrends(SOFA_model , identity ~ `Total SOFA Score`, var="Total SOFA Score", adjust='none')
```

Appendix 1.3 Trajectory analysis focusing on outcome (Small molecules)

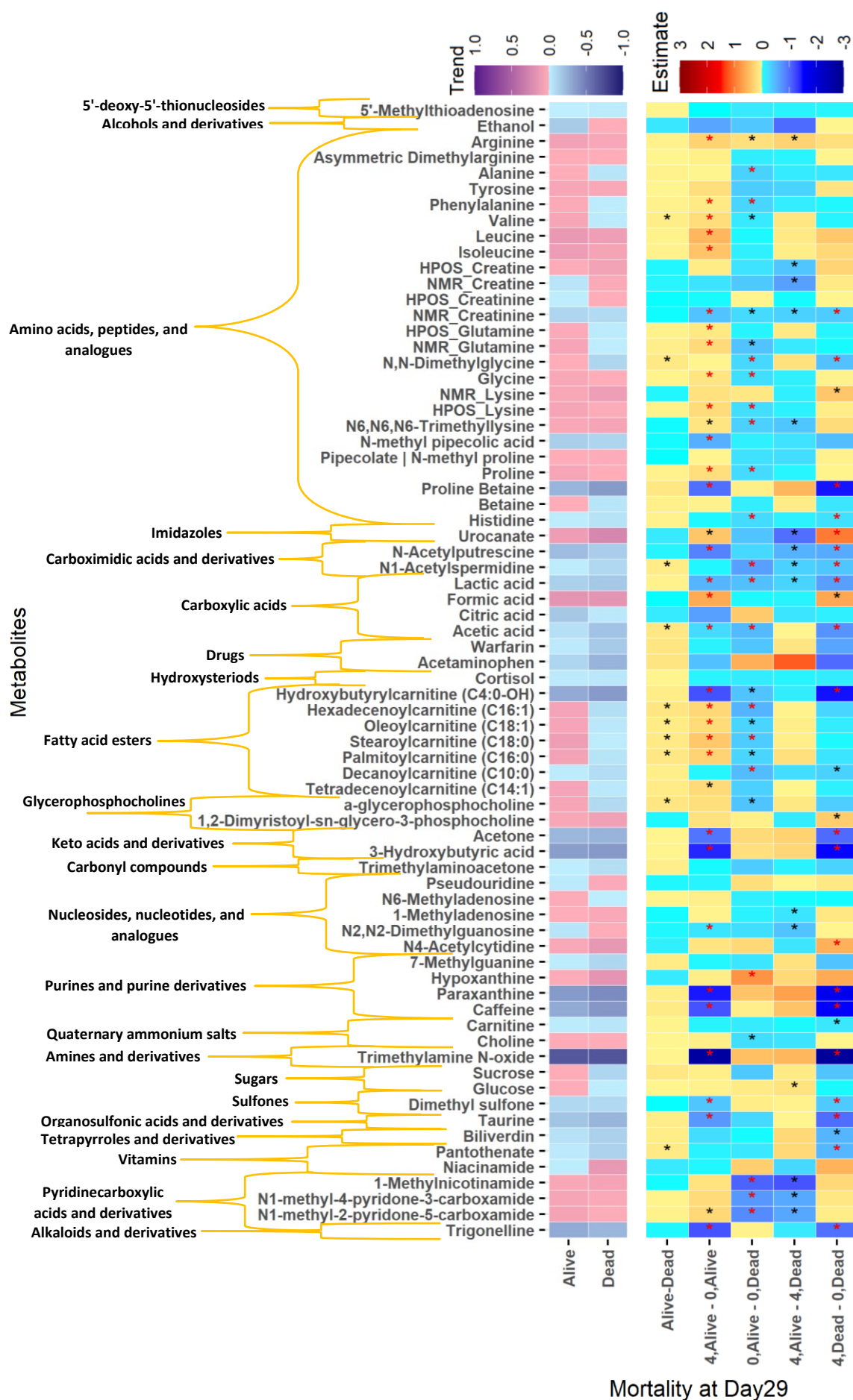


Figure A1.1: The results of LME models focusing on mortality for combined SMoIESY-select and HPOS ppR log transformed data with age and sex as confounders for VANISH. Various contrasts were performed via contrast coding during the trajectory analysis and their outcomes are shown in this figure. LME model outputs including slope (i.e., change in metabolite levels over time), estimate (i.e., the difference between the estimated marginal mean levels of metabolites) are presented in different colour scales. Metabolites which are significantly different between groups from various pairwise comparisons are highlighted in black and red asterisks. Metabolites that were only significant before correction for multiple comparisons are shown with a black asterisk whilst those that remained significant after correction are shown in red. The slopes for alive (i.e., survivors) and dead (i.e., non-survivors) are shown as well as the difference between their slopes and whether this difference in the overall slopes is significant between the two groups. The scale for slope goes from positive to negative. Positive values for slope indicate that the metabolites increase with time whilst the negative values indicate that the metabolites decrease over time and the stronger the colour the bigger the magnitude for both positive and negative slopes. The difference between the slopes for alive and dead is presented in different colour scale (i.e., estimate). Estimated marginal means were also calculated for each group at days 0 and day 4 using the LME model and the difference between the EMMs for four pairwise comparisons are presented here. The four pairwise comparisons include: 4,Alive-0,Alive = Difference between the EMMs of alive at day 4 and day 0 based on the LME model; 4,Dead-0,Dead = Difference between the EMMs of dead at day 4 and day 0 based on the LME model; 0,Alive-0,Dead = Difference between the EMMs of alive and dead at day 0 based on the LME model; 4,Alive-4,Dead = Difference between the EMMs of alive and dead at day 4 based on the LME model.

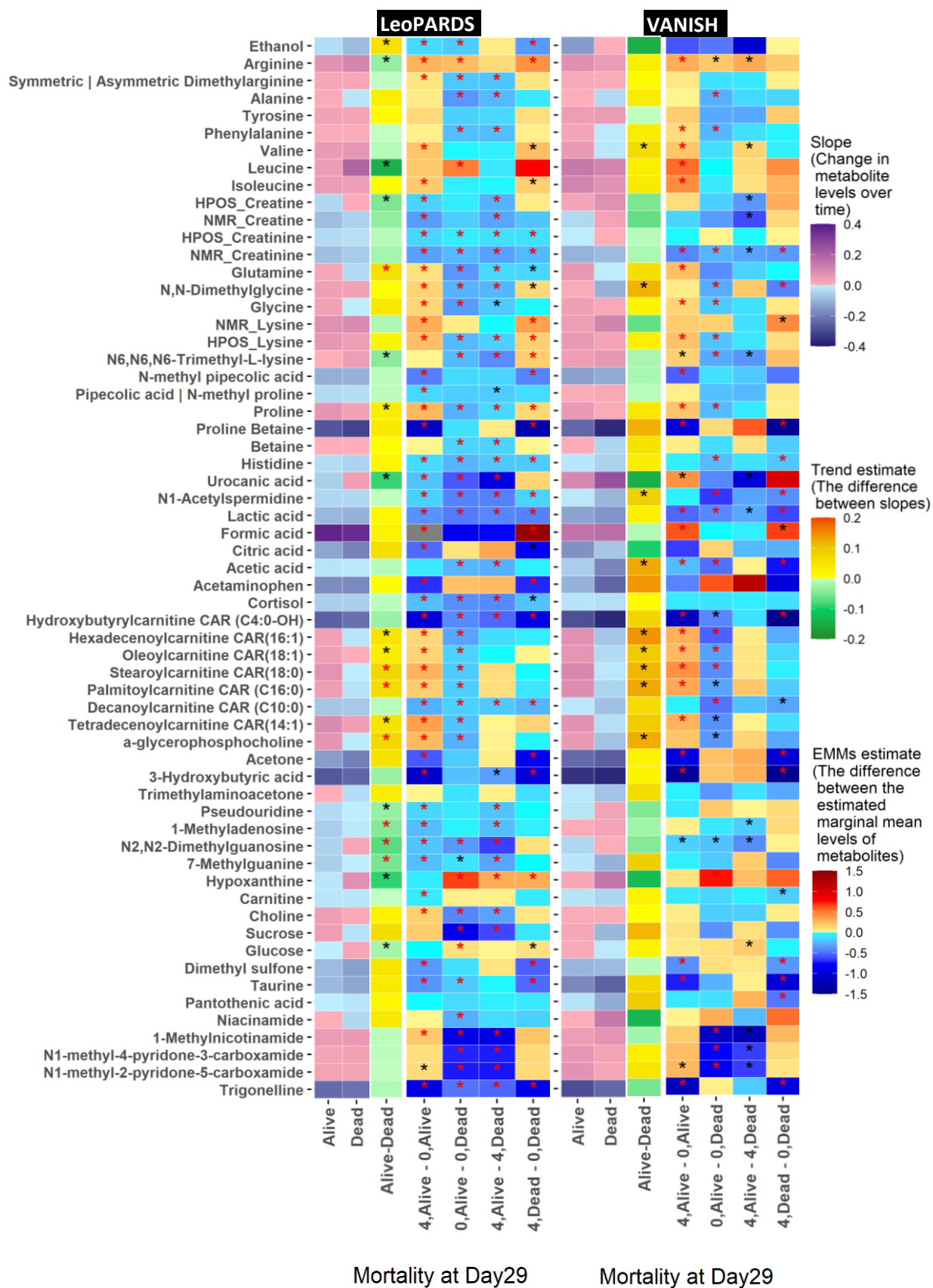


Figure A1.2: LME model results of combined SMoIESY-select and HPOS ppR log transformed data with age, sex, and BMI as confounders for LeoPARDS and VANISH. Various contrasts were performed via contrast coding during the trajectory analysis and their outcomes are shown in this figure. LME model outputs

including slope (i.e., change in metabolite levels over time), trend estimate (i.e., the difference between slopes) and EMMs estimate (i.e., the difference between the estimated marginal mean levels of metabolites) are presented in different colour scales. Metabolites which are significantly different between groups from various pairwise comparisons are highlighted in black and red asterisks. Unadjusted p-values calculated with Satterthwaite approximation method for degrees of freedom are shown in black asterisk whilst adjusted p-values calculated with B-H correction are shown in red asterisk. The slopes for alive (i.e., survivors) and dead (i.e., non-survivors) are shown as well as the difference between their slopes and whether this difference in the overall slopes is significant between the two groups. The scale for slope goes from positive to negative. Positive values for slope indicate that the metabolites increase with time whilst the negative values indicate that the metabolites decrease over time and the stronger the colour the bigger the magnitude for both positive and negative slopes. The difference between the slopes for alive and dead is presented in different colour scale (i.e., Trend estimate). Estimated marginal means were also calculated for each group at days 0 and day 4 using the LME model and the difference between the EMMs for four pairwise comparisons are shown in this figure. The four pairwise comparisons include: 4,Alive-0,Alive = Difference between the EMMs of alive at day 4 and day 0 based on the LME model; 4,Dead-0,Dead = Difference between the EMMs of dead at day 4 and day 0 based on the LME model; 0,Alive-0,Dead = Difference between the EMMs of alive and dead at day 0 based on the LME model; 4,Alive-4,Dead = Difference between the EMMs of alive and dead at day 4 based on the LME model.

Table A1.1: Confusion matrices for TP0TP2 foldchange mortality predictive models for VANISH and LeoPARDS SMoIESY-select datasets. Note: TP0TP2_{WC} = within cohort for TP0TP2 and TP0TP2_{EXCO} = with external cohort for TP0TP2.

Validation of VANISH within cohort			
VANISH TP0TP2 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	62	20
	Non-survivors	8	2
Validation of VANISH in LeoPARDS			
VANISH TP0TP2 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	215	56
	Non-survivors	13	2
Validation of LeoPARDS within cohort			
LeoPARDS TP0TP2 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	216	49
	Non-survivors	12	9
Validation of LeoPARDS in VANISH			
LeoPARDS TP0TP2 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	59	18
	Non-survivors	11	4

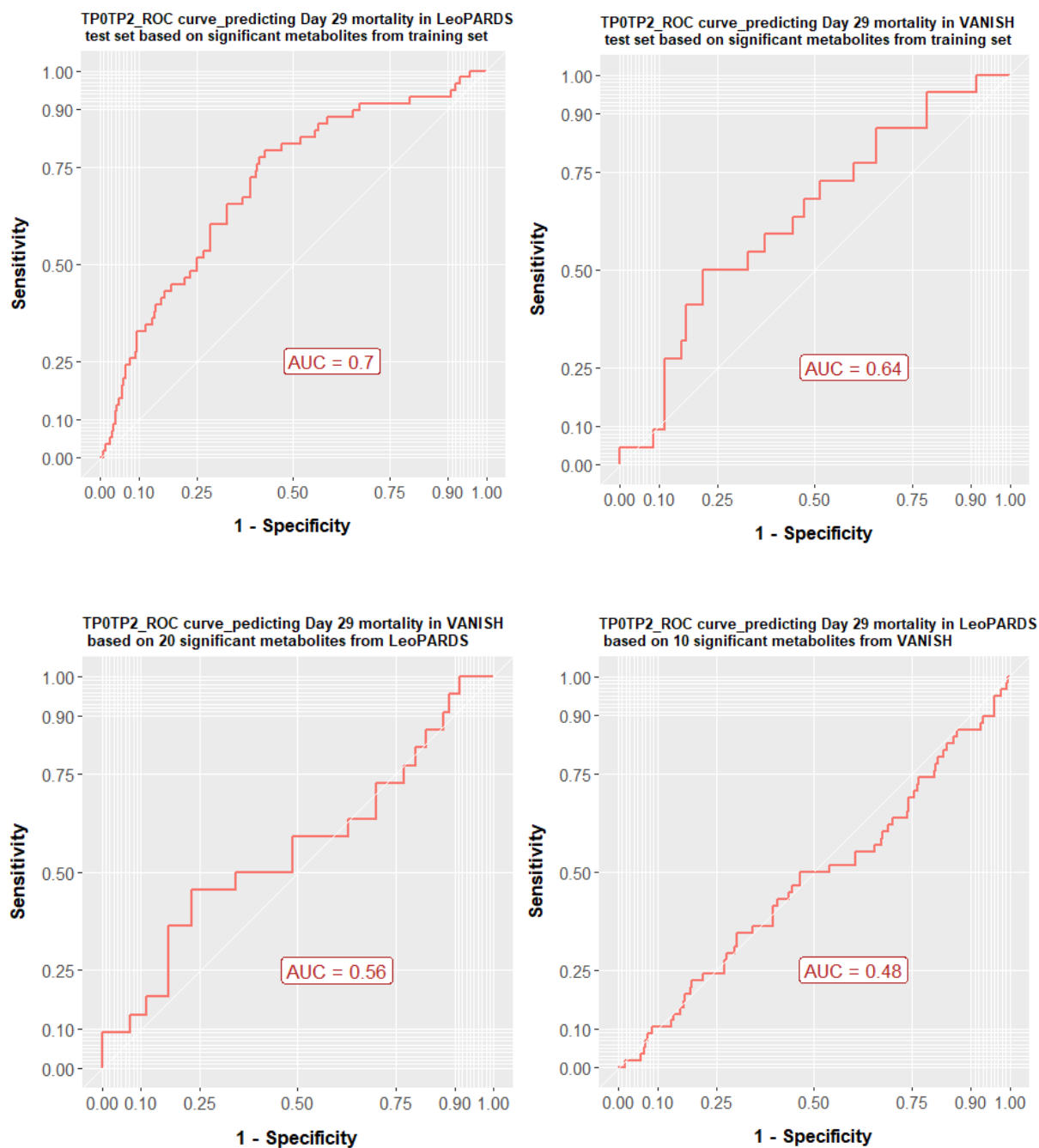


Figure A1.3: ROC curves for the predictive models of mortality on combined SMoESY-select and HPOS ppR dataset for VANISH and LeoPARDS for TP0TP2 samples with fold-changes.

