

Metabolic Phenotyping of Acute and Chronic Liver disease characterised by ^1H NMR spectroscopy and UPLC-MS analysis

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Declaration of Originality

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Abstract

Liver disease is a major cause of premature mortality, the incidence of which continues to rise steadily in the UK. Cirrhosis is the most common form, with a significant risk to patients who can deteriorate from acute decompensation (AD) to acute-on-chronic liver failure (ACLF). Mechanisms of cell death and immune dysfunction drive disease pathogenesis and have been linked to metabolic perturbations associated with the progression of cirrhosis to ACLF and short-term mortality. In this thesis, metabolic phenotypes of plasma and serum from patients with AD, ACLF and acute liver failure (ALF) were characterised using both ^1H NMR spectroscopy and ultra-performance liquid chromatography – mass spectrometry (UPLC-MS). Untargeted ^1H NMR spectroscopy and targeted UPLC-MS analysis of amino compounds, confirmed perturbations of metabolic pathways related to energy metabolism and the urea cycle, which were common to ALF, AD and ACLF. In addition, targeted UPLC-MS analysis of amino compounds identified unique metabolic perturbations for ACLF and ALF, which included increased concentrations of alanine, asparagine, glutamine, glycine, histidine, lysine, proline, threonine and tyrosine in ALF together with a reduced concentration of serine in ACLF. Furthermore, the quantitative amino compound data also indicated a greater metabolic perturbation in ALF compared to ACLF.

Untargeted UPLC-MS lipid profiling also identified metabolic perturbations that were both common and discriminant for ACLF, AD and ALF. Lipids including lysophosphatidylcholines (LysoPCs), sphingomyelins (SMs) and triglycerides (TGs) were depleted in these groups relative to healthy controls (HCs), whilst lysophosphatidic acids (LPA) were elevated and phosphatidylcholines (PC) were both elevated and depleted. Furthermore, lipid profiles generated from AD and ACLF patients were superior to standard clinical scores including CLIF-SOFA and MELD for predicting 30-day mortality based on AUROC analysis, with SM and LysoPC depletion associated with 30-day mortality in AD and ACLF patients.

In conclusion, this thesis identifies discriminant metabolic phenotypes of ACLF vs ALF and AD vs ACLF for the first time. In addition, metabolic changes associated with mortality risk are also identified, which highlights the potential for metabolic phenotyping to drive personalised predictive models of outcome in the clinical setting.

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Contributions of others

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Abbreviations

¹H NMR – Proton Nuclear Magnetic Resonance
AA – Amino Acid
AAA – Aromatic Amino Acid
AABA – α -Aminoisobutyric Acid
AARS – Aminoacyl-tRNA Synthetase
ACLF – Acute-on-Chronic Failure
ACN – Acetonitrile
AD – Acute Decompensation
ALF – Acute Liver Failure
ALI – Acute Liver Injury
ALP – Alkaline Phosphatase
ALT – Alanine Aminotransferase
APACHE II – Acute Physiology, Age, Chronic Health Evaluation II
APAP – Acetaminophen
AUC – Area Under the Curve
AST – Aspartate Transaminase
ATX – Autotaxin
BAIBA – β -Aminoisobutyric Acid
BCAA – Branched Chain Amino Acid
CARs – Compensatory Anti-inflammatory Response Syndrome
CLIF-SOFA – Chronic Liver Failure-Sequential Organ Failure Assessment
CPMG – Carr Purcell Meiboom Gill
CRP – C-Reactive Protein
CV-ANOVA – Cross-Validated Residuals – Analysis Of Variance
CV – Coefficient of Variation
DAG – Diacylglycerol
DDA – Data Dependent Acquisition
DILI – Drug Induced Liver Injury
DRC – Drug Related Compound
ESI – Electrospray Ionisation
ELT – Emergency Liver Transplantation
FA – Fatty Acid
FDR – False Discovery Rate
FiO₂ – Fraction of inspired Oxygen
GCS – Glasgow Coma Scale
GGT – Gamma-glutamyltransferase
GPC – Glycerophosphocholine
Hb – Haemoglobin
HC – Healthy Control
HCA – Hierarchical Cluster Analysis
HE – Hepatic Encephalopathy
HILIC – Hydrophilic Interaction Chromatography
INR – International Normalized Ratio
KCC – Kings College Criteria
KEGG – Kyoto Encyclopedia of Genes and Genomes
LDH – Lactate Dehydrogenase

LDL – Low-Density Lipoprotein
 LLOQ – Lower Limit Of Quantification
 LOESS – Locally Estimated Scatterplot Smoothing
 LPA – Lysophosphatidic Acid
 LPCAT – Lysophosphatidylcholine Acyltransferase
 LPS – Lipopolysaccharide
 LysoPC – Lysophosphatidylcholine
 MAT- Methionine Adenosyltransferase
 MELD – Model for End stage Liver Disease
 MRM – Multiple Reaction Monitoring
 MSEA – Metabolite Set Enrichment Analysis
 NAFLD – Non-Alcoholic Fatty Liver Disease
 NASH – Non-Alcoholic Steatohepatitis
 NH₄ – Ammonia
 NOESY – Nuclear Overhauser Effect Spectroscopy
 OPLS-DA – Orthogonal Partial Least Squares- Discriminant Analysis
 PA – Phosphatidic Acid
 PBMC – Peripheral Blood Mononuclear Cell
 PC – Phosphatidylcholine
 PDH – Pyruvate Dehydrogenase
 PE – Phosphatidylethanolamine
 PEMT – Phosphatidylethanolamine-N-methyltransferase
 PCA – Principal Component Analysis
 PLA-1 – Phospholipase A1
 PLA-2 – Phospholipase A2
 PLS – Partial Least Squares
 PPM – Parts per million
 PSPH – Phosphoserine Phosphatase
 QC – Quality Control
 Q-TOF – Quadrupole-Time Of Flight
 ROC – Receiver Operating Curve
 RSD – Relative Standard Deviation
 SAME – S-adenosyl-L-methionine (SAME)
 SC – Stable Cirrhosis
 SHMT1 – Serine Hydroxymethyltransferase 1
 SIRS – Systemic Inflammatory Response Syndrome
 SM – Sphingomyelin
 SOFA – Sequential Organ Failure Assessment
 STOCYSY – Statistical Total Correlation Spectroscopy
 TCA – The Citric Acid
 TG – Triglyceride
 TQ-MS – Triple Quadrupole-Mass Spectrometer
 TSP – (3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionic acid
 ULOQ – Upper Limit Of Quantification
 UKELD – United Kingdom Model for End-Stage Liver Disease
 UPLC-MS –Ultra-Performance Liquid Chromatography-Mass Spectrometry
 VIP – Variable Importance in the Projection
 vLDL – Very Low-Density Lipoprotein