Appendix 1.4 Metabolic changes with organ failure scores (Small Molecules)

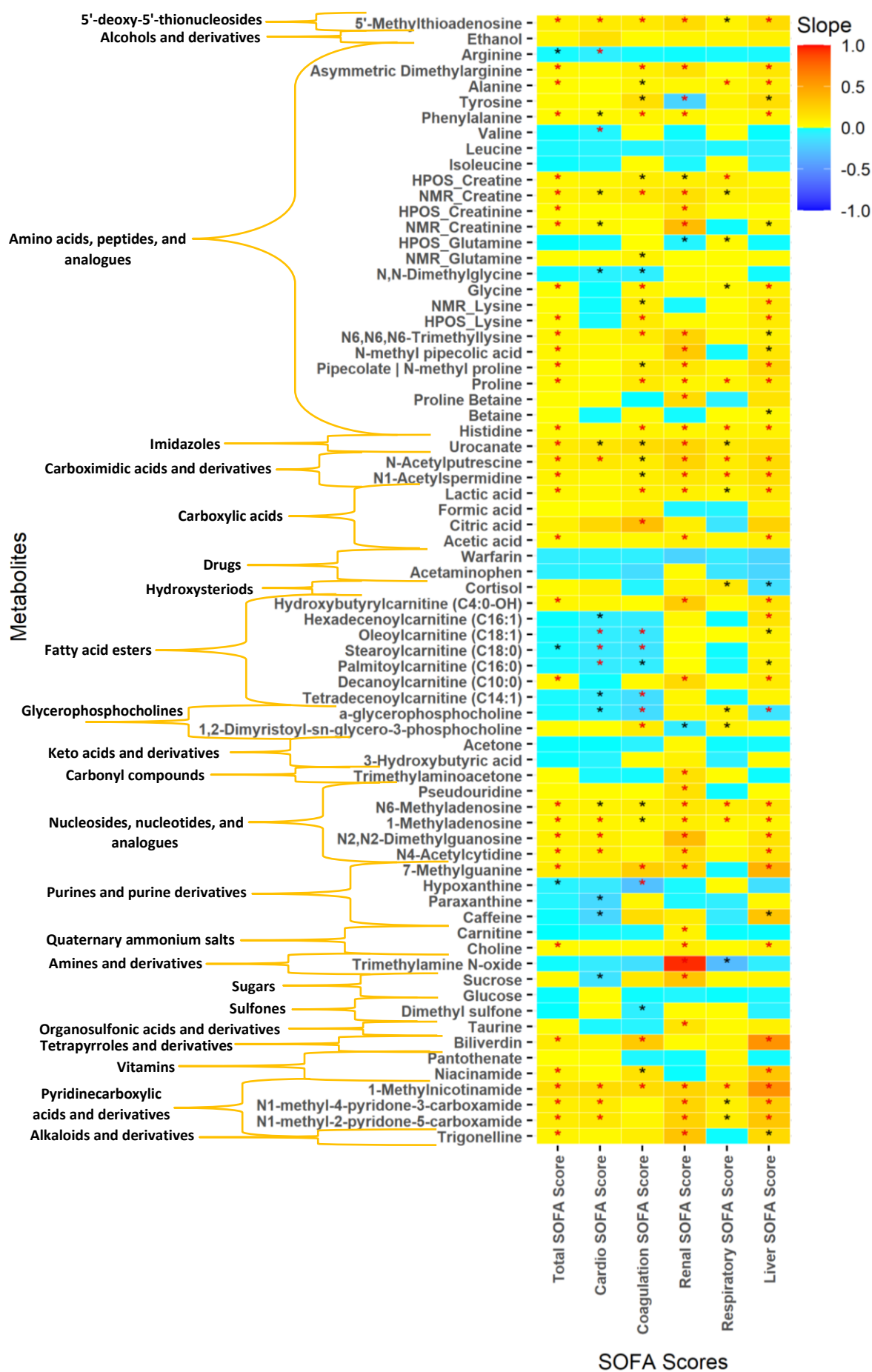


Figure A1.4: The results of LME models based on the SOFA scores for combined SMoIESY-select and HPOS ppR log transformed data with age and Sex as confounders for VANISH. Identity contrast was performed via contrast coding and the outcomes are shown here. LME model output slope (i.e., change in metabolite levels with SOFA scores) is presented for each SOFA score. The scale for slope ranges from positive to negative values, where positive values indicate higher levels of metabolites when the SOFA score is high whilst negative values indicate lower levels of metabolites when the SOFA score is high and stronger the colour the more the levels of metabolites with higher SOFA scores. Unadjusted p-values calculated with Satterthwaite approximation method for degrees of freedom are shown in black asterisk and represent whether the slope for metabolites for each SOFA score is different from 0 or not. Adjusted p-values calculated with B-H correction are shown in red asterisk.

Metabolites

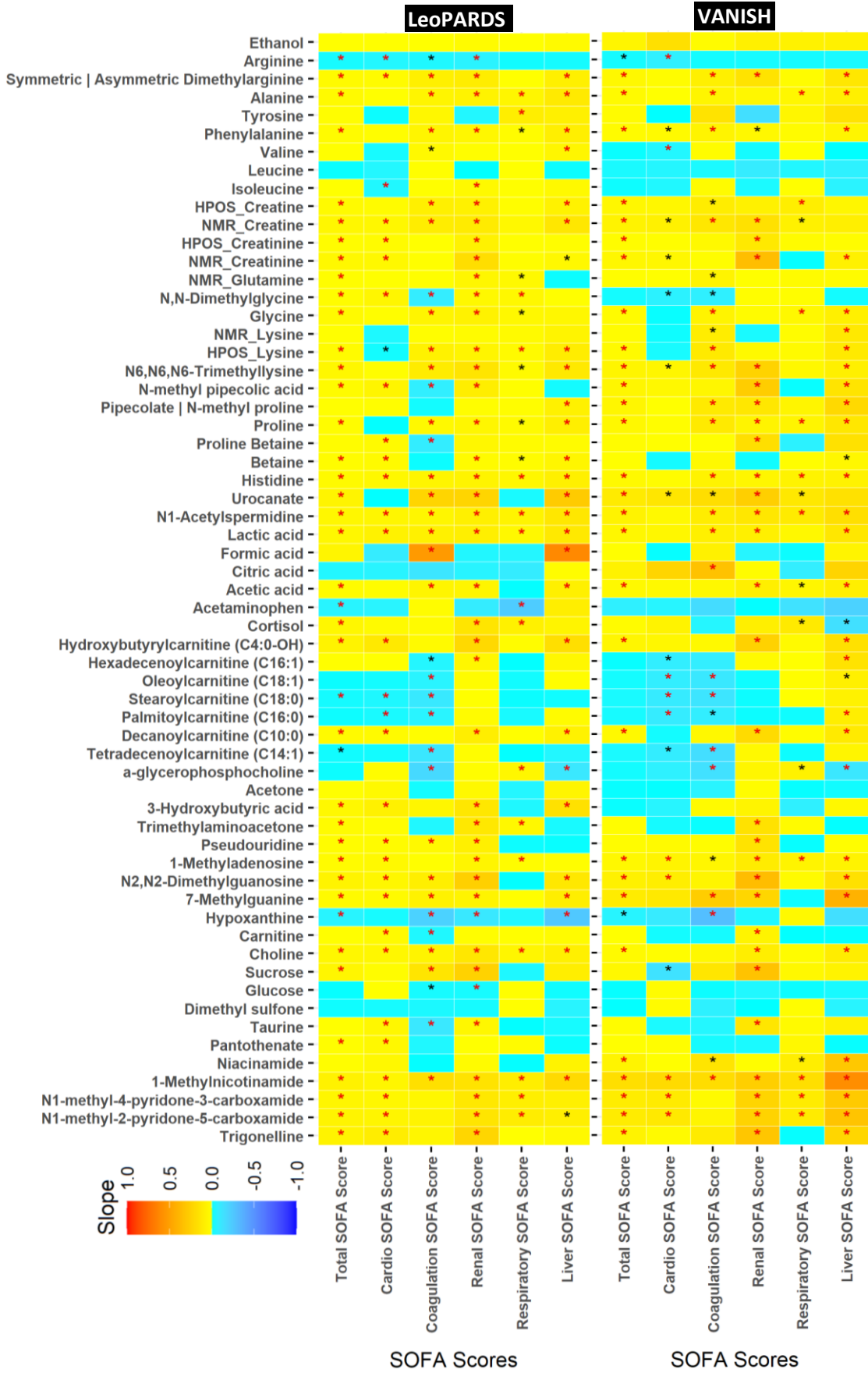


Figure A1.5: The results of LME models based on the SOFA scores for combined SMoIESY-select and HPOS ppR log transformed data with age, sex, and BMI as confounders for LeoPARDS and VANISH. Identity contrast was performed via contrast coding and the outcomes are shown here. LME model output slope (i.e., change in the metabolite levels with SOFA scores) is presented for each SOFA score. The scale for slope ranges from positive to negative values, where positive values indicate higher levels of metabolites when the SOFA score is high whilst negative values indicate lower levels of metabolites when the SOFA score is high and stronger the colour the more the levels of metabolites with higher SOFA scores. Unadjusted p-values calculated with Satterthwaite approximation method for degrees of freedom are shown in black asterisk and represent whether the slope for metabolites for each SOFA score is different from 0 or not. Adjusted p-values calculated with B-H correction are shown in red asterisk.

Appendix 1.5 Univariate analysis of lipids and lipoproteins and validation of results.

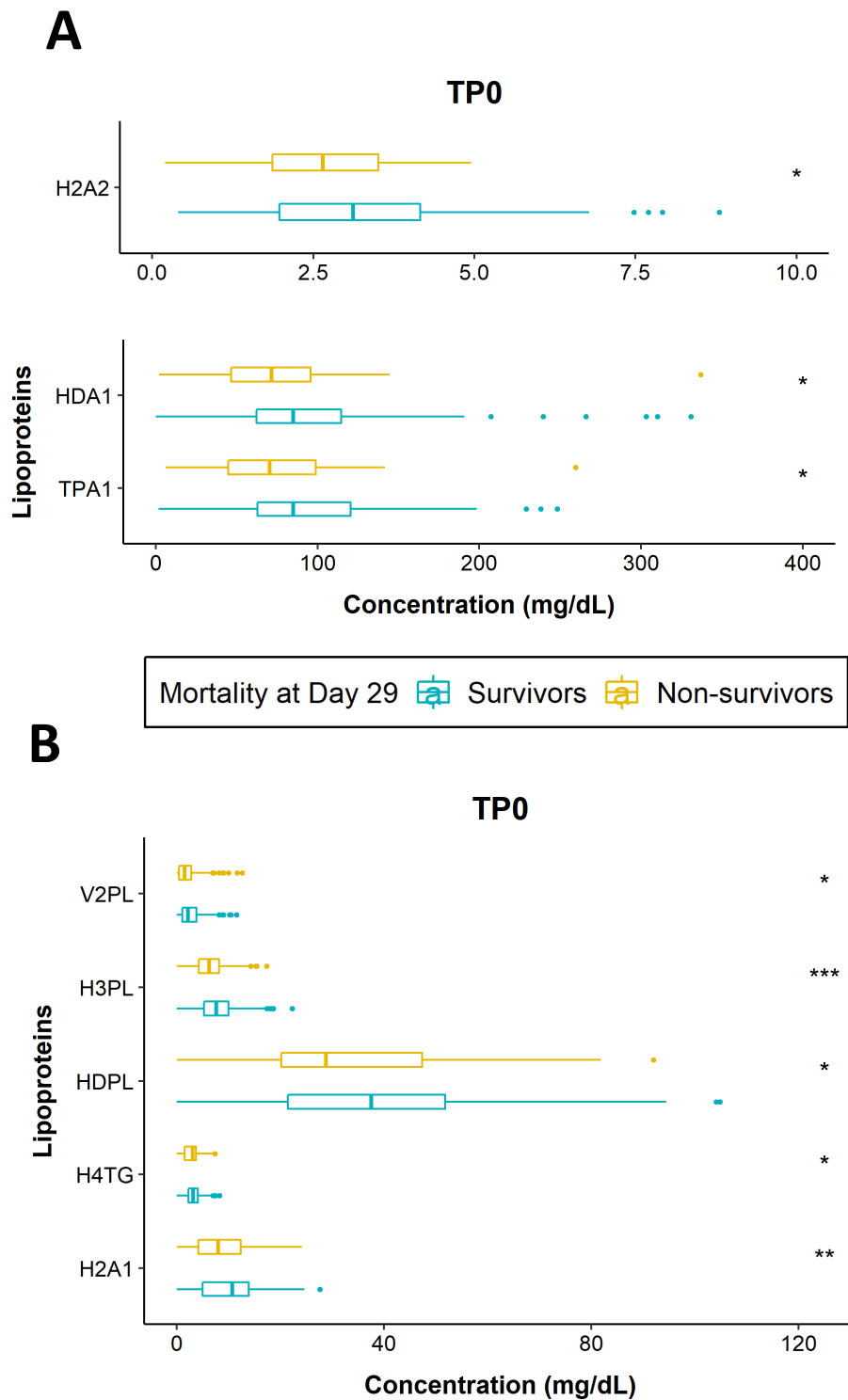


Figure A1.6: Box plots for lipoproteins which were significantly different between survivors and non-survivors at TP0 in VANISH (A) and LeoPARDS (B) datasets.