Thesis Structure

Chapter 1: Introduction

- Acute and chronic liver disease: definitions and aetiology
- Key mechanisms: cell death, immune dysfunction and metabolic disturbances
- Metabonomics
- Metabonomics studies on acute and chronic liver disease

Chapter 2: Methods

- ^1H NMR spectroscopic profiling
- UPLC-MS analyses
- Metabolite identification
- Chemometrics

Chapter 3: ^1H NMR spectroscopy

- Demographics and clinical characteristics of the clinical cohort phenotyped in this thesis
- ^1H NMR analysis of plasma

Chapter 4: Targeted UPLC-MS analysis of amino compounds

- UPLC-MS analysis of amino compounds from plasma

Chapter 5: Untargeted UPLC- MS analysis of lipids

- Untargeted UPLC-MS lipid analysis of serum

Chapter 6: Discussion

- Key findings
- Limitations
- Future work



Chapter 1

INTRODUCTION

1.1 Introduction

The incidence of liver disease continues to increase rapidly in the UK, and has now become the third leading cause of premature death, with the three leading causes of liver disease being alcoholism, obesity and viral hepatitis.¹ Mortality rates for liver disease continue to rise and are associated with significant healthcare costs estimated at £2.1 billion annually to date.¹

Cirrhosis is the main cause of liver disease in the UK and often results in liver failure. It is increasing in incidence in the UK in line with rising rates of obesity and alcohol consumption.² The latter factors have the potential to drive progression of cirrhosis from stable cirrhosis (SC) to acute decompensation (AD) and finally acute-on-chronic liver failure (ACLF). ACLF has a high mortality estimated between 30-40% at 28 days post admission³ and accounts for a significant proportion of people requiring emergency liver transplantation (ELT).

Acute liver failure (ALF), on the other hand is a rarer disease and has a lower mortality risk, estimated at 22%, compared to ACLF. It is mainly caused by drug overdose (chiefly paracetamol/acetaminophen (APAP) induced).⁴ Hence, ALF is an important comparator to ACLF and serves as an effective positive control in a number of studies.⁵⁻⁷ Moreover, the exact metabolic differences between the two forms of liver failure are poorly characterised and require further investigation. Additionally, the metabolic differences that explain the progression of AD to ACLF have the potential to improve stratification and identify new therapeutic targets to improve mortality risk.

Furthermore, the systemic inflammatory response syndrome (SIRS) and compensatory anti-inflammatory response syndrome (CARs) have been shown to drive mortality in ACLF and ALF.^{8,9} Identifying global metabolic changes associated with immune dysfunction (immunometabolism),¹⁰ may improve our understanding of key pathways driving disease progression in these different liver failure syndromes. Therefore, an important research area for improving mortality risk and enhancing our mechanistic understanding of processes driving immune dysfunction, will be identifying more sensitive biomarkers that can predict disease progression and mortality in ACLF.

1.2 Aims

The overarching aim of this thesis is to improve understanding of global metabolic changes in acute and chronic liver disease and also metabolic changes associated with disease progression and short-term mortality.

The main aim of this chapter is to provide an overview of our current understanding of the main metabolic disturbances associated with liver dysfunction and the key mechanisms which drive disease pathogenesis. A further aim is to summarise the main findings from previous metabolic phenotyping studies on acute and chronic liver disease syndromes.

1.3 The liver

The liver is the second largest organ in the human body and is responsible for regulating many vital vascular, immunological, metabolic, secretory and excretory functions, as well as, the detoxification and modification of xenobiotic compounds. In particular, the liver is a highly metabolically active organ and is the central hub of the major metabolic processes in the body. Hence, it provides an important source of metabolites for other organs and is also vital to maintaining metabolic homeostasis.¹¹

As a highly vascularized organ the liver receives up to 25% of cardiac output, of which 30% is received through the hepatic artery and 70% through the hepatic portal vein. The portal vein supplies blood containing products of digestion, which include major dietary macromolecules – carbohydrates, lipids and proteins. These are metabolised by the liver and the metabolites produced are then secreted and taken up by other major organs. Furthermore, drugs and other xenobiotics ingested are also received through this route and are detoxified and modified hepatically.¹²

1.4 Liver disease

As its central function is maintaining metabolic homeostasis, liver failure is associated with serious health complications and is considered a medical emergency. The leading cause of liver failure in the UK is cirrhosis, mainly triggered by alcoholism and NAFLD,¹³ frequently resulting in immediate admittance to intensive care. Due to the increasing prevalence of cirrhosis and cases of liver failure, the number of organ donors and liver intensive care facilities

are progressively limited and therefore pose a significant burden on healthcare services in the UK.¹⁴

Both ALF and ACLF are associated with high mortality rates - particularly from sepsis, as well as, multi-organ failure and high clinical care costs, as patients are generally treated in the critical care setting, with some requiring emergency liver transplantation (ELT).^{15,16} These patients often require physiological support such as fluid replacement and maintenance, nutritional support to maintain metabolic homeostasis, and often additional organ support.^{17,18} Although, these patients frequently require ELT, it is still a challenge to accurately predict patient suitability for transplantation,^{15,19} and clinicians rely on clinical scoring systems to prognosticate. These utilise standard blood derived markers of hepatic dysfunction such as bilirubin, INR (international normalized ratio - measure of blood coagulation), creatinine and albumin levels. A schematic overview of AD, ACLF and ALF development is provided in Figure 1.