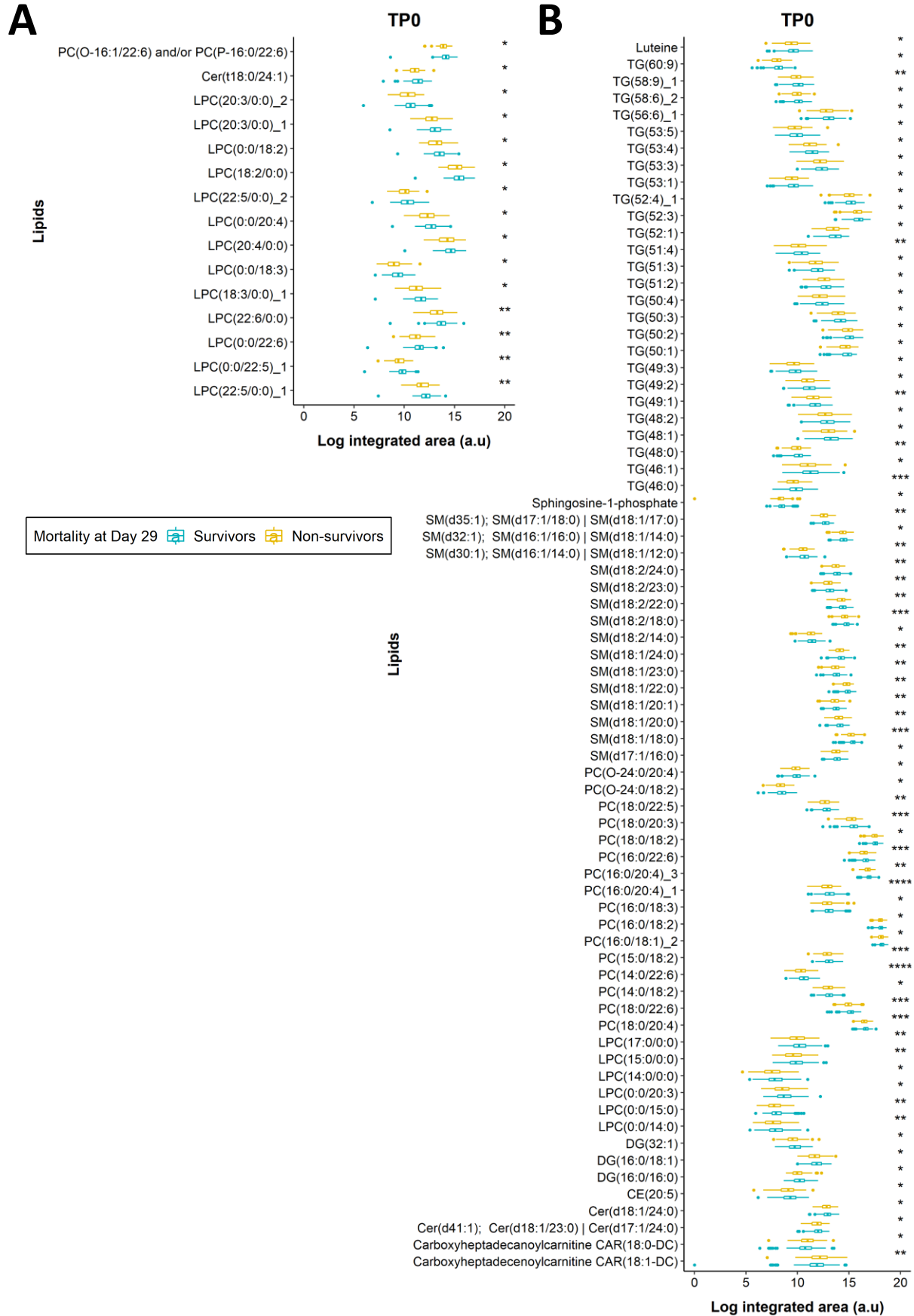
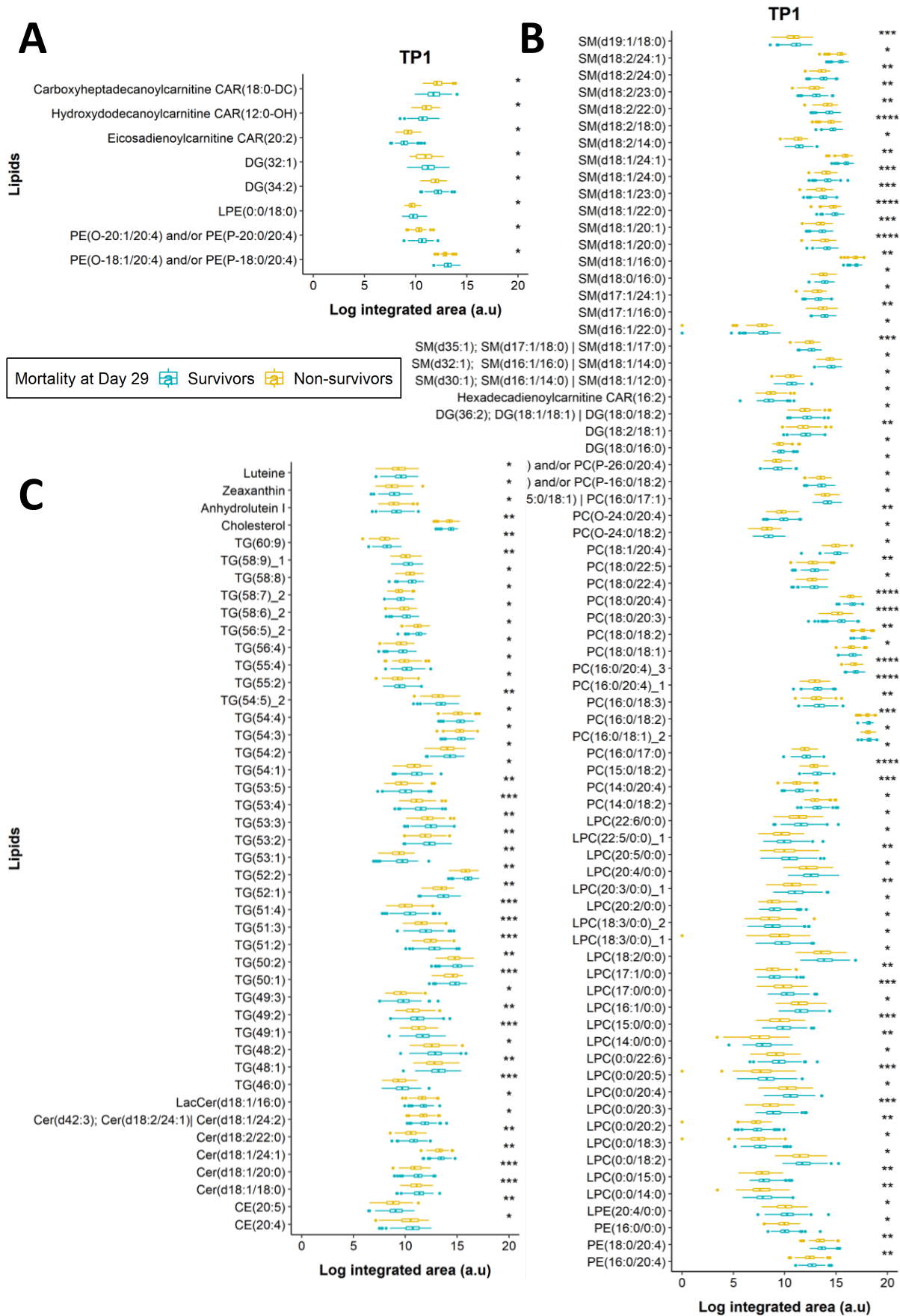


Figure A1.7: Box plots for lipids which were significantly different between survivors and non-survivors at TP0 in VANISH (A) and LeoPARDS (B) LPOS ppR datasets.



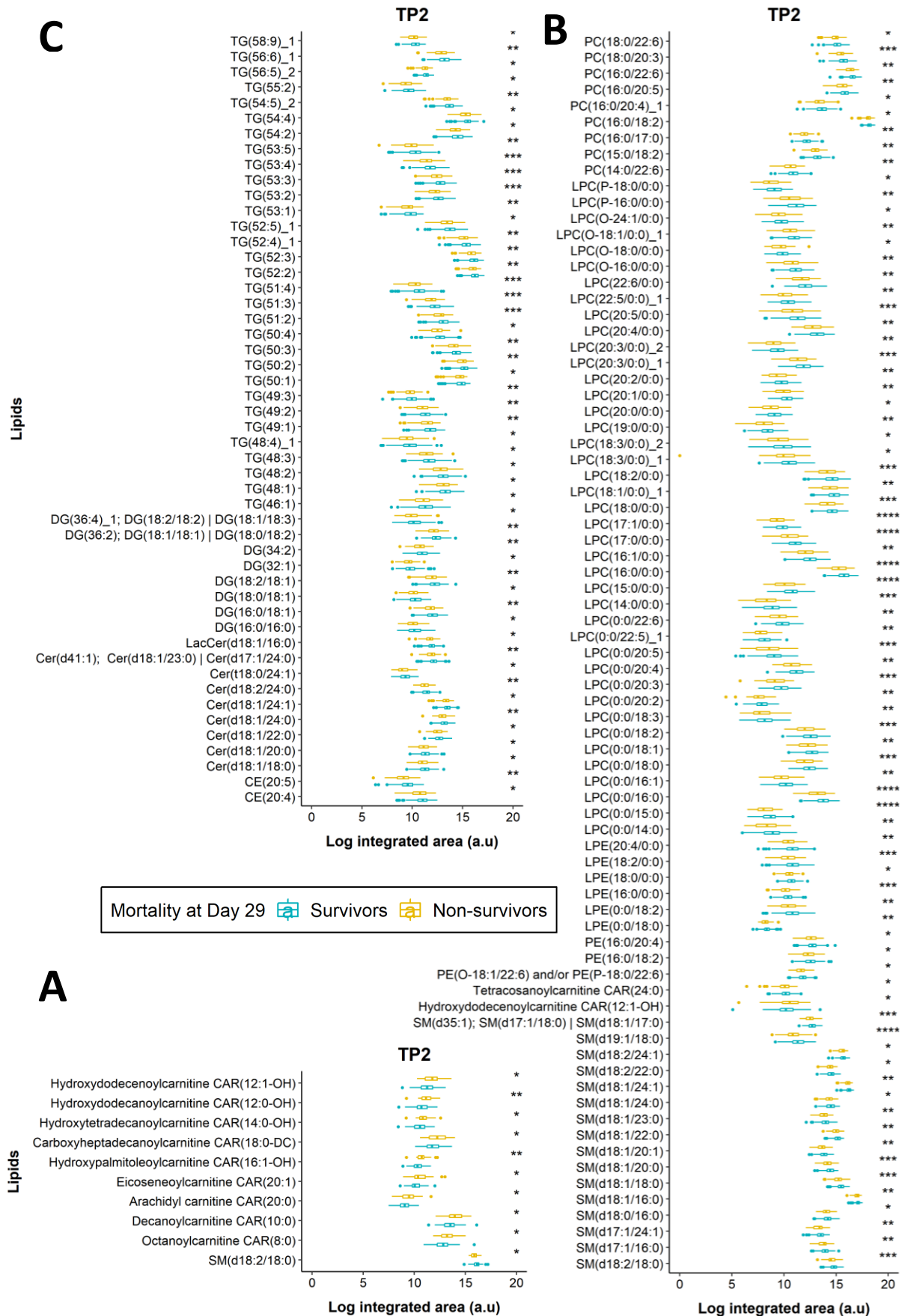


Figure A1.9: Box plots for lipids which were significantly different between survivors and non-survivors at TP2 in VANISH (A) and LeoPARDS (B & C) LPOS ppR data.

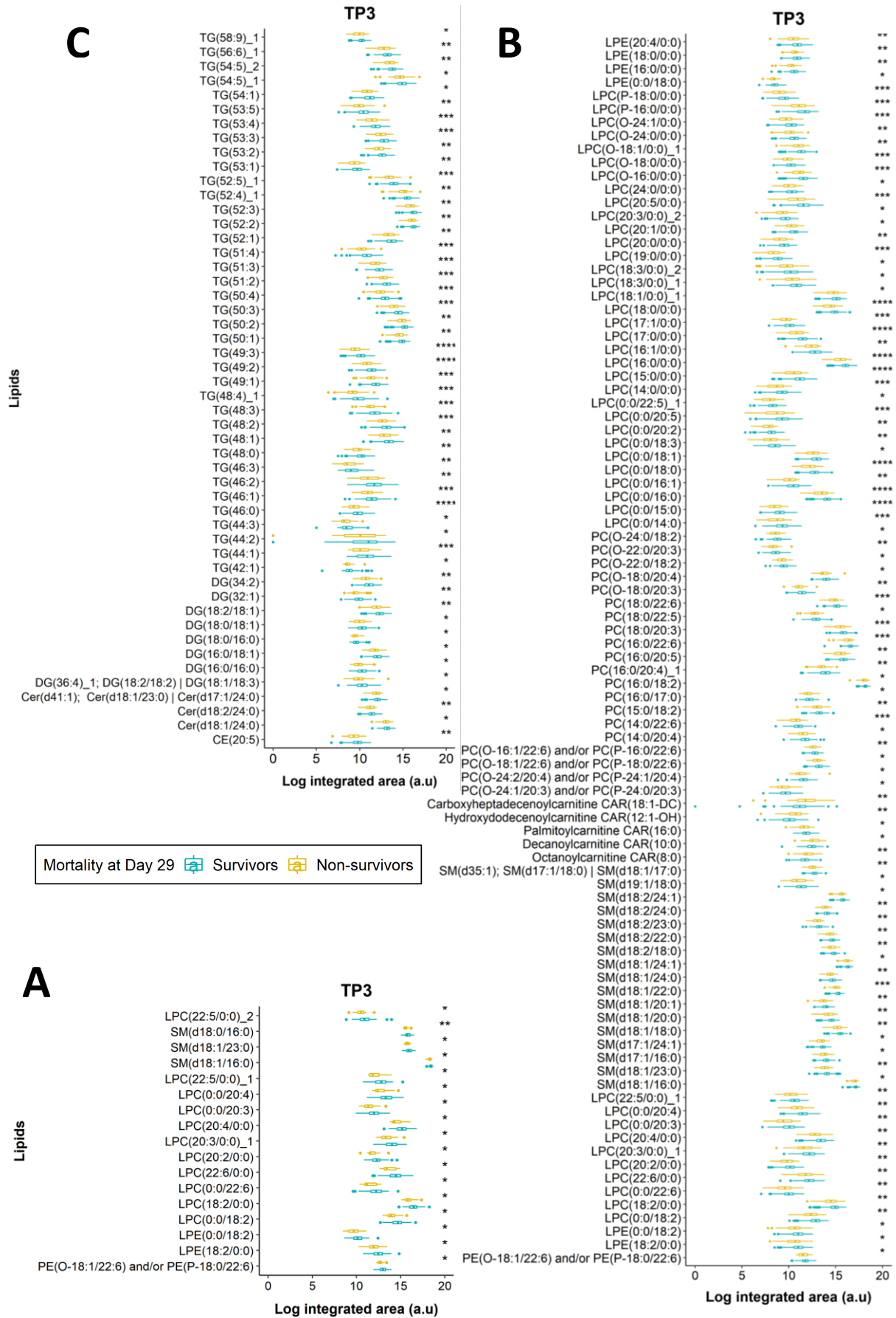


Figure A1.10: Box plots for lipids which were significantly different between survivors and non-survivors at TP3 in VANISH (A) and LeoPARDS (B & C) LPOS ppR data.

Table A1.3: Confusion matrices for TP0 mortality predictive models for VANISH and LeoPARDS NMR targeted (BI LISA) datasets. Note: TP0_{WC} = within cohort for TP0 and TP0_{EXCO} = with external cohort for TP0.