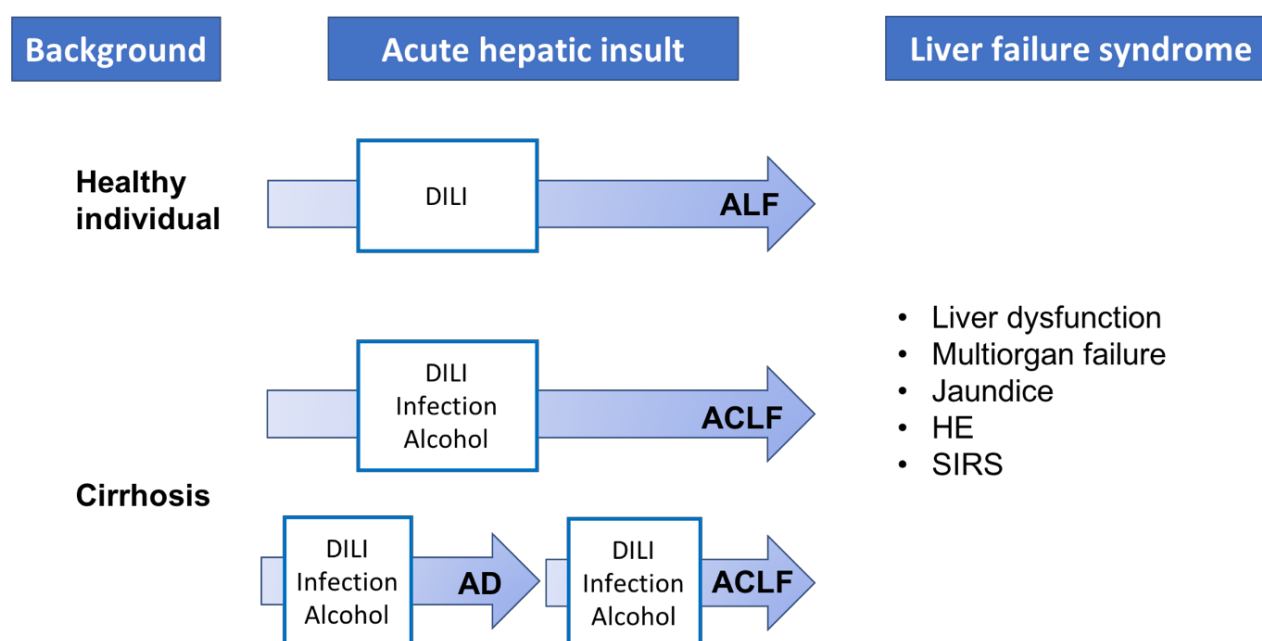


Figure 1. An overview of AD, ACLF and ALF development

ALF develops in individuals who have no background of liver disease, whilst AD and ACLF develop in individuals with pre-existing cirrhosis. ALF – acute liver failure; AD – acute decompensation; ACLF – acute-on-chronic liver failure; DILI – drug induced liver injury; HE – hepatic encephalopathy; SIRS – systemic inflammatory response syndrome;

1.4.1 Acute liver disease

Acute liver disease in this thesis included patients with ALF and acute liver injury (ALI). ALF is characterised by the rapid onset of hepatocellular injury, hepatic encephalopathy (HE)

and coagulopathy, with the absence of pre-existing liver disease and results in a loss of hepatic metabolic and immunological function,¹⁸ whilst in ALI there is an absence of HE.²⁰ In this study the main cause of ALI was drug overdose, predominantly acetaminophen (APAP) overdose. ALI is an early form of ALF and in the case of APAP overdose is reversible if treated immediately with a recovery rate of up to 93%.²⁰ In this study, the ALI group was a small group and served as the main comparator for progression of acute liver disease to ALF. The key focus was on ALF and comparisons to ACLF, hence ALF is discussed in more detail below.

ALF is defined as the onset of liver symptoms within eight weeks of the initial insult in patients with no prior liver injury.¹⁶ The ALF patients in this thesis were defined according to the “O’Grady” criteria.²¹ In the developed world, the predominant aetiology of ALF is APAP overdose, with its incidence reported to be increasing in America and Western Europe.¹⁹

The Kings College Criteria (KCC), is the prognostic scoring system currently used to determine disease severity in patients with ALF.²² The sensitivity of a number of additional prognostic scoring systems have also been investigated for ALF. These include MELD (model for end stage liver disease) and the Child-Pugh scores, which are generally used to assess severity of chronic liver disease to aid clinical decision making for liver transplantation. Nevertheless, these scores have often proven to be inaccurate in determining patient outcomes and suitability of patients for ELT, hence, a strong reliance on these scores is not recommended.²³

1.4.2 Chronic liver disease

Cirrhosis is a fibrotic disease, whereby healthy liver tissue is progressively replaced by abnormal fibrous scar tissue.²⁴ Recently, it was reported that cirrhosis accounts for 1.7% of mortality in Europe, highlighting the significant burden of this disease in the developed world.²⁵ The most common cause of cirrhosis is excessive alcohol consumption²⁶ which is a more significant problem in the UK compared to the rest of Europe.²⁷ There are various forms of cirrhosis as recently defined by the CANONIC criteria³ which include stable cirrhosis (SC), where the liver is scarred but there is enough healthy tissue for the liver to function adequately. AD occurs in individuals with pre-existing cirrhosis who, following an acute hepatic insult, present with complications such as ascites, HE, jaundice and bleeding varices, and require critical care support and are often in urgent need of a liver transplant. ACLF, occurs following an acute insult to the liver, in an individual who already has pre-existing cirrhosis causing them to suffer liver

failure. Hence, ACLF is a medical emergency, where the patient requires immediate physiological support in an intensive care unit, as they are at high risk of multi organ failure.²⁸

A number of clinical scoring systems have been used to prognosticate cirrhosis including UKELD, MELD and the Child Pugh Score, where, MELD was previously considered the most superior prognostic model.²⁹ However, these scores do not take into account organ failure which is the main factor driving short term mortality in AD and ACLF.²⁸ The UKELD score is widely used to determine patient suitability for liver transplantation in the case of chronic liver disease in the UK.³⁰ Whilst, the CLIF-SOFA score which is adapted from the SOFA score, incorporates multi organ failure, can also determine disease severity for AD and ACLF³¹ which are associated with high short term mortality at 28 day and 90 days post admission.³

1.5 Key mechanisms of liver disease

Cell death, immune dysfunction and metabolic disturbance are common pathophysiological mechanisms in AD, ACLF and ALF. These mechanisms advance disease progression and exacerbate liver injury and are therefore associated with mortality. Mechanisms of mitochondrial oxidative stress caused by injurious stimuli, such as toxic drug metabolites in ALF and alcohol or lipotoxicity in cirrhosis, initiate cell death mechanisms.³² Furthermore, there is significant cross talk between mechanisms of cell death and inflammation.³³ However, the precise mechanistic links between metabolic perturbation and cell death or inflammation in liver disease are less clear and have been mainly associated with dysfunctional lipid metabolism.^{34–36}

1.5.1 Cell death

Apoptosis and necrosis are the main forms of hepatocyte death reported in AD, ACLF and ALF.^{37,38} The specific contribution of each of these forms of cell death to liver dysfunction remains a controversial subject.³⁹ The mode of cell death is dependent on a number of factors, including aetiology and the duration and magnitude of liver injury which is hypervariable in both acute and chronic liver disease.⁴⁰ Whilst acute liver disease is characterised by excessive cell death, which is central to liver damage, in chronic liver disease sustained cell death is associated with the development of cirrhosis from initial fibrosis.⁴¹

Apoptosis, is a highly regulated mechanism which proceeds via two signalling pathways that are controlled by a group of cysteine proteases called caspases.³⁹ Whilst necrosis occurs as a result of signal transduction pathways activating RIP (receptor interacting protein)-1 and RIP-3, which cause mitochondrial dysfunction resulting in cell necrosis.⁴² The key differentiating factor between apoptosis and necrosis is believed to be the involvement of the caspase cascade in apoptosis.⁴¹ Although, apoptosis and necrosis are governed by different signalling pathways, a recent review suggested that there is cross talk between these pathways and indicates that it is the balance of these cell death mechanisms that may determine the extent of cell death and hence the survival of cells and organs in pathophysiological states.⁴³

In order to monitor apoptosis and necrosis, circulating cytokeratin-18 levels which are biomarkers of cell death, can be measured. Caspase cleaved cytokeratin-18 (M30) is indicative of apoptosis and total intact cytokeratin-18 (M65) is released from cells undergoing necrosis.⁴⁴ M30 has been proposed as a prognostic biomarker in ALF for predicting outcome alongside current clinical biomarkers.⁴⁵ In addition, apoptosis in ALF is also hypothesized to occur extra-hepatically and is linked to subsequent multi-organ dysfunction and early mortality.³⁷ These markers have also been detected in AD and ACLF patients, where M65 correlated with severity of disease and both M30 and M65 improved prediction by CLIF-SOFA of the progression of AD to ACLF.³⁸

Furthermore, high rates of cell death are implicated in the development of dysfunctional inflammatory signalling. This occurs due to the upregulated release of damage associated molecular patterns (DAMPs) from ruptured membranes of necrotized cells, which trigger sterile inflammation.⁴⁶

1.5.2 Immune dysfunction

The role of systemic inflammation and immune dysfunction in advancing disease progression and exacerbating liver injury in both ALF and ACLF is now widely accepted.^{8,47} During the inflammatory response, the release of cytokines and chemoattractants activates additional inflammatory cells, causing a cytokine cascade of both pro and anti-inflammatory cytokines and hepatic infiltration by neutrophils promoting further tissue damage.⁴⁸ It has been reported that the clinical features observed in patients with septic shock are comparable to patients with ALF and ACLF.^{8,49} Immune dysfunction in cirrhosis, bacterial overgrowth in the gut and ongoing liver damage, trigger immune activation leading to immune dysfunction.⁵⁰

Whereas, the existence of the systemic inflammatory response syndrome (SIRS), which is characterised by the elevation of pro-inflammatory cytokines, has been observed in patients with ALF and has been correlated with worsening of encephalopathy and outcome.⁸ Furthermore, the emergence of a compensatory anti-inflammatory response syndrome (CARS), which is characterised by an elevation of anti-inflammatory cytokines in response to SIRS, leads to fulminant liver failure, multiple organ dysfunction syndrome (MODS) and functional immunoparesis.⁸ Consequently, a number of immune cell types including T-cells, monocytes, macrophages and neutrophils become dysfunctional in ALF and ACLF.^{6,51–54}

1.5.3 Metabolic disturbance

A dysfunctional liver loses its ability to maintain metabolic homeostasis and subsequently leads to major disturbances in carbohydrate, lipid and protein metabolism, as well as, a disruption of energy metabolism.¹² Hence, metabolic markers of liver function including glucose, urea, creatinine, AST (aspartate transaminase), ALT (alanine transaminase), ALP (alkaline phosphatase), GGT (gamma-glutamyltransferase), bilirubin, INR, albumin, ammonia, lactate and LDH (lactate dehydrogenase) are routinely monitored in liver intensive care units and impact organ support and critical care therapies.⁵⁵

An elevation in circulating concentrations of liver transaminases AST and ALT correlate with liver dysfunction. During end stage liver disease, a rise in ALT and AST is indicative of liver damage, as these enzymes leak from necrotic hepatocytes into the circulation.⁵⁶ These enzymes regulate amino acid pathways linked to the urea cycle and gluconeogenesis.⁵⁷ Both ALT and AST assist with nitrogen clearance, by transferring amine groups from excess amino acids, contributing to the generation of ammonia, which is subsequently metabolised to urea for excretion.⁵⁸

During liver failure, the urea cycle is disrupted leading to hyperammonemia and a reduction in urea and aminoacidemia as the liver loses capacity to remove excess amino acids, which consequently leak into the circulation. Aminoacidemia is well recognised in liver disease and a number of early studies document this metabolic disturbance.^{59–62} Furthermore, hyperammonemia, is associated with the development of HE which is a key feature of liver failure, as high concentrations of circulating ammonia build up in the brain leading to cognitive disturbance.⁶³ On the other hand, branched chain amino acids (BCAAs) including isoleucine, leucine and valine are depleted, and these correlate with an elevation of the aromatic amino acids (AAAs) tyrosine and phenylalanine also defined as the Fischer ratio.⁶⁴ The Fischer ratio is

also associated with HE development, as it leads to the increased influx of AAA into the brain.⁶⁵ Whilst, a reduction in circulating BCAAs have been associated with muscle wasting and malnutrition⁶⁶ which are further hallmarks of liver failure, and supplementation with BCAAs have proven to prevent the progression of cirrhosis to liver failure.⁶⁷

Acidosis is a further major metabolic perturbation present in severe cirrhosis or liver failure and encompasses three main disorders including metabolic alkalosis, ketoacidosis or ketogenesis and lactic acidosis.⁶⁸ These disorders mean a loss of acid base balance which is fundamental to normal cellular and organ function. Metabolic alkalosis, occurs as a result of impaired hepatic albumin synthesis, whilst, ketoacidosis and lactic acidosis occur as a result of elevated concentrations of ketone bodies and lactate due to a disruption of hepatic energy metabolism.⁶⁸

Finally, abnormal lipid metabolism is a further metabolic disturbance associated with liver dysfunction. In both acute and chronic liver disease, a significant depletion of circulating cholesterol and triglyceride concentration is observed as a consequence of impaired lipoprotein synthesis.^{69,70}

1.6 Metabolic phenotyping of liver disease

Metabolic phenotyping or metabonomics, measures metabolic changes in the host metabolome or microbiome in response to disease, changes in physiology or a genetic modification.^{71,72} It uses a 'top-down' systems approach that enables study of multiparametric alterations in metabolism.⁷³ This is in contrast to traditional "bottom-up" approaches testing *a priori* hypotheses, focusing on isolated signalling pathways.

The human metabolome is comprised of a diverse and complex range of metabolites, which are interconnected by multiple pathways that form a network. These vary according to changes in the host genetics, lifestyle, demographic, environment and health status.⁷⁴ Hence, metabolic phenotyping studies focus on large groups of individuals to counteract confounders arising from these influences and to bring focus to phenotypic differences between the variables of interest.⁷⁴ The main principle behind metabolic phenotyping studies in the clinical setting, is that localised disturbances from a particular disease will cause large-scale changes in the host which will present systemically across biofluids and tissues.⁷³ These can then be modelled against healthy individuals to identify precise metabolic changes that occur in response to disease.⁷⁵

The presence of particular metabolites in biofluids such as plasma relate to physiological processes, and by detecting changes in such metabolite concentrations across different biological compartments, novel mechanistic insight can be gained on pathological mechanisms.⁷⁴ Highly sensitive analytical chemistry techniques used in metabolic profiling of biological samples and tissues, include ¹H NMR spectroscopy and ultra-performance liquid chromatography (UPLC), which is coupled to mass spectrometry (UPLC-MS).⁷⁶ These techniques are able to quantify and profile diverse sets of small molecule metabolites (up to <1kDa) at a systems level simultaneously, providing a broad coverage of the metabolome.⁷⁴