Validation of VANISH within cohort			
VANISH TP0 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	116	41
	Non-survivors	6	3
Validation of VANISH in LeoPARDS			
VANISH TP0 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	304	146
	Non-survivors	0	0
Validation of LeoPARDS within cohort			
LeoPARDS TP0 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	289	141
	Non-survivors	15	5
Validation of LeoPARDS in VANISH			
LeoPARDS TP0 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	120	44
	Non-survivors	2	0

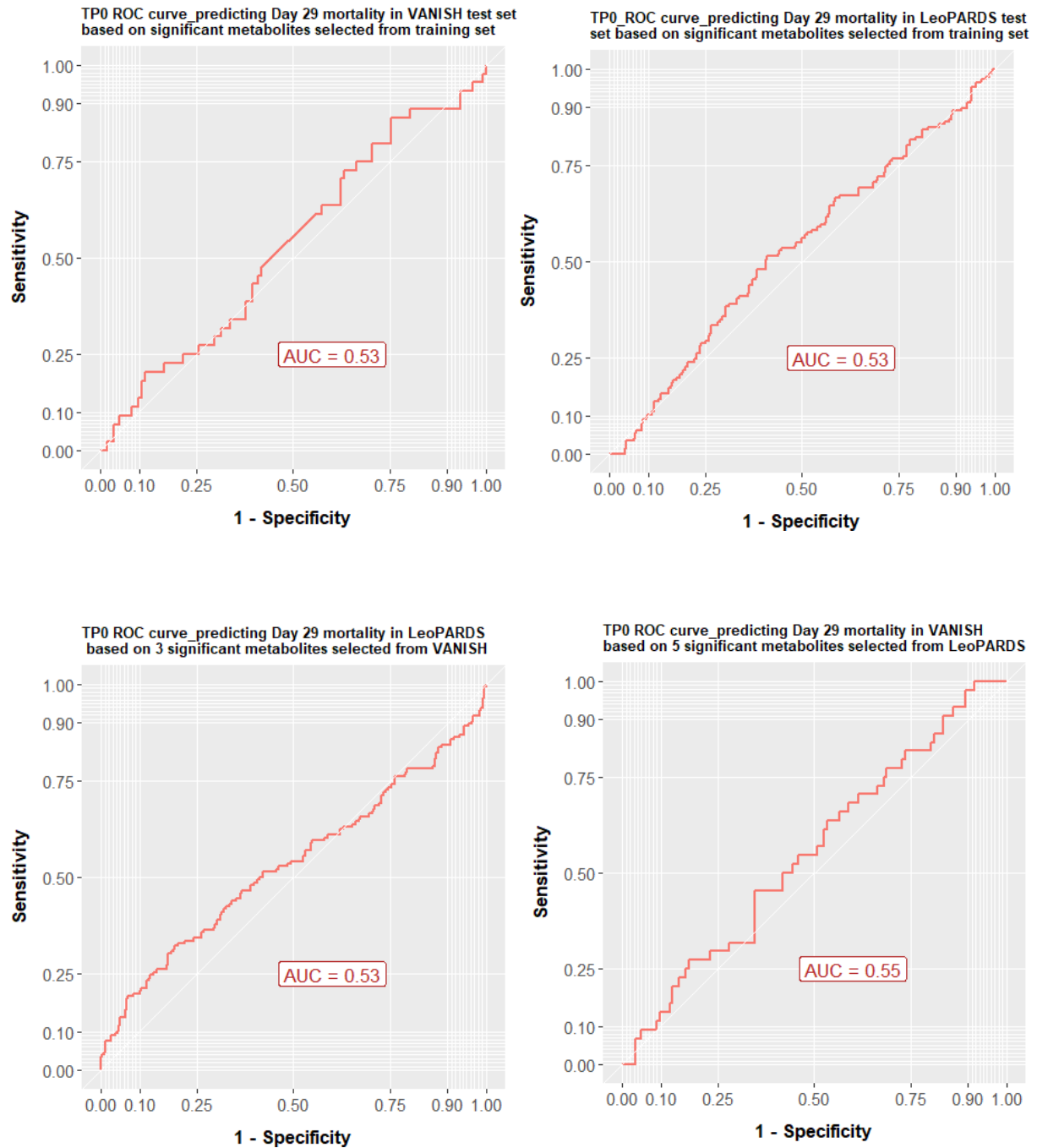


Figure A1.11: ROC curves for the predictive models of mortality on NMR targeted dataset (BI LISA) for VANISH and LeoPARDS at TP0.

Table A1.4: Confusion matrices for TP1 mortality predictive models for VANISH and LeoPARDS LPOS ppR datasets. Note: TP1_{WC} = within cohort for TP1 and TP1_{EXCO} = with external cohort for TP1.

Validation of VANISH within cohort			
VANISH TP1 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	85	23
	Non-survivors	20	9
Validation of VANISH in LeoPARDS			
VANISH TP1 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	55	12
	Non-survivors	243	98
Validation of LeoPARDS within cohort			
LeoPARDS TP1 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	227	58
	Non-survivors	71	52
Validation of LeoPARDS in VANISH			
LeoPARDS TP1 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	105	32
	Non-survivors	0	0

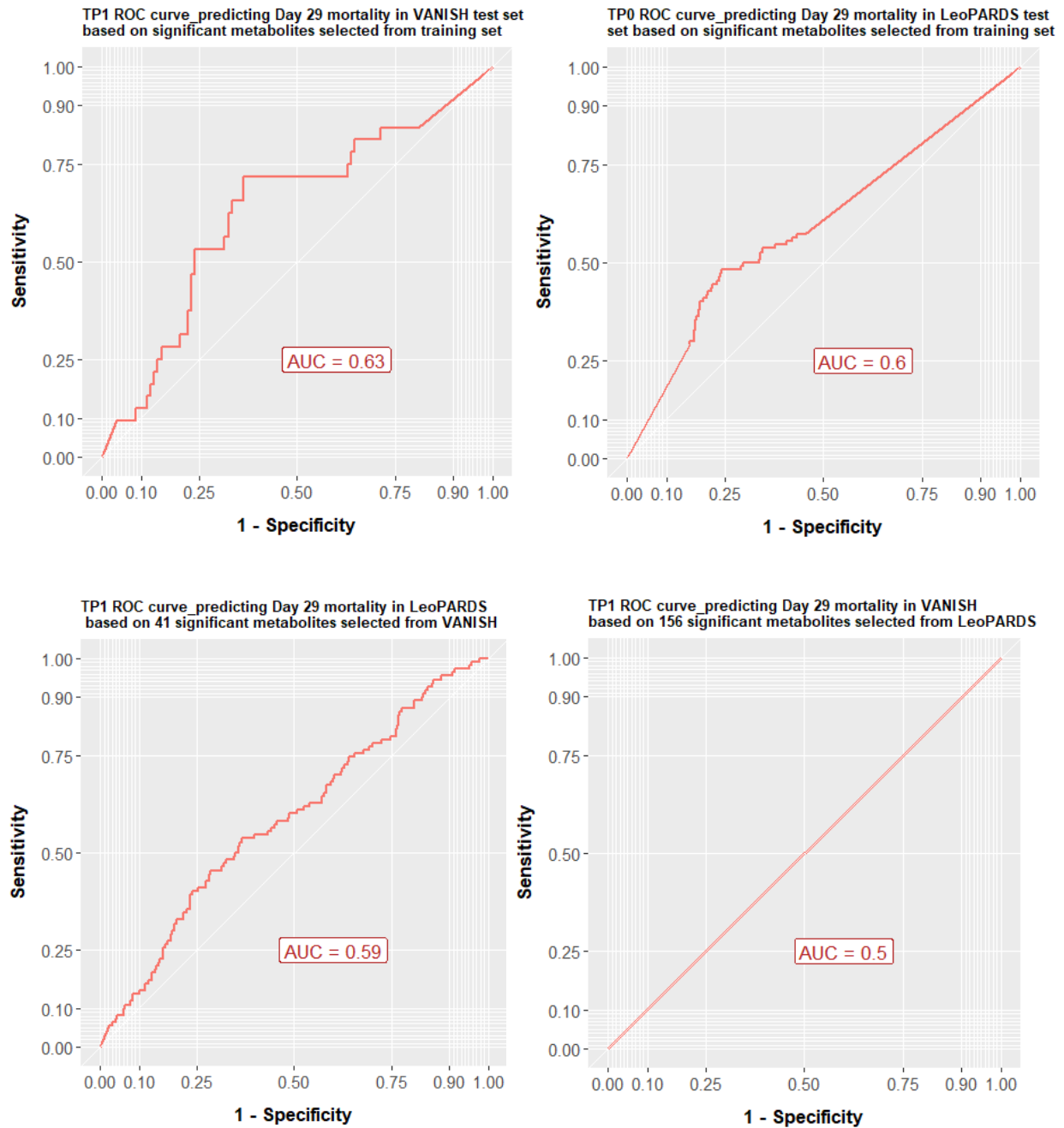
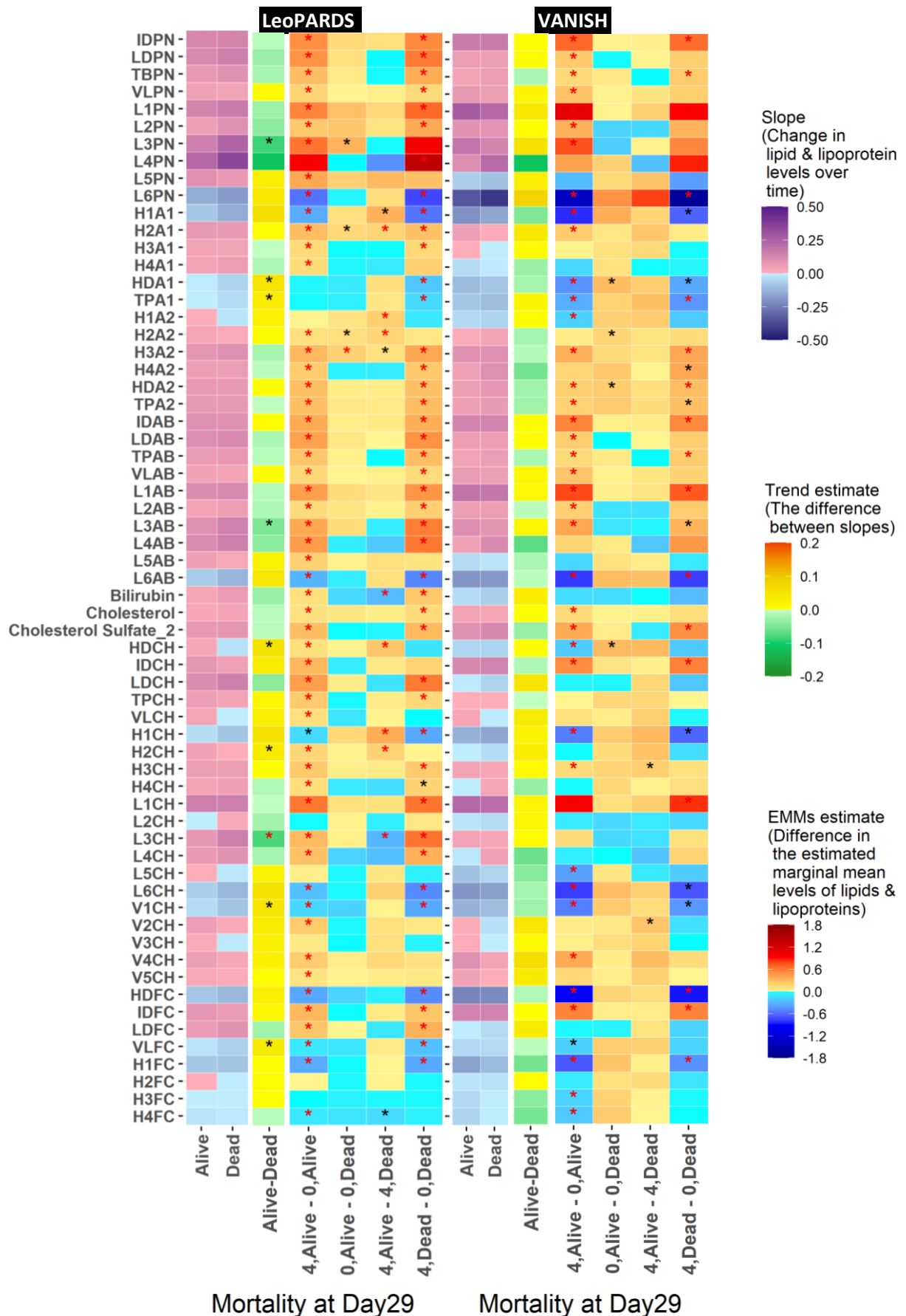
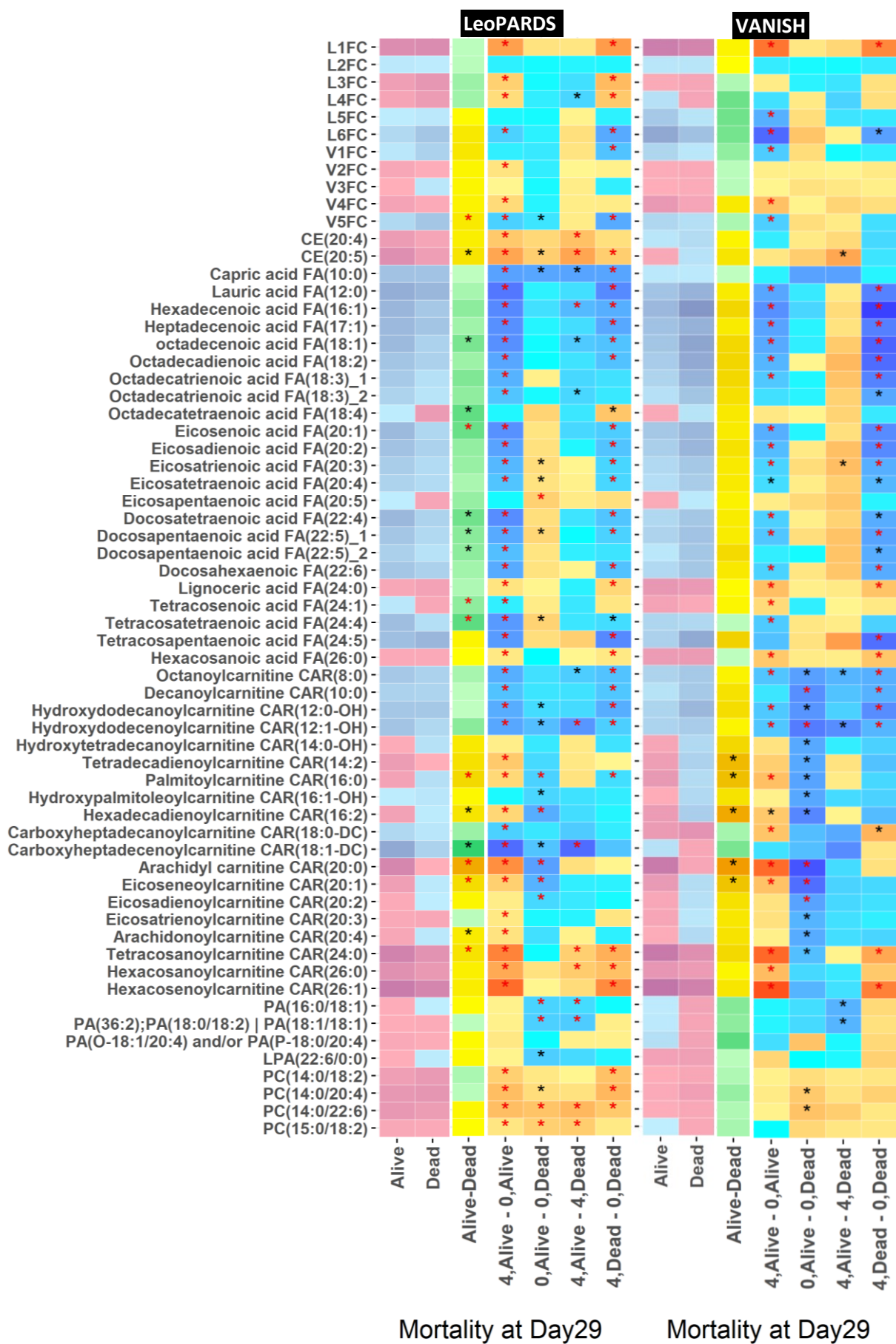


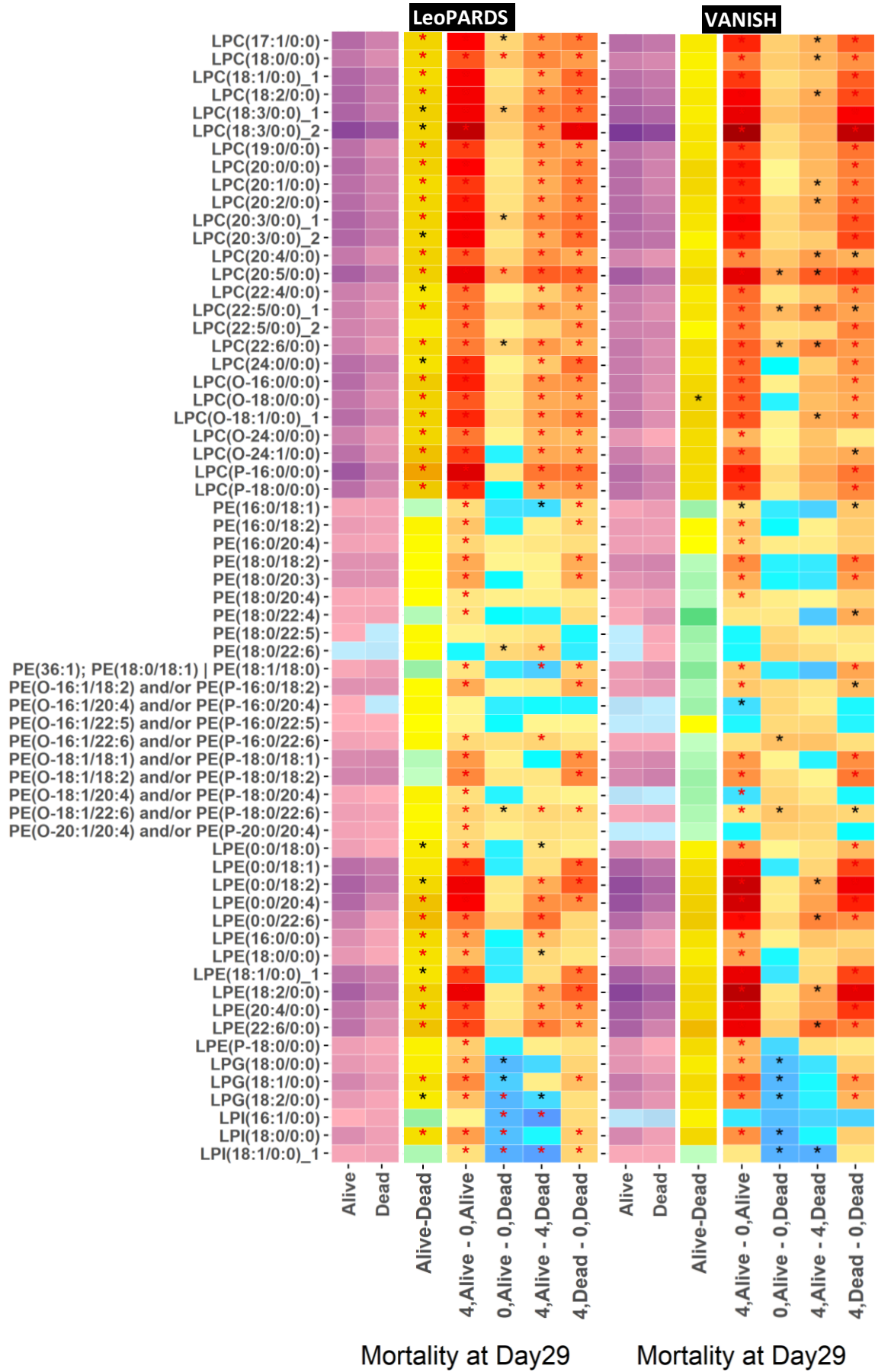
Figure A1.12: ROC curves for the predictive models of mortality on LPOS ppR dataset for VANISH and LeoPARDS at TP1.

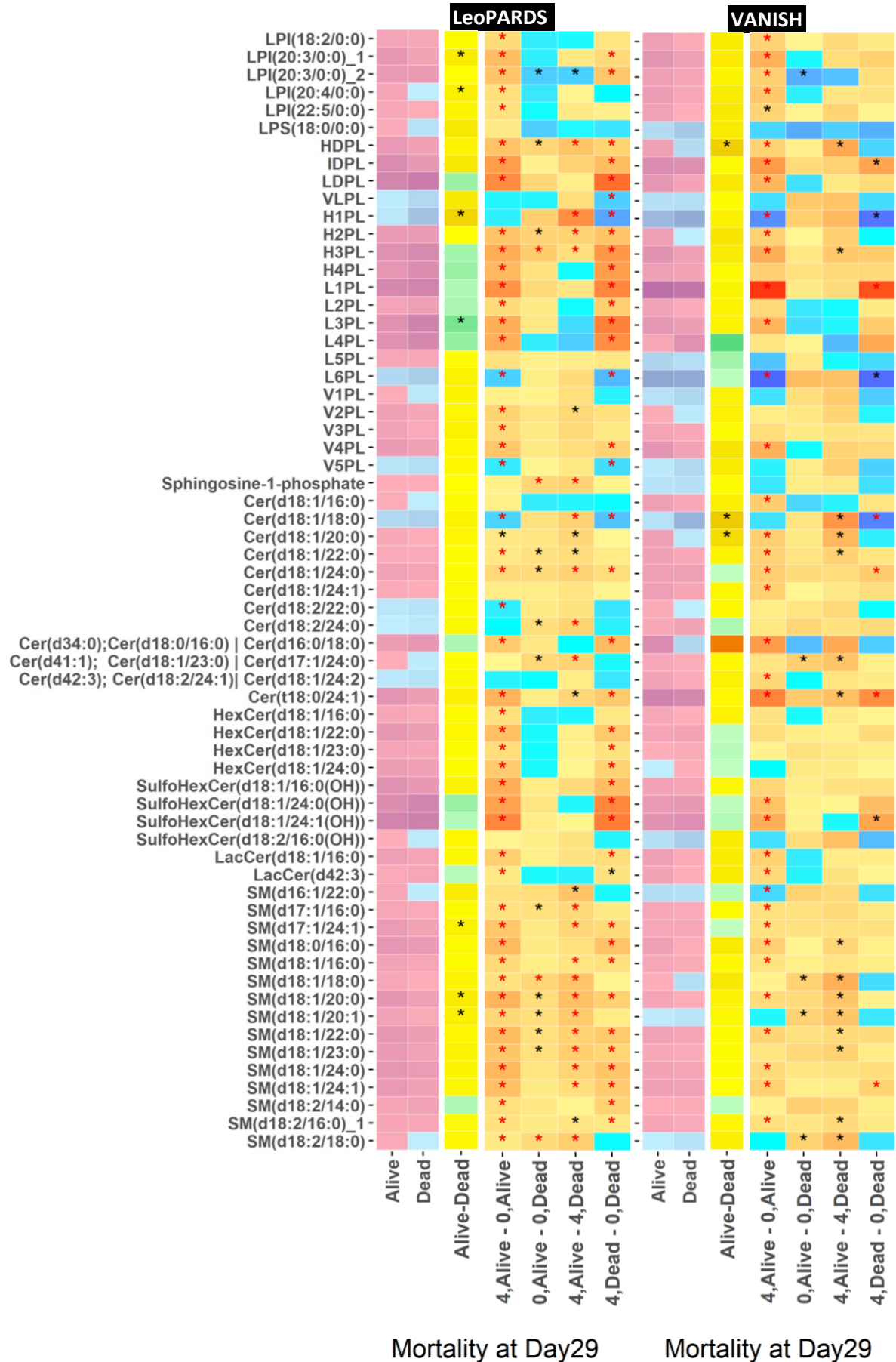
Appendix 1.6 Metabolic trajectories focusing on outcome (Lipids and Lipoproteins) and validation of results.

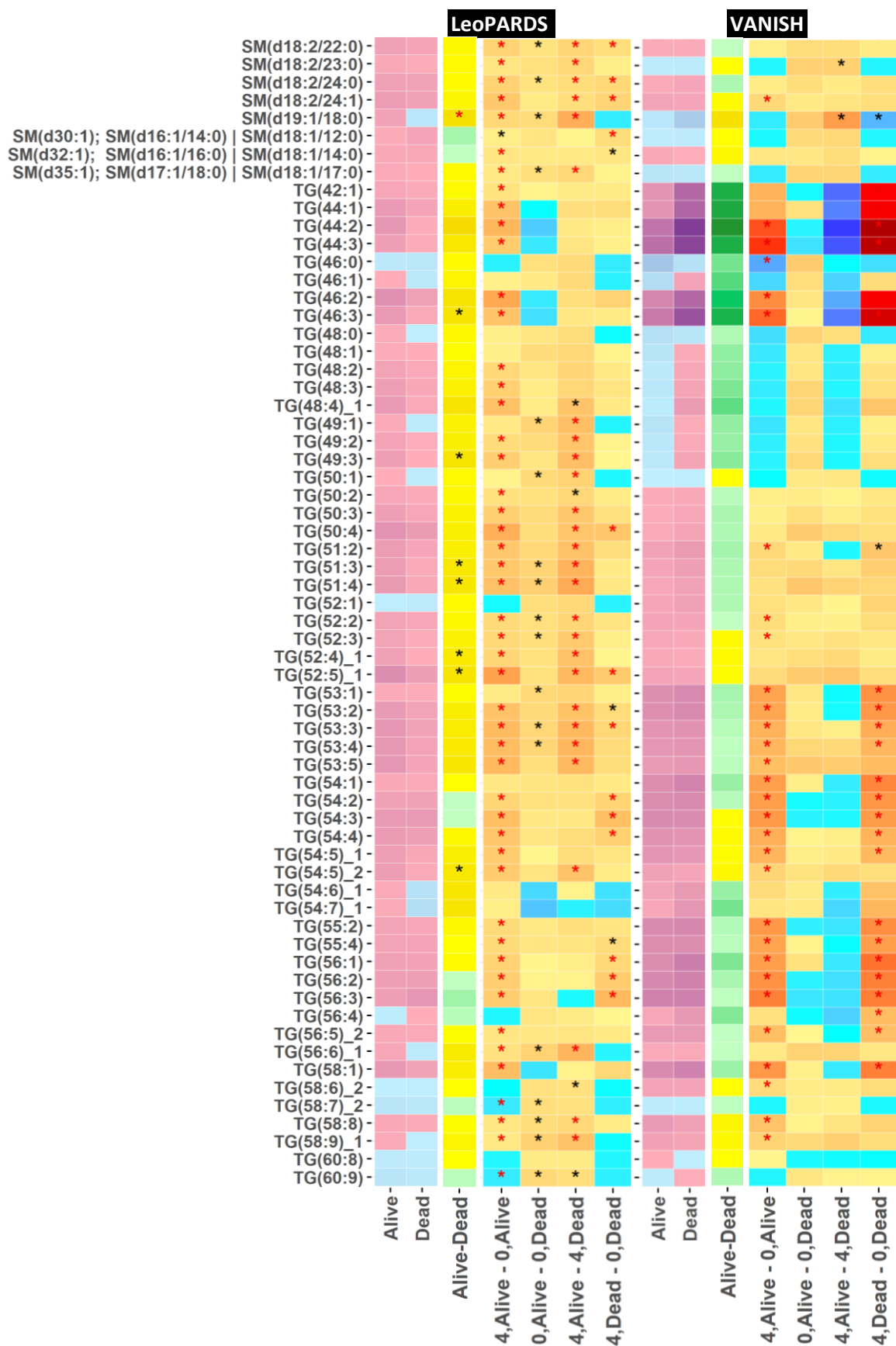












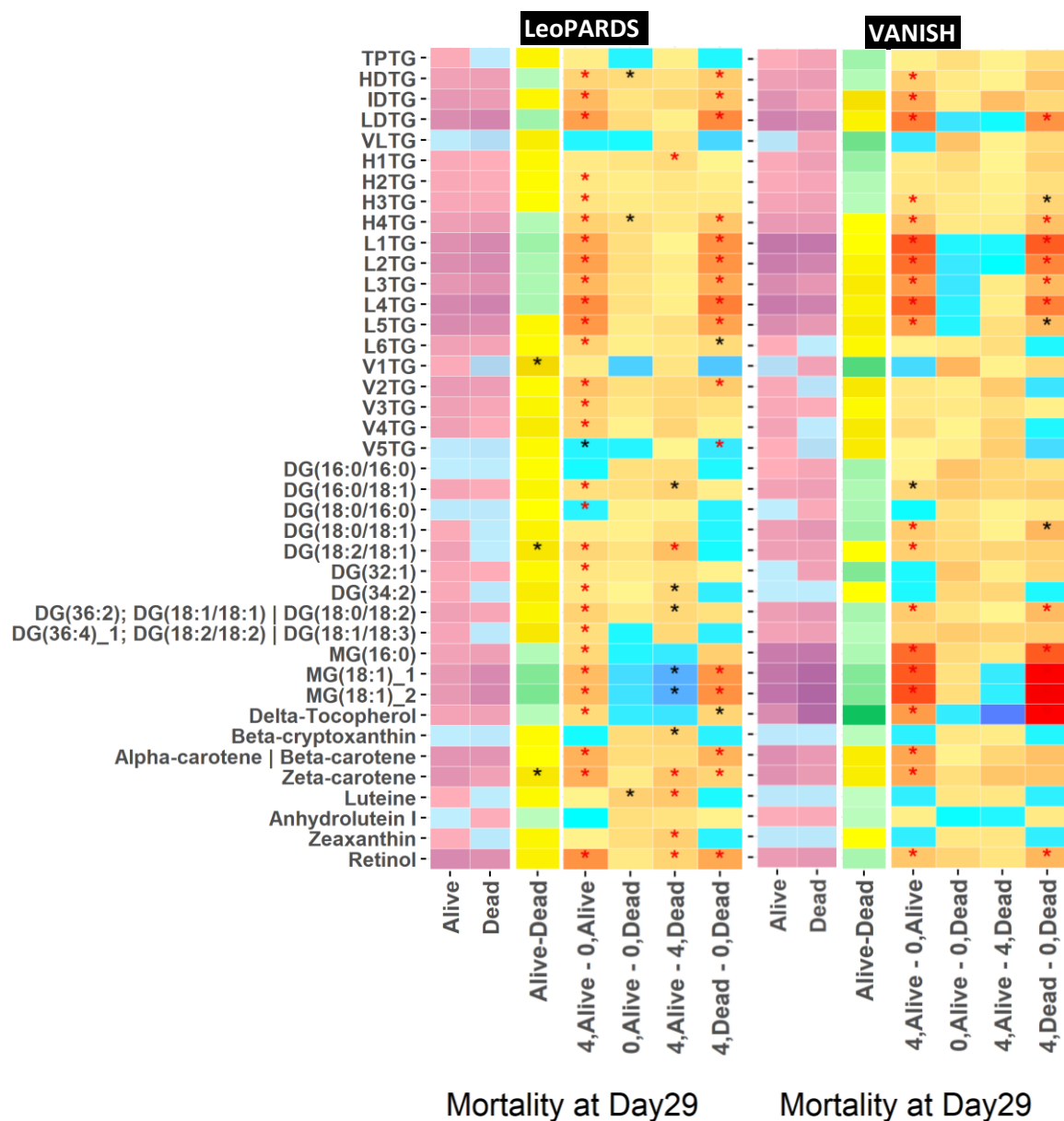


Figure A1.13: The results of LME models focusing on mortality for combined BI LISA, LPOS ppR and LNEG ppR log transformed data with age, sex and BMI as confounders for VANISH and LeoPARDS. Various contrasts were performed via contrast coding during the trajectory analysis and their outcomes are shown in this figure. LME model outputs including slope (i.e., change in lipid and lipoprotein levels over time), estimate (i.e., the difference between the estimated marginal mean levels of lipids and lipoproteins) are presented in different colour scales. Lipids and lipoproteins which are significantly different between groups from various pairwise comparisons are highlighted in black and red asterisks. Metabolites that were only significant before correction for multiple comparisons are shown with a black asterisk whilst those that remained significant after correction are shown in red. The slopes for alive (i.e., survivors) and dead (i.e., non-survivors) are shown as well as the difference between their slopes and whether this difference in the overall slopes is significant between the two groups. The scale for slope goes from positive to negative. Positive values for slope indicate that the metabolites increase with time whilst the negative values indicate that the metabolites decrease over time and the stronger the colour the bigger the magnitude for both positive and negative slopes. The difference between the slopes for alive and dead is presented in different colour scale (i.e., estimate). Estimated marginal means were also calculated for each group at days 0 and

day 4 using the LME model and the difference between the EMMs for four pairwise comparisons are presented here. The four pairwise comparisons include: 4,Alive-0,Alive = Difference between the EMMs of alive at day 4 and day 0 based on the LME model; 4,Dead-0,Dead = Difference between the EMMs of dead at day 4 and day 0 based on the LME model; 0,Alive-0,Dead = Difference between the EMMs of alive and dead at day 0 based on the LME model; 4,Alive-4,Dead = Difference between the EMMs of alive and dead at day 4 based on the LME model. Abbreviations: CH = Cholesterol; FC = Free Cholesterol; PL = Phospholipid; TG = Triglycerides; A1 = Apo-A1; A2 = Apo-A2 , AB = Apo-B; HD = High density lipoprotein (HDL); VL = Very low density lipoprotein (VLDL); ID = Intermediate density lipoprotein (IDL); LD = Low density lipoprotein; TPCH = Total plasma Cholesterol; VLCH = VLDL Cholesterol; V1-V5CH = VLDL Cholesterol subfractions 1-5; IDCH = IDL Cholesterol; LDCH = LDL Cholesterol; L1-6CH = LDL Cholesterol subfractions 1-6; HDCH = HDL Cholesterol; H1-4CH = HDL Cholesterol subfractions 1-4; VLFC = VLDL Free Cholesterol; V1-V5FC = VLDL Free Cholesterol subfractions 1-5; IDFC = IDL Free Cholesterol; LDFC = LDL Free Cholesterol; L1-L6FC = LDL Free Cholesterol subfractions 1-6; HDFC = HDL Free Cholesterol; H1-H4FC = HDL Free Cholesterol subfractions 1-4; TPA1 = Total Plasma A1; HDA1 = HDL Apo-A1; H1-H4A1 = HDL Apo A1 subfractions 1-4; TPA2 = Total plasma A2; H1-H4A2 = HDL Apo A2 subfractions 1-4; TPAB = Total plasma Apo-B; LDAB = LDL Apo-B; L1-L6AB = LDL Apo-B subfractions 1-6; IDAB = IDL Apo-B; TPBN: Total blood particle number; VLPN = VLDL Particle Number; IDPN = IDL Particle Number; LDPN = LDL Particle Number; L1-L6PN = LDL Particle number subfractions 1-6; TPTG = Total Plasma Triglycerides; VLTG = VLDL Triglycerides; V1-V5TG = VLDL Triglycerides subfractions 1-5; IDTG = IDL Triglycerides; LDTG = LDL Triglycerides; L1-L6TG = LDL Triglycerides subfractions 1-6; HDTG = HDL Triglycerides; H1-H4TG = HDL Triglycerides subfractions 1-4; VLPL = HDL Phospholipids; V1-V5PL = VLDL Phospholipid subfractions 1-5; IDPL = IDL Phospholipids; LDPL = LDL Phospholipids; L1-L6PL = LDL Phospholipids subfractions 1-6; HDPL = HDL Phospholipids; H1-H4PL = HDL Phospholipids subfractions 1-4; PC = Phosphatidylcholine; LPC = Lysophosphatidylcholine; PE = Phosphatidylethanolamine; LPE = Lysophosphatidylethanolamine; PI = Phosphatidylinositol; LPI = Lysophosphatidylinositol; LPG = Lysophosphatidylglycerol; PA = Phosphatidic acid; LPA = Lysophosphatidic acid; LPS = Lysophosphatidylserine; SM = Sphingomyelin; Cer = Ceramide; LacCer = Lactosylceramide; HexCer = Hexosylceramide; SulfoHexCer = Sulfohexosylceramide; CE = Cholesteryl esters; DG = Diacylglycerol; MG = Monoacylglycerols; TG = Triglycerides; CAR = Carnitine; FA = Fatty acid. Note: Carbon atoms and double bonds in all lipid species are represented as (C atoms:double bond); d indicates 2 hydroxyl groups in the sphenoid base of ceramides and sphigomyelins; DC denotes for dicarboxylic acid; Prefixes "OH", "O" and "P" represents different substituents/linkages in the lipid species: "OH" denotes for hydroxyl group, "O" represents an alkyl ether linkage whilst "P" refers to the plasmalogen link. Lipid species with abbreviations _1 and _2 indicate same m/z value but different retention times for those lipids.

Table A1.5: Confusion matrices for TP0TP2 foldchange mortality predictive models for VANISH and LeoPARDS BI LISA, LPOS and LNEG ppR combined datasets. Note: TP0TP2_{WC} = within cohort for TP0TP2 samples with fold changes and TP0TP2_{EXCO} = with external cohort for TP0TP2 samples with fold changes.

Validation of VANISH within cohort			
VANISH TP0TP2 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	60	18
	Non-survivors	11	4
Validation of VANISH in LeoPARDS			
VANISH TP0TP2 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	208	54
	Non-survivors	18	4
Validation of LeoPARDS within cohort			
LeoPARDS TP0TP2 _{WC}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	175	37
	Non-survivors	51	21
Validation of LeoPARDS in VANISH			
LeoPARDS TP0TP2 _{EXCO}		Actual	
		Survivors	Non-survivors
Predicted	Survivors	56	16
	Non-survivors	15	6

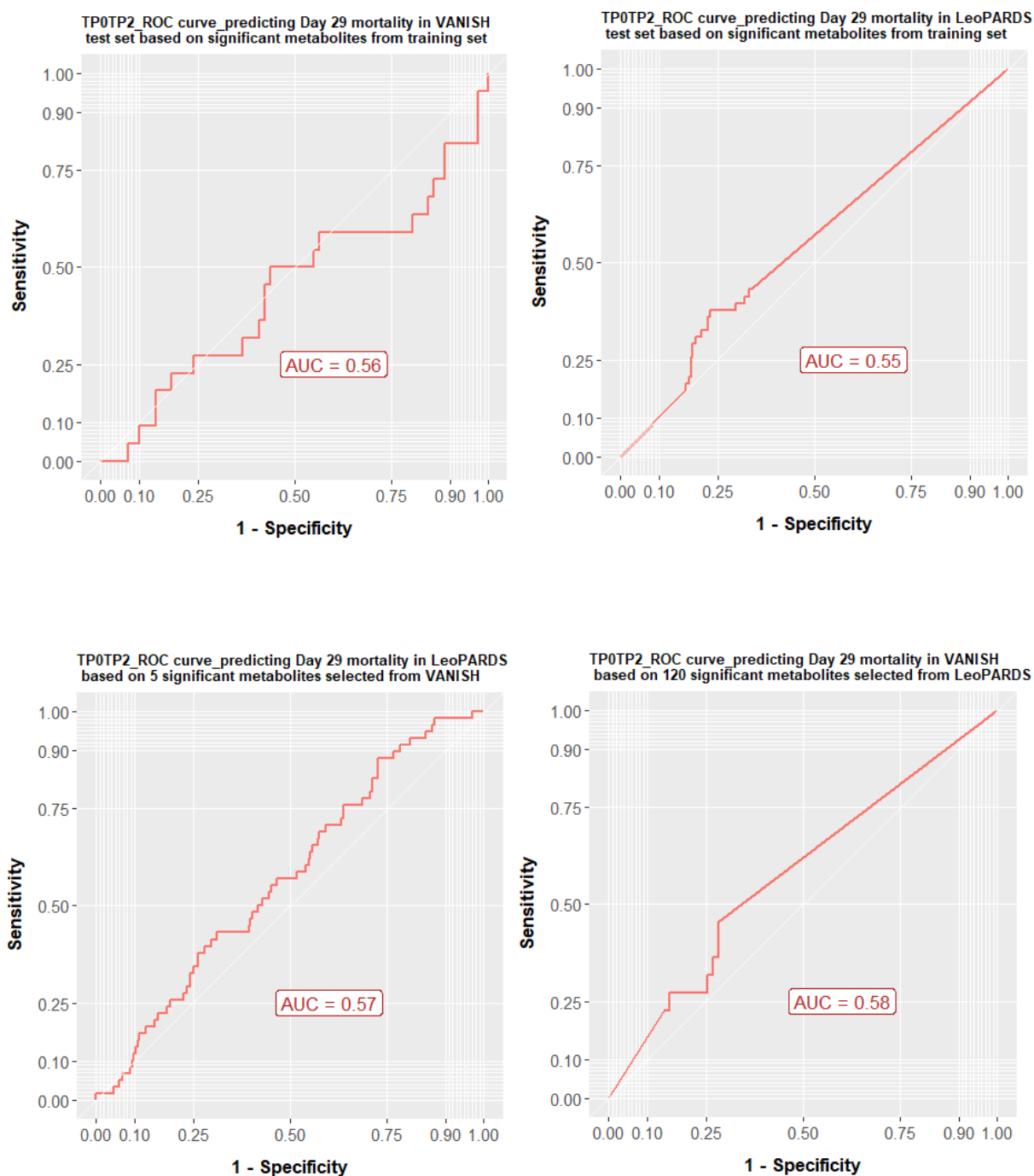
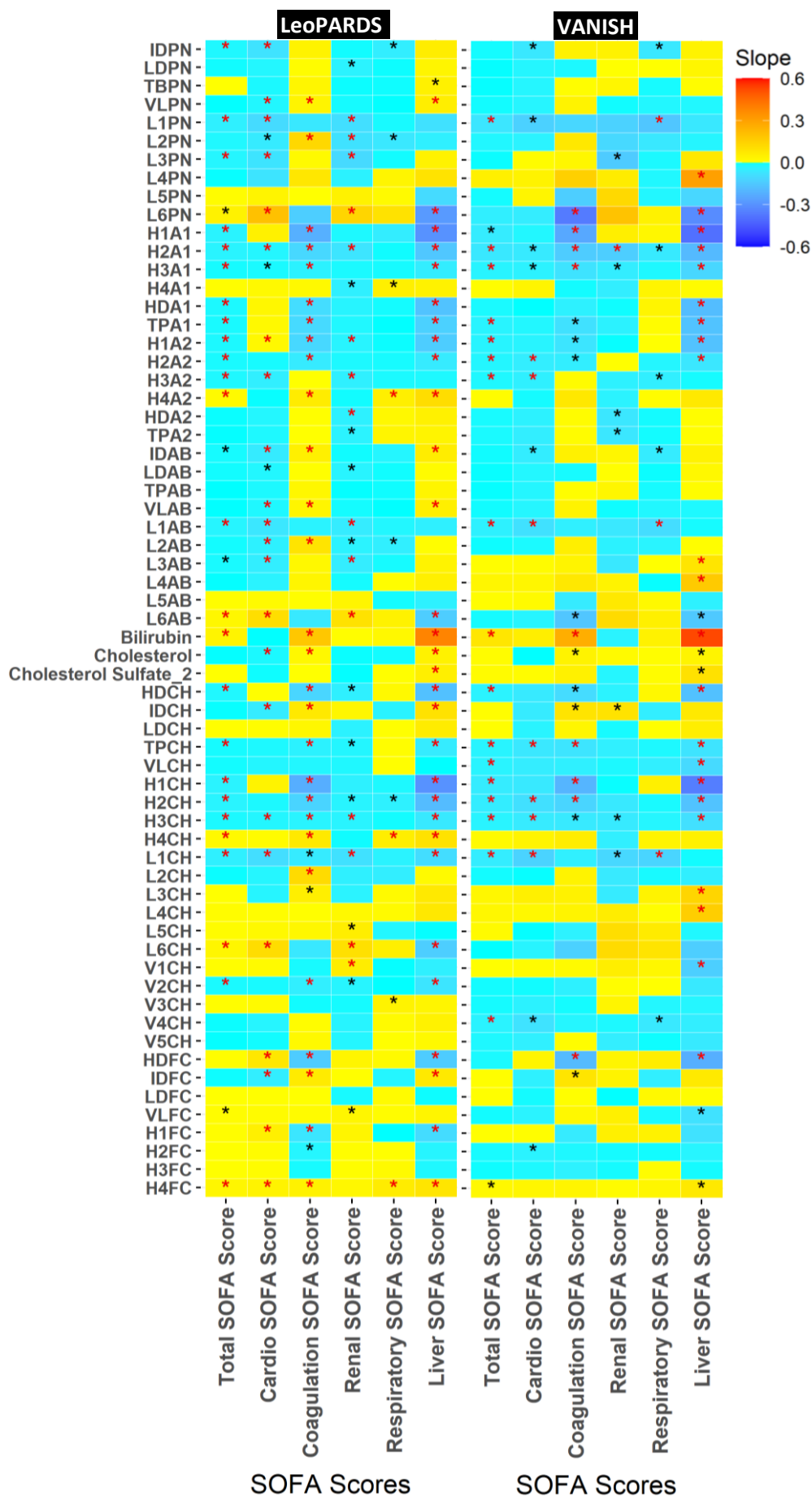
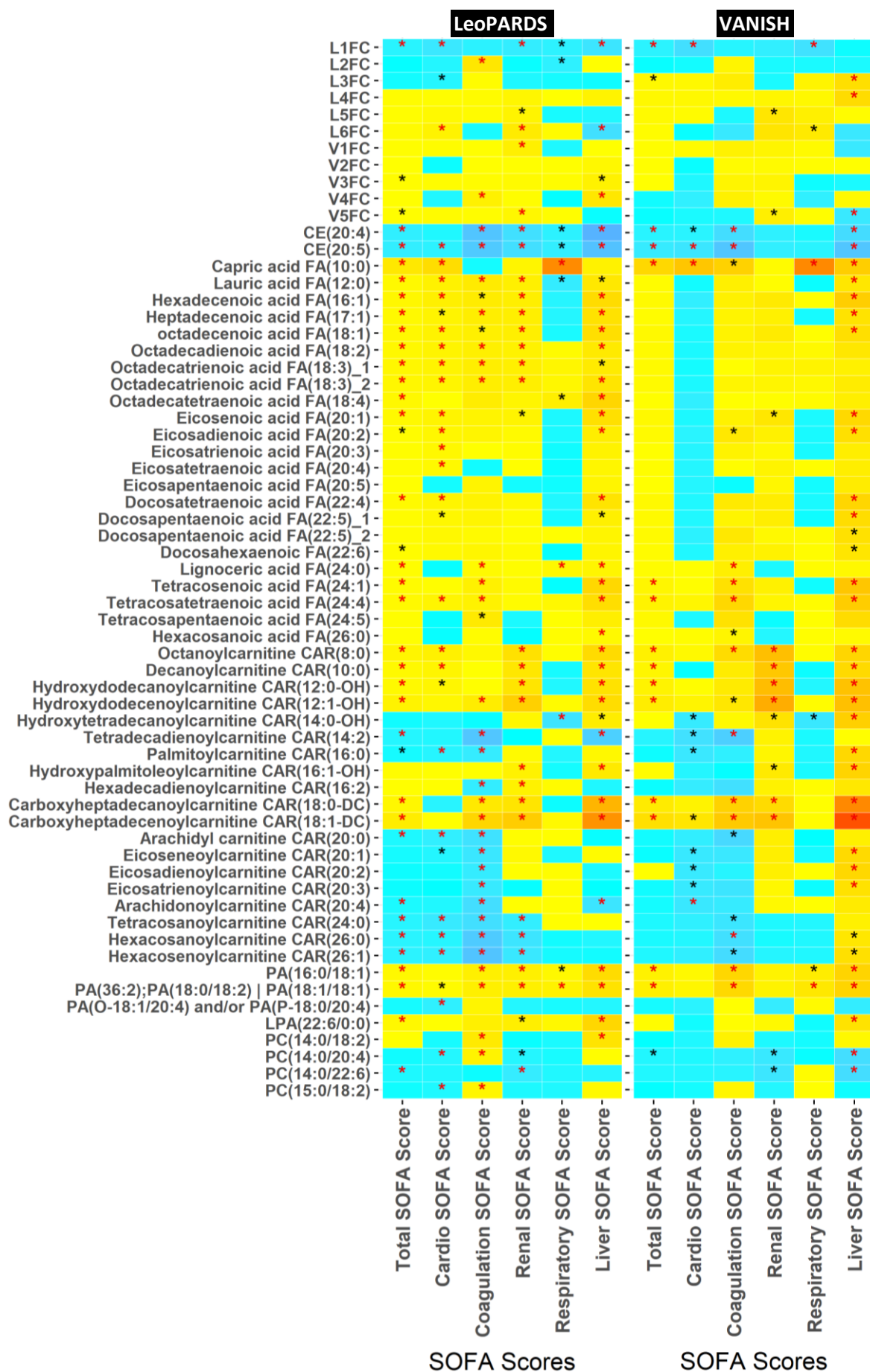


Figure A1.14: ROC curves for the predictive models of mortality on combined BI LISA, LPOS and LNEG ppR dataset for VANISH and LeoPARDS for TP0TP2 samples with fold-changes.