Given the sensitivity of metabolic phenotyping techniques, these have the potential to drive forward novel personalised healthcare and dynamic patient stratification strategies.⁷⁷ Better patient stratification is required for patients with liver disease, where prognostication and characterisation of disease progression require improvement.⁷⁸ Furthermore, metabonomics also has the potential to enhance our mechanistic understanding of metabolic differences between acute and chronic forms of liver disease and uncover novel biomarkers of disease.⁷⁹

1.6.1 Metabolic phenotyping studies on acute and chronic liver disease

Metabolic phenotyping studies of plasma or serum in drug induced acute liver disease including ALF and ALI are limited, and focus on small numbers of people and are mainly compared to healthy individuals.^{80–82} The main metabolic perturbations identified from these studies for ALF include aminoacidemia relating to perturbed deamination and transamination and also glutamine elevation, which are both linked to the impairment of the urea cycle.^{80,81} Further perturbations are also observed in the energy metabolites citrate and lactate and also in lipids.⁸⁰ Whereas, the main studies on ALI focus on detection and comparison of drug metabolites and conjugates,⁸³ and one study reveals an elevation of ophthalmic acid, which is present in the sulphur pathway, and is associated with mortality.⁸²

Metabolic phenotyping studies on cirrhosis and ACLF, however, are limited to a lesser extent compared to ALF and ALI.⁸³ Studies focused on cirrhosis and ACLF identify elevation of amino acids including a rise in alanine, methionine, glutamine, phenylalanine, proline, tyrosine, and depletion of BCAAs isoleucine, leucine, valine and serine and a rise in energy metabolites pyruvate.^{84–86} These changes also correlate with the progression of compensated cirrhosis to decompensated cirrhosis.⁸⁵ Furthermore, a depletion of lysophosphatidylcholines (LysoPCs)

relative to healthy controls have also been observed in cirrhosis,⁸⁷ and have been associated with mortality in decompensated cirrhosis.⁸⁸

In addition to the limited study on metabolic profiling of ALF, none of these studies compare ACLF to ALF. Furthermore, metabolic phenotyping studies on the progression of AD to ACLF are also limited. It is therefore, that the study of these comparisons, that forms the main basis of this thesis.



Chapter 2

METHODOLOGY

2.1 Introduction

Metabolic phenotyping typically employs ^1H NMR spectroscopy and liquid chromatography (LC) coupled to mass spectrometry (MS), to generate metabolic profiles from tissues or biological fluids.⁷⁶ Although there are advantages and disadvantages associated with each technique, these techniques are complimentary when used in combination as they can collectively provide a higher coverage of the metabolome by detecting a diverse range of chemical classes.⁸⁹ The key differences between the two analytical platforms include the greater sensitivity and detection range of UPLC-MS, which can therefore profile a greater span of metabolites in contrast to ^1H NMR spectroscopy. Whilst ^1H NMR spectroscopy generates data with superior reproducibility, structural information per metabolite and analysis of data is simpler.⁹⁰ In addition, UPLC-MS analysis can be performed using a targeted approach to analyse a specific group of metabolites, whilst an untargeted approach analyses all measurable metabolites in a sample.⁹¹

In this thesis, a multiplatform approach was applied to phenotype plasma and serum from a clinical cohort of patients and healthy controls. Prior to the preparation of the pooled quality controls (QCs), all serum samples were randomised with respect to class and 90-day survival. The random sample order was generated by the CPC (Clinical Phenome Centre) using in-house software.

^1H NMR spectroscopy was initially used to profile plasma to identify the main metabolite groups perturbed in acute and chronic liver disease. In addition, a targeted UPLC-MS method⁹² for the quantification of amino compounds, was also applied to a subset of plasma samples phenotyped by ^1H NMR analysis. Following these analyses, a UPLC-MS approach was employed using an untargeted method to generate lipid profiles from serum (serum was used as there was insufficient plasma remaining). Serum samples from the same cohort of patients (profiled by ^1H NMR spectroscopy) were analysed by UPLC-MS in both positive and negative ionisation modes. A schematic providing an overview of the methodology applied in this thesis is presented in Figure 2.

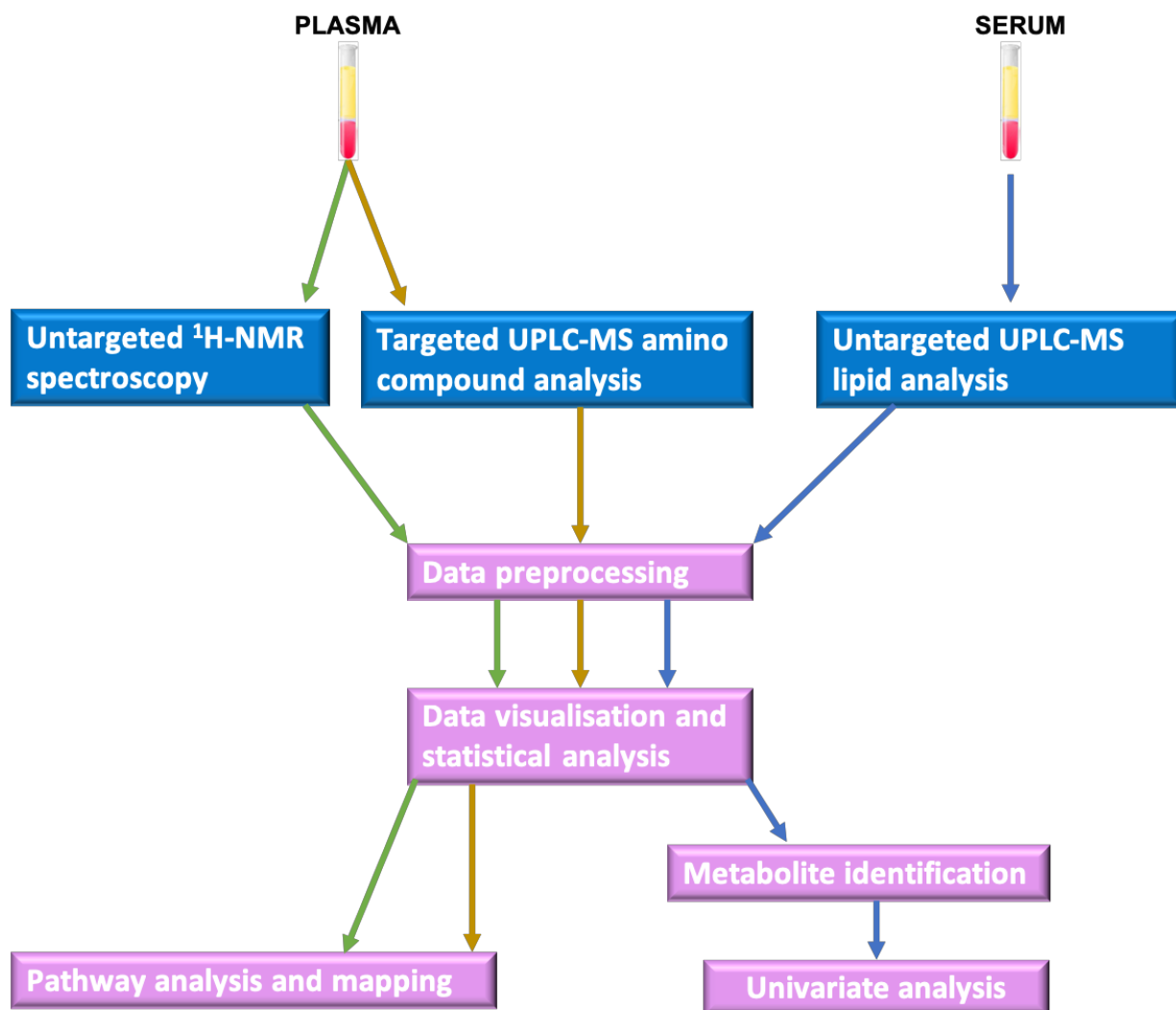


Figure 2. A schematic overview of the methodology undertaken in this thesis

2.1.1 Aims

The main aim of this chapter is to provide an overview of the methodologies applied in this thesis. These include ¹H NMR spectroscopy and UPLC-MS analysis, as well as, subsequent data preprocessing, multivariate statistical analyses and metabolite identification.

2.2 ¹H NMR spectroscopy

The use of high resolution ¹H NMR spectroscopy for profiling biofluids such as urine, plasma or serum is now well established.^{93,94} A structurally diverse range of metabolites can be detected simultaneously using this method in a relatively short time. ¹H NMR spectroscopy provides detailed structural information based on proton (¹H) centred nuclear interactions in

chemical structures.⁹⁵ The metabolic profiles generated by this technique are presented as spectra which comprise of peaks that relate to chemical structures.⁹⁶ Signals are generated in a spectrum by applying a high frequency pulse to a sample placed within a magnetic field, these cause protons in the sample to shift between energy states.⁹⁷ The signal produced is then Fourier transformed generating a chemical shift (the resonance frequency of a nucleus relative to a known standard in a magnetic field), which is then displayed against signal intensity, producing a spectrum.

In this thesis, plasma profiled by ^1H NMR spectroscopy was performed using a 1-dimensional ^1H NMR pulse sequence which is the Carr Purcell-Meiboom-Gill (CPMG). This pulse sequence is applied to enhance signals from small molecule metabolites, whilst attenuating signals from large molecules such as proteins and lipoproteins present in plasma, these tend to overlap and obscure signals from small molecules.⁹³

Individual metabolites were identified by both comparing chemical shift values to published data and also identifying correlation patterns between specific peaks in the spectra. The correlation patterns were identified by performing statistical total correlation spectroscopy (STOCSY).⁹⁸ STOCSY identifies correlation patterns in the spectra which can correspond to metabolite class or structural correlations within a single metabolic compound depending on the strength of the correlation. A colour scale, which ranges from high correlation (1) to low correlation (0), indicates correlation between peaks to a selected peak of interest.⁹⁸ Further details on the ^1H NMR spectroscopic methods applied in this thesis are summarised in Chapter 3.

2.3 Ultra performance liquid chromatography

Liquid chromatography (LC) is typically used in tandem with mass spectrometry (MS) to perform additional separation before MS analysis in metabonomics.⁹¹ Prior separation by LC is performed to improve detection and quantification of metabolites, as well as, reducing ion suppression – where ionisation is disrupted by the presence of additional compounds.⁹⁹ LC is performed using a derivatised silica packed column which consists of the stationary phase, whilst the mobile phase is comprised of two phases made up of organic and aqueous solvents.⁸⁹ In this thesis, ultra-performance liquid chromatography (UPLC) was performed which utilises columns packed with a small particle size of $1.7\mu\text{m}$ under high pressure ranging between

10,000-15,000psi. These parameters increase the chromatographic resolution, as well as, shortening the run time in contrast to standard LC systems.¹⁰⁰

The chromatography performed for both the targeted and untargeted analyses in this thesis used reversed phase chromatography, a commonly used technique in metabolomics studies.⁸⁹ This uses a hydrophobic stationary phase (silica), and the solvent gradient is run through a high aqueous to an organic phase, which allows metabolites to elute in order of polarity, beginning with high polarity metabolites and finishing with low polarity metabolites.⁸⁹

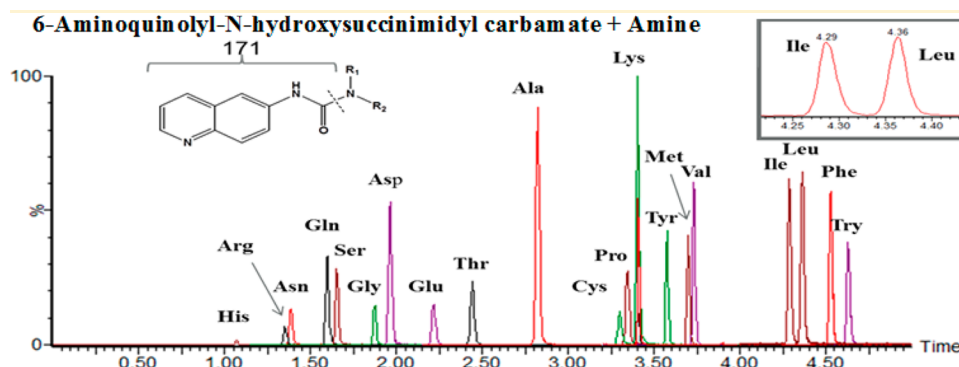


Figure 3. Chromatographic separation of amino-compounds

Adapted from Gray et al¹⁰¹

For untargeted UPLC-MS analyses, the chromatography applied aims to characterise the entire detectable profile of a biological sample. The chromatography for the untargeted lipid analysis in this thesis was developed to detect lipids and therefore the chromatographic gradient for this method is aimed at separating lipids and bypasses polar or aqueous metabolites.¹⁰² Furthermore, for targeted UPLC-MS analyses such as the targeted amine method applied in this thesis, a chromatographic method is developed specifically to measure a specific panel of compounds.¹⁰¹ The chromatography in targeted UPLC-MS analyses aim to maximise separation of the target compounds and ensure clean elution of each analyte. Ultimately, the aim of the method is to incorporate the detection and quantitation of each metabolite from the target panel in a single analysis of a sample. The chromatographic peaks per target analyte elute in specific retention time windows (see Figure 3).

2.4 Mass Spectrometry

Mass spectrometry is an analytical chemistry technique which determines the mass-to-charge ratio (m/z) of molecules from a sample. Briefly, following LC separation, the sample is ionised and passed through a mass analyser. The mass analyser separates the ions according to m/z and passes them to a detector for detection and converts the reading into a digital output. Ionisation is performed by electrospray ionisation (ESI), which uses a needle applied with a high voltage to ionise and spray the analytes into the MS (see Figure 4). This process produces charged liquid droplets that turn gaseous as the solvent evaporates. The dispersed ionised charged droplets are then drawn towards the MS by plate with an opposing electronic charge to the ionised particles.¹⁰³

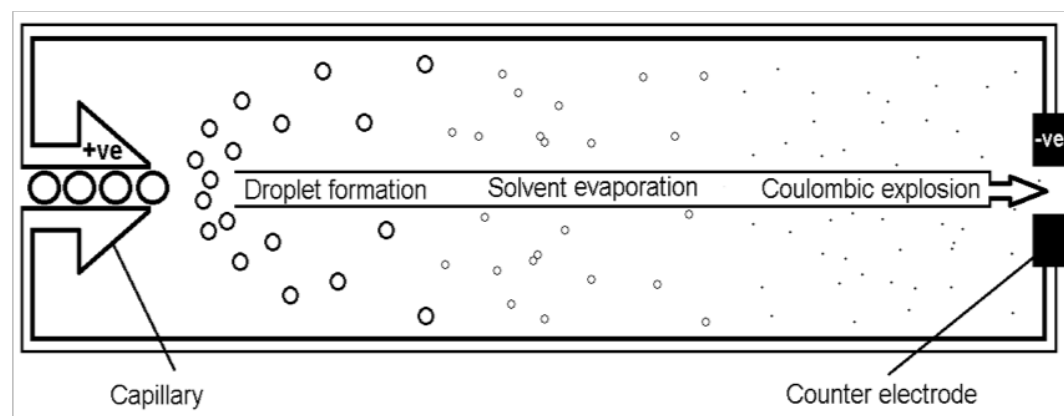


Figure 4. Electrospray ionisation

The two main forms of mass analysers used in this thesis were the quadrupole-time of flight (Q-TOF) and the triple quadrupole (TQ) see Figure 5. The Q-TOF was used for untargeted UPLC-MS lipid profiling whilst the TQ was used for the targeted UPLC-MS amine method.

The Q-TOF measures the time taken for ions to reach the detector using a fixed distance and energy to accelerate the ion to determine the m/z of the metabolites. For untargeted MS analyses of lipids, as a broader mass range is acquired in a single scan, Q-TOF-MS instruments are typically used due to their greater resolution and mass accuracy.¹⁰⁴ A greater resolution improves separation and differentiation of signals from compounds, which have identical or similar m/z values. This analytical quality is crucial for untargeted analyses of biofluids which typically contain lipids with similar nominal masses.

A TQ-MS contains a collision cell in the central quadrupole, where ions are selected and fragmented for further quantification by tandem mass spectrometry (see in section 2.4.1 below). The TQ-MS is often used for targeted analyses due its higher selectivity of compound ions meaning there is less overall interference from any co-eluting compounds and a greater accuracy for quantification. Furthermore, the TQ-MS has a higher sensitivity allowing lower detection and quantitation limits and therefore a linear quantitation range, as well as, good reproducibility making it ideal for targeted analyses.^{90,104}

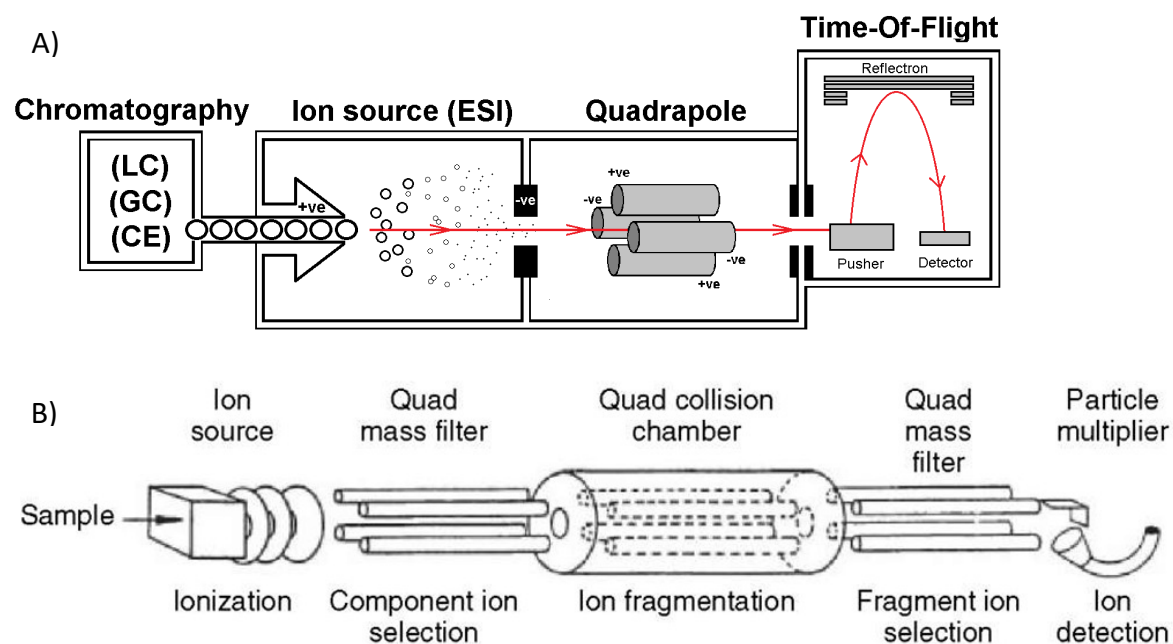


Figure 5. Mass Analysers

A) quadrupole-time of flight (Q-TOF); B) triple quadrupole-mass spectrometer (TQ-MS)

2.4.1 Tandem mass spectrometry

Tandem mass spectrometry also known as MS/MS, is a method employed to target specific ions for fragmentation in the collision cell. This technique was applied for both the untargeted UPLC-MS lipid analysis and also the targeted UPLC-MS amine analysis.

For the targeted amine analysis, tandem mass spectrometry performed was for multiple reaction monitoring (MRM) experiments. MRM aids quantification of preselected metabolites, whereby known precursor ions are selected and then fragmented in the collision cell.¹⁰⁵ Known product ions generated are then selected for quantification, which extrapolate unknown concentrations, from a standard curve and spiked internal standards with known concentrations.⁹¹

MS/MS experiments were performed for the untargeted UPLC-MS analysis to aid metabolite identification. In addition to MS/MS experiments, a further experiment performed included MS^E. MS^E experiments generate precursor and fragment ion information for multiple metabolites in a single run by applying collision energy which oscillates between high and low energy.¹⁰⁶ MS^E is a complimentary method to the standard MS/MS acquisition and the fragmentation data generated provides a useful comparator when analysing fragmentation data. A further method called DDA (data dependent acquisition) can also be used to complement MS/MS data. DDA identifies high intensity parent ions across the entire run by performing an initial MS scan and then subsequently fragments these ions.¹⁰⁷

2.4.2 Sample blanks and instrument conditioning for untargeted UPLC-MS lipid analysis

Prior to the untargeted UPLC-MS lipid run a number of instrument checks, conditioning and sample blanks injections for checking the analytical performance of the instrument are performed. A series of sample blanks which are made up of water are initially injected at the start of a UPLC-MS run to check the analytical performance of the instrument and verify the instrument is fit for purpose. Furthermore, the blanks also help to identify any potential contaminants and artefacts present in the instrument, column or solvents.¹⁰⁸ Following satisfactory instrument performance checks using the blanks, conditioning of the instrument is undertaken.

Pooled QCs (quality controls) which are made up of a small amount of all samples being analysed in the run are injected up to 9-10 times (depending on the instrument and method) and are used to “condition” or coating of active sites which normally absorb metabolites. This process allows the instrument to equilibrate and subsequently improves the reproducibility of the data acquired during the run.¹⁰⁸ Following injection of sample blanks and conditioning of the instrument the main analysis of the study samples can be performed. See Chapter 5 for further details on the feature selection and data preprocessing.

2.4.3 Metabolite identification of lipids

Mass accuracy is an important aspect of metabolite identification and refers to the accuracy of the m/z measurement of an analysed ion and is described in the equation below (Figure 6).

$$\text{ppm} = \left(\frac{\text{measured mass} - \text{theoretical mass}}{\text{theoretical mass}} \right) \times 10^6$$

Figure 6. Mass error calculation

ppm – parts per million

Metabolite identification performed using existing fragmentation data, which provided information on characteristic fragments generated by MS/MS per lipid class and fragment size according to fatty acid chain length and saturation. An example of a lysophosphatidylcholine (LysoPC) identified from MS/MS data is shown in Figure 7.

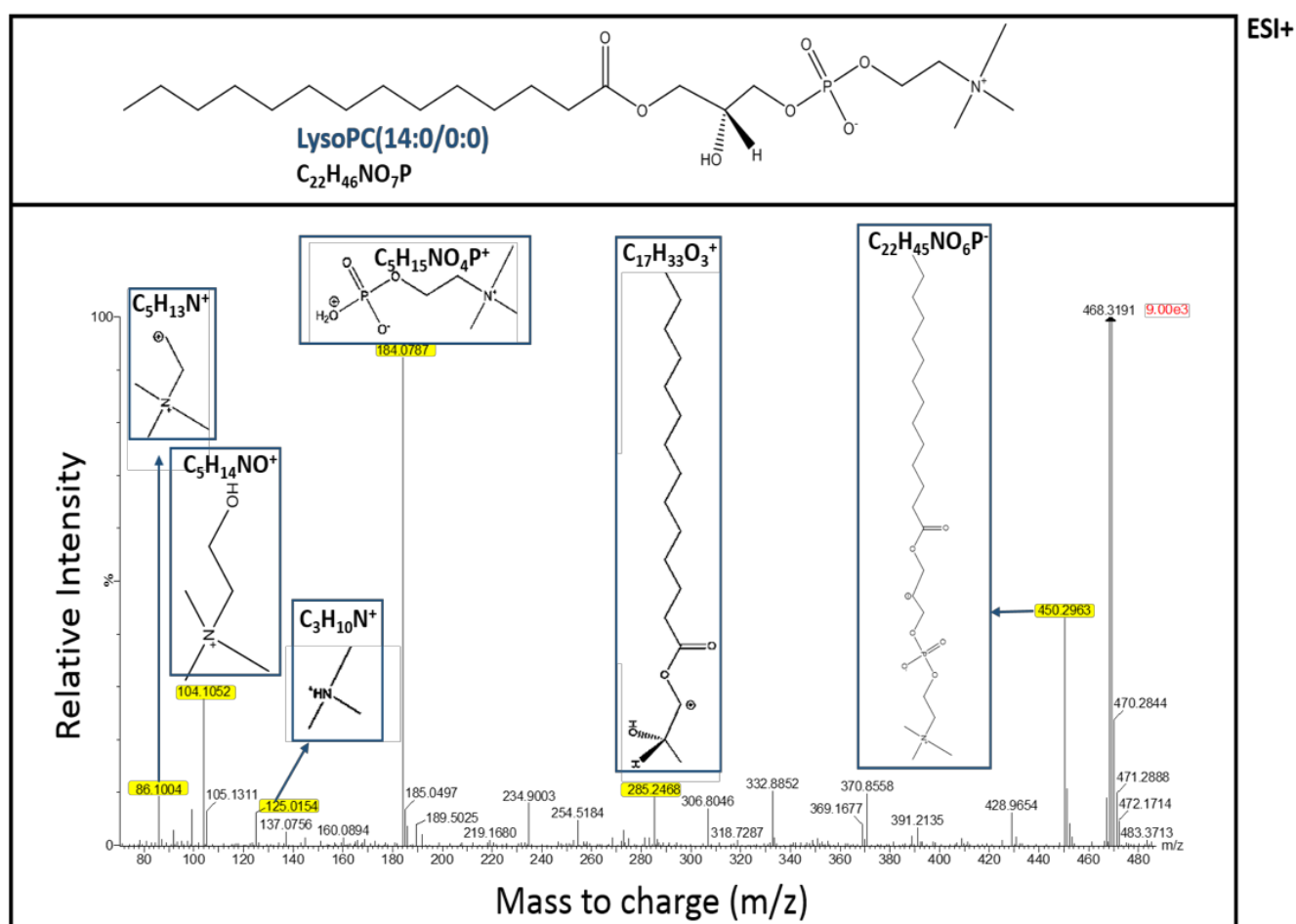


Figure 7. Identification of a LysoPC by tandem mass spectrometry

2.5 Data preprocessing of untargeted UPLC-MS data

Data produced by untargeted UPLC-MS profiling is preprocessed, which allows extraction of information from the raw data. This is carried out by automated peak picking software. In this thesis, XCMS was used to carry out preprocessing of the untargeted UPLC-MS lipid data. XCMS is peak picking software available as a free R package, and allows all parameters to be specifiable and tailored to each dataset and also allows efficient information extraction in contrast to commercial peak picking software.¹⁰⁹ The main steps undertaken in XCMS include peak alignment, peak picking, peak grouping and filtering. The parameters for XCMS were determined by visual examination of the raw data. The key parameters are summarised in Table 1. Further details on preprocessing parameters used for the untargeted UPLC-MS data are presented in the methods section of Chapter 5.

Parameter	Description
ppm tolerance	The maximum relative ppm difference permitted between masses of the same peak in consecutive scans
Peak width	Peak width range to include the narrowest peak width to the largest
m/z tolerance	The maximal tolerated m/z deviation in consecutive scans (ppm)
Intensity prefilter	Minimum