Appendix 1.7: Metabolic changes with organ failure (Lipids and Lipoproteins)

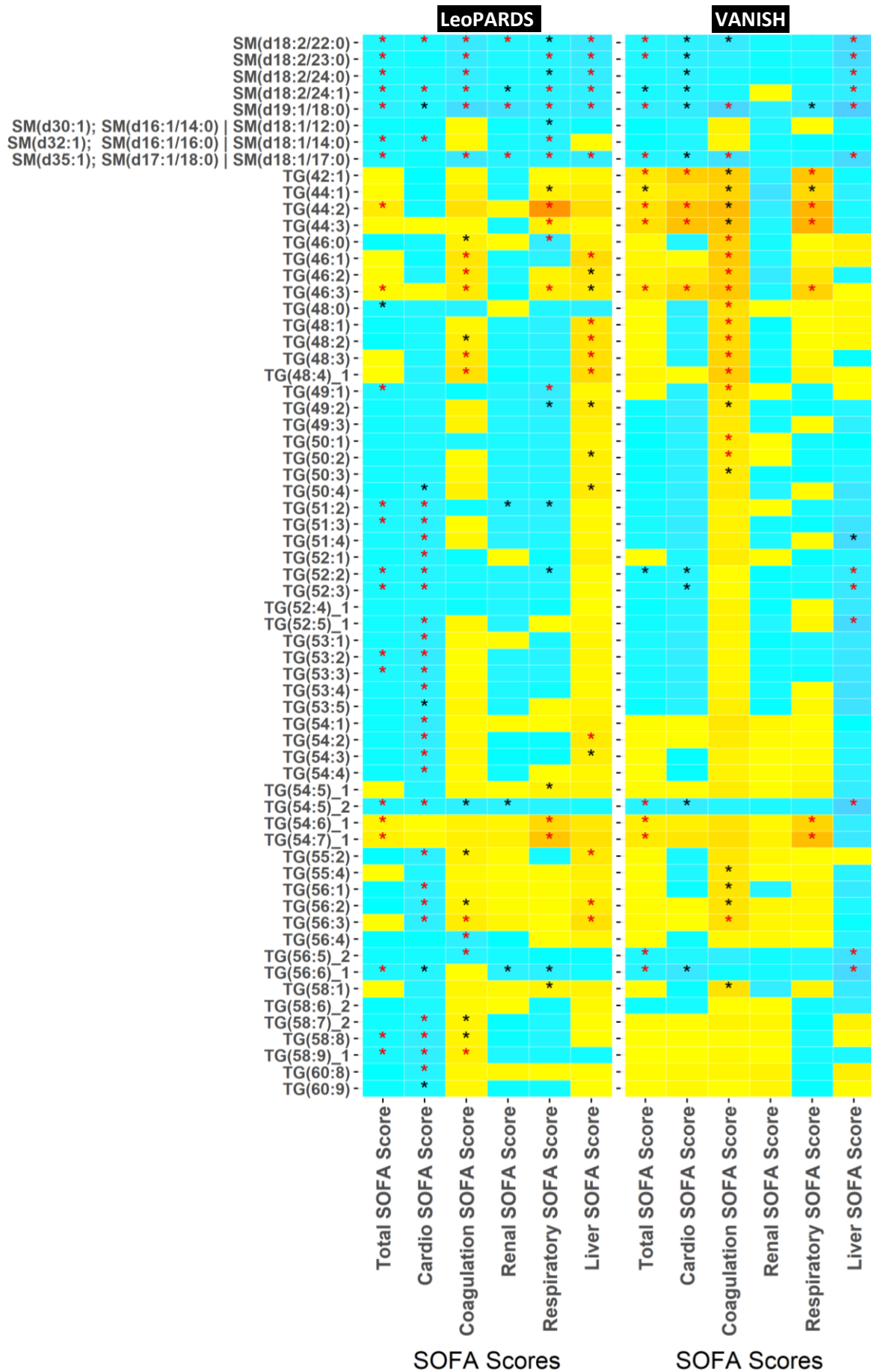




	LeoPARDS						VANISH						
PC(16:0/16:0)-			*		*	*	*		*				*
PC(16:0/17:0)-					*	*			*				*
PC(16:0/18:1)- 2			*			*		*					
PC(16:0/18:2)-		*	*						*				
PC(16:0/18:3)-		*	*			*			*				
PC(16:0/20:4)- 1	*	*											
PC(16:0/20:4)- 3	*			*		*							*
PC(16:0/20:5)-	*	*		*			*						*
PC(16:0/22:4)-	*		*	*		*	*						*
PC(16:0/22:6)-	*					*							*
PC(18:0/18:0)-		*											*
PC(18:0/18:1)-	*		*		*	*	*		*		*		*
PC(18:0/18:2)-		*	*			*	*		*		*		*
PC(18:0/20:3)-	*		*	*	*								*
PC(18:0/20:4)-				*									*
PC(18:0/22:4)-			*	*									
PC(18:0/22:5)-			*		*						*		
PC(18:0/22:6)-	*		*	*		*							*
PC(18:1/20:4)-	*	*		*									*
PC(32:1); PC(14:0/18:1) PC(16:0/16:1)-	*		*			*	*		*				*
PC(33:1); PC(15:0/18:1) PC(16:0/17:1)-			*			*	*		*				*
PC(34:0); PC(18:0/16:0) PC(16:0/18:0)-		*	*			*	*		*				*
PC(40:3)-			*	*									
PC(O-16:0/14:0)-					*	*	*		*				*
PC(O-16:0/15:0)-					*	*	*						*
PC(O-16:0/16:0)-		*				*	*		*				*
PC(O-16:0/18:2)-	*	*			*	*			*				*
PC(O-16:1/18:2) and/or PC(P-16:0/18:2)-	*	*		*	*			*					
PC(O-16:1/22:6) and/or PC(P-16:0/22:6)-	*	*		*	*	*							
PC(O-18:0/16:0)-		*				*	*						*
PC(O-18:0/20:3)-	*		*	*		*			*				
PC(O-18:0/20:4)-	*		*	*									
PC(O-18:0/22:4)-					*								
PC(O-18:1/18:1) and/or PC(P-18:0/18:1)-	*	*		*				*					
PC(O-18:1/22:6) and/or PC(P-18:0/22:6)-	*	*	*	*		*	*		*				
PC(O-22:0/18:2)-	*		*	*									
PC(O-22:0/20:3)-	*		*	*		*							
PC(O-24:0/18:2)-	*		*	*				*					
PC(O-24:0/20:4)-	*				*	*							
PC(O-24:1/20:3) and/or PC(P-24:0/20:3)-	*		*			*							
PC(O-24:1/20:4) and/or PC(P-24:0/20:4)-	*		*										
PC(O-24:1/22:4) and/or PC(P-24:0/22:4)-	*		*										
PC(O-24:2/20:4) and/or PC(P-24:1/20:4)-	*		*										
PC(O-26:1/20:4) and/or PC(P-26:0/20:4)-		*	*						*				
LPC(0:0/14:0)-	*	*	*	*		*	*	*	*	*	*		*
LPC(0:0/15:0)-	*	*	*	*	*	*	*	*	*	*	*		*
LPC(0:0/16:0)-	*	*	*	*		*	*	*	*	*	*		*
LPC(0:0/16:1)-	*	*	*	*		*	*	*	*	*	*		*
LPC(0:0/18:0)-	*	*	*	*		*	*	*	*	*	*		*
LPC(0:0/18:1)-	*	*	*	*		*	*	*	*	*	*		*
LPC(0:0/18:2)-	*	*	*	*		*	*	*	*	*	*	*	*
LPC(0:0/18:3)-	*	*	*	*		*	*	*	*	*	*		*
LPC(0:0/20:2)-	*	*	*	*		*	*	*	*	*	*		*
LPC(0:0/20:3)-	*	*	*	*		*	*	*	*	*	*	*	*
LPC(0:0/20:4)-	*	*	*	*	*	*	*	*	*	*	*		*
LPC(0:0/20:5)-	*	*	*	*	*	*	*	*	*	*	*	*	*
LPC(0:0/22:5)- 1	*	*	*	*	*	*	*	*	*	*	*		*
LPC(0:0/22:6)-	*	*	*	*		*	*	*	*	*	*		*
LPC(14:0/0:0)-	*	*	*	*		*	*	*	*	*	*		*
LPC(15:0/0:0)-	*	*	*	*	*	*	*	*	*	*	*		*
LPC(16:0/0:0)-	*	*	*	*		*	*	*	*	*	*		*
LPC(16:1/0:0)-	*	*	*	*		*	*	*	*	*	*		*
LPC(17:0/0:0)-	*	*	*	*		*	*	*	*	*	*		*
	Total SOFA Score	Cardio SOFA Score	Coagulation SOFA Score	Renal SOFA Score	Respiratory SOFA Score	Liver SOFA Score	Total SOFA Score	Cardio SOFA Score	Coagulation SOFA Score	Renal SOFA Score	Respiratory SOFA Score	Liver SOFA Score	
	SOFA Scores						SOFA Scores						

	LeoPARDS						VANISH					
LPC(17:1/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(18:0/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(18:1/0:0)_1	*	*	*	*	*	*	*	*	*	*	*	*
LPC(18:2/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(18:3/0:0)_1	*	*	*	*	*	*	*	*	*	*	*	*
LPC(18:3/0:0)_2	*	*	*	*	*	*	*	*	*	*	*	*
LPC(19:0/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(20:0/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(20:1/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(20:2/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(20:3/0:0)_1	*	*	*	*	*	*	*	*	*	*	*	*
LPC(20:3/0:0)_2	*	*	*	*	*	*	*	*	*	*	*	*
LPC(20:4/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(20:5/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(22:4/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(22:5/0:0)_1	*	*	*	*	*	*	*	*	*	*	*	*
LPC(22:5/0:0)_2	*	*	*	*	*	*	*	*	*	*	*	*
LPC(22:6/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(24:0/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(O-16:0/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(O-18:0/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(O-18:1/0:0)_1	*	*	*	*	*	*	*	*	*	*	*	*
LPC(O-24:0/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(O-24:1/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(P-16:0/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPC(P-18:0/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
PE(16:0/18:1)-	*	*	*	*	*	*	*	*	*	*	*	*
PE(16:0/18:2)-	*	*	*	*	*	*	*	*	*	*	*	*
PE(16:0/20:4)-	*	*	*	*	*	*	*	*	*	*	*	*
PE(18:0/18:2)-	*	*	*	*	*	*	*	*	*	*	*	*
PE(18:0/20:3)-	*	*	*	*	*	*	*	*	*	*	*	*
PE(18:0/20:4)-	*	*	*	*	*	*	*	*	*	*	*	*
PE(18:0/22:4)-	*	*	*	*	*	*	*	*	*	*	*	*
PE(18:0/22:5)-	*	*	*	*	*	*	*	*	*	*	*	*
PE(18:0/22:6)-	*	*	*	*	*	*	*	*	*	*	*	*
PE(36:1); PE(18:0/18:1) PE(18:1/18:0)-	*	*	*	*	*	*	*	*	*	*	*	*
PE(O-16:1/18:2) and/or PE(P-16:0/18:2)-	*	*	*	*	*	*	*	*	*	*	*	*
PE(O-16:1/20:4) and/or PE(P-16:0/20:4)-	*	*	*	*	*	*	*	*	*	*	*	*
PE(O-16:1/22:5) and/or PE(P-16:0/22:5)-	*	*	*	*	*	*	*	*	*	*	*	*
PE(O-16:1/22:6) and/or PE(P-16:0/22:6)-	*	*	*	*	*	*	*	*	*	*	*	*
PE(O-18:1/18:1) and/or PE(P-18:0/18:1)-	*	*	*	*	*	*	*	*	*	*	*	*
PE(O-18:1/18:2) and/or PE(P-18:0/18:2)-	*	*	*	*	*	*	*	*	*	*	*	*
PE(O-18:1/20:4) and/or PE(P-18:0/20:4)-	*	*	*	*	*	*	*	*	*	*	*	*
PE(O-18:1/22:6) and/or PE(P-18:0/22:6)-	*	*	*	*	*	*	*	*	*	*	*	*
PE(O-20:1/20:4) and/or PE(P-20:0/20:4)-	*	*	*	*	*	*	*	*	*	*	*	*
LPE(0:0/18:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPE(0:0/18:1)-	*	*	*	*	*	*	*	*	*	*	*	*
LPE(0:0/18:2)-	*	*	*	*	*	*	*	*	*	*	*	*
LPE(0:0/20:4)-	*	*	*	*	*	*	*	*	*	*	*	*
LPE(0:0/22:6)-	*	*	*	*	*	*	*	*	*	*	*	*
LPE(16:0/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPE(18:0/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPE(18:1/0:0)_1	*	*	*	*	*	*	*	*	*	*	*	*
LPE(18:2/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPE(20:4/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPE(22:6/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPE(P-18:0/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPG(18:0/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPG(18:1/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPG(18:2/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPI(16:1/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPI(18:0/0:0)-	*	*	*	*	*	*	*	*	*	*	*	*
LPI(18:1/0:0)_1	*	*	*	*	*	*	*	*	*	*	*	*
	Total SOFA Score	Cardio SOFA Score	Coagulation SOFA Score	Renal SOFA Score	Respiratory SOFA Score	Liver SOFA Score	Total SOFA Score	Cardio SOFA Score	Coagulation SOFA Score	Renal SOFA Score	Respiratory SOFA Score	Liver SOFA Score
	SOFA Scores						SOFA Scores					

	LeoPARDS						VANISH					
LPI(18:2/0:0)-					*	*	*					*
LPI(20:3/0:0)_1	*	*	*				*	*	*			*
LPI(20:3/0:0)_2												
LPI(20:4/0:0)-			*				*		*			*
LPI(22:5/0:0)-						*	*					*
LPS(18:0/0:0)-	*	*	*	*	*		*		*	*	*	*
HDPL-	*	*	*	*	*		*	*	*	*	*	*
IDPL-		*					*					
LDPL-	*	*		*					*			
VLPL-					*							*
H1PL-	*		*	*	*	*	*		*			*
H2PL-	*	*	*	*	*	*	*	*	*	*	*	*
H3PL-	*	*	*	*	*	*	*	*	*	*	*	*
H4PL-				*	*	*			*	*	*	*
L1PL-	*	*	*	*	*		*	*		*	*	
L2PL-		*	*	*	*	*						
L3PL-		*		*								*
L4PL-												*
L5PL-												
L6PL-		*	*	*		*		*				*
V1PL-												*
V2PL-			*	*	*							*
V3PL-				*								
V4PL-		*		*			*	*				
V5PL-												*
Sphingosine-1-phosphate	*		*	*		*	*		*			*
Cer(d18:1/16:0)-		*	*			*	*	*			*	*
Cer(d18:1/18:0)-							*	*		*		*
Cer(d18:1/20:0)-		*					*	*				*
Cer(d18:1/22:0)-		*					*	*				*
Cer(d18:1/24:0)-	*	*		*			*	*				*
Cer(d18:1/24:1)-		*	*									*
Cer(d18:2/22:0)-		*	*				*					*
Cer(d18:2/24:0)-	*	*	*				*					*
Cer(d34:0);Cer(d18:0/16:0)		*	*			*						*
Cer(d41:1); Cer(d18:1/23:0)	*	*	*	*			*	*				*
Cer(d42:3); Cer(d18:2/24:1)	*	*	*			*						*
Cer(t18:0/24:1)-	*	*	*		*							*
HexCer(d18:1/16:0)-						*	*		*			*
HexCer(d18:1/22:0)-	*	*	*	*								*
HexCer(d18:1/23:0)-				*								*
HexCer(d18:1/24:0)-			*									*
SulfoHexCer(d18:1/16:0(OH))	*	*										
SulfoHexCer(d18:1/24:0(OH))												
SulfoHexCer(d18:1/24:1(OH))	*	*										
SulfoHexCer(d18:2/16:0(OH))	*	*			*	*						*
LacCer(d18:1/16:0)-	*	*	*		*							*
LacCer(d42:3)-	*	*	*	*	*	*						*
SM(d16:1/22:0)-	*	*	*	*	*	*	*	*	*		*	*
SM(d17:1/16:0)-	*	*	*	*	*	*	*	*		*		*
SM(d17:1/24:1)-	*	*	*	*	*	*	*	*		*		*
SM(d18:0/16:0)-	*	*		*	*	*		*				*
SM(d18:1/16:0)-	*	*		*	*	*		*				*
SM(d18:1/18:0)-	*	*	*	*	*	*	*	*	*	*	*	*
SM(d18:1/20:0)-	*	*	*	*	*	*	*	*	*	*	*	*
SM(d18:1/20:1)-	*	*	*	*	*	*	*	*	*	*	*	*
SM(d18:1/22:0)-	*	*	*	*	*	*	*	*	*	*	*	*
SM(d18:1/23:0)-	*	*	*	*	*	*	*	*	*	*	*	*
SM(d18:1/24:0)-	*	*	*	*	*	*	*	*	*	*	*	*
SM(d18:1/24:1)-	*	*	*	*	*	*	*	*	*	*	*	*
SM(d18:2/14:0)-	*	*	*	*	*	*	*	*	*	*	*	*
SM(d18:2/16:0)_1	*	*	*	*	*	*	*	*	*	*	*	*
SM(d18:2/18:0)-	*	*	*	*	*	*	*	*	*	*	*	*
	Total SOFA Score	Cardio SOFA Score	Coagulation SOFA Score	Renal SOFA Score	Respiratory SOFA Score	Liver SOFA Score	Total SOFA Score	Cardio SOFA Score	Coagulation SOFA Score	Renal SOFA Score	Respiratory SOFA Score	Liver SOFA Score
	SOFA Scores						SOFA Scores					



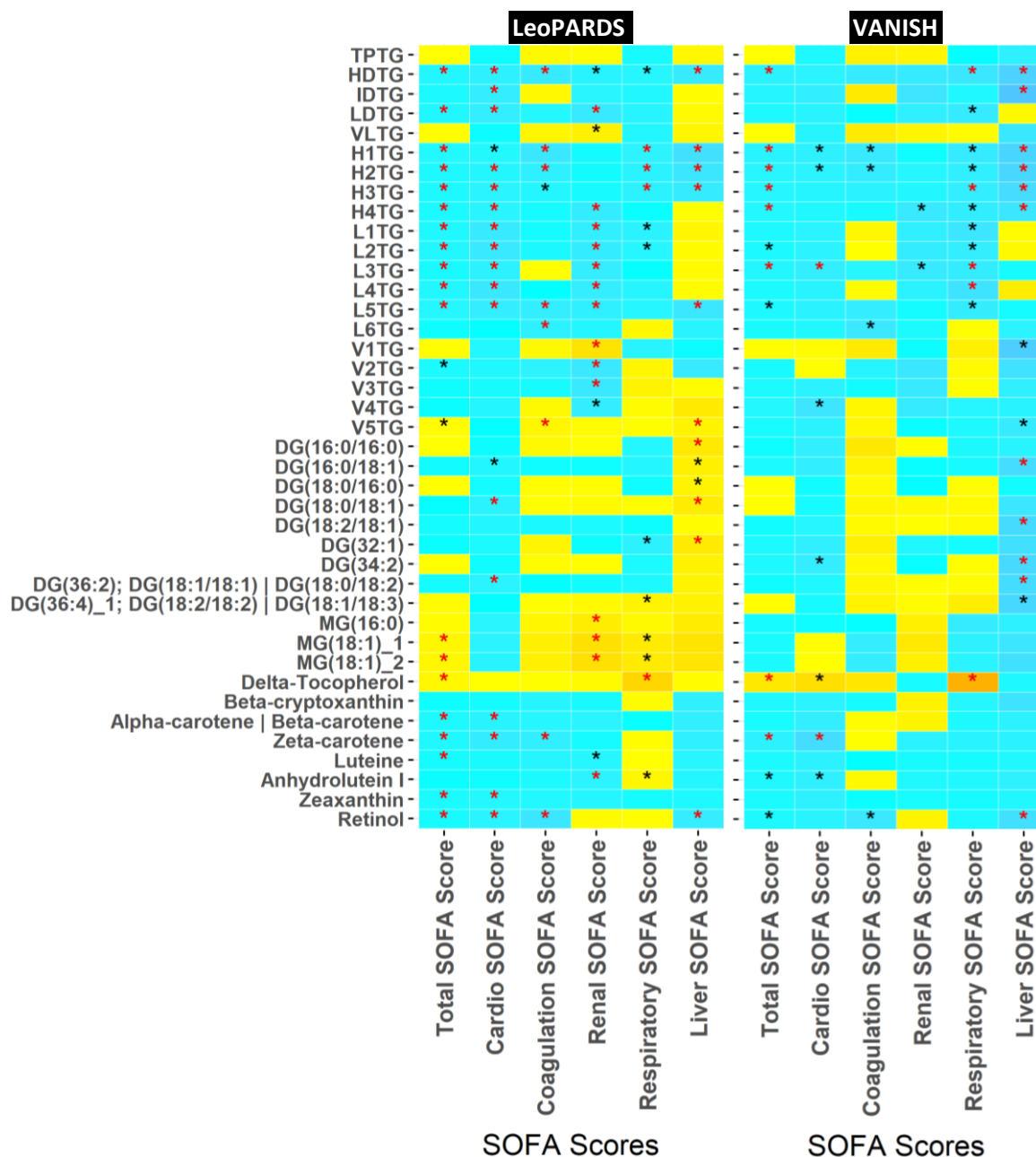


Figure A1.15: The results of LME models based on the SOFA scores for combined BI LISA, LPOS ppR and LNEG ppR log transformed data with age, sex and BMI as confounders for LeoPARDS and VANISH. Identity contrast was performed via contrast coding and the outcomes are shown here. LME model output slope (i.e., change in metabolite levels with SOFA scores) is presented for each SOFA score. The scale for slope ranges from positive to negative values, where positive values indicate higher levels of metabolites when the SOFA score is high whilst negative values indicate lower levels of metabolites when the SOFA score is high and stronger the colour the more the levels of metabolites with higher SOFA scores. Unadjusted p-values calculated with Satterthwaite approximation method for degrees of freedom are shown in black asterisk and represent whether the slope for metabolites for each SOFA score is different from 0 or not. Adjusted p-values calculated with B-H correction are shown in red asterisk. Abbreviations: CH = Cholesterol; FC = Free Cholesterol; PL = Phospholipid; TG = Triglycerides; A1 = Apo-A1; A2 = Apo-A2, AB = Apo-B; HD = High density lipoprotein (HDL); VL = Very low density lipoprotein (VLDL); ID = Intermediate density lipoprotein (IDL); LD = Low density lipoprotein; TPCH = Total plasma Cholesterol; VLCH = VLDL Cholesterol; V1-V5CH = VLDL Cholesterol subfractions 1-5; IDCH = IDL Cholesterol; LDCH = LDL Cholesterol; L1-6CH = LDL Cholesterol subfractions 1-6; HDCH = HDL Cholesterol; H1-4CH = HDL Cholesterol subfractions 1-4; VLFC =

VLDL Free Cholesterol; V1-V5FC = VLDL Free Cholesterol subfractions 1-5; IDFC = IDL Free Cholesterol; LDFC = LDL Free Cholesterol; L1-L6FC = LDL Free Cholesterol subfractions 1-6; HDFC = HDL Free Cholesterol; H1-H4FC = HDL Free Cholesterol subfractions 1-4; TPA1 = Total Plasma A1; HDA1 = HDL Apo-A1; H1-H4A1 = HDL Apo A1 subfractions 1-4; TPA2 = Total plasma A2; H1-H4A2 = HDL Apo A2 subfractions 1-4; TPAB = Total plasma Apo-B; LDAB = LDL Apo-B; L1-L6AB = LDL Apo-B subfractions 1-6; IDAB = IDL Apo-B; TPBN: Total blood particle number; VLPN = VLDL Particle Number; IDPN = IDL Particle Number; LDPN = LDL Particle Number; L1-L6PN = LDL Particle number subfractions 1-6; TPTG = Total Plasma Triglycerides; VLTG = VLDL Triglycerides; V1-V5TG = VLDL Triglycerides subfractions 1-5; IDTG = IDL Triglycerides; LDTG = LDL Triglycerides; L1-L6TG = LDL Triglycerides subfractions 1-6; HDTG = HDL Triglycerides; H1-H4TG = HDL Triglycerides subfractions 1-4; VLPL = HDL Phospholipids; V1-V5PL = VLDL Phospholipid subfractions 1-5; IDPL = IDL Phospholipids; LDPL = LDL Phospholipids; L1-L6PL = LDL Phospholipids subfractions 1-6; HDPL = HDL Phospholipids; H1-H4PL = HDL Phospholipids subfractions 1-4; PC = Phosphatidylcholine; LPC = Lysophosphatidylcholine; PE = Phosphatidylethanolamine; LPE = Lysophosphatidylethanolamine; PI = Phosphatidylinositol; LPI = Lysophosphatidylinositol; LPG = Lysophosphatidylglycerol; PA = Phosphatidic acid; LPA = Lysophosphatidic acid; LPS = Lysophosphatidylserine; SM = Sphingomyelin; Cer = Ceramide; LacCer = Lactosylceramide; HexCer = Hexosylceramide; SulfoHexCer = Sulfohexosylceramide; CE = Cholesteryl esters; DG = Diacylglycerol; MG = Monoacylglycerols; TG = Triglycerides; CAR = Carnitine; FA = Fatty acid. Note: Carbon atoms and double bonds in all lipid species are represented as (C atoms:double bond); d indicates 2 hydroxyl groups in the sphenoid base of ceramides and sphigomyelins; DC denotes for dicarboxylic acid; Prefixes "OH", "O" and "P" represents different substituents/linkages in the lipid species: "OH" denotes for hydroxyl group, "O" represents an alkyl ether linkage whilst "P" refers to the plasmalogen link. Lipid species with abbreviations _1 and _2 indicate same m/z value but different retention times for those lipids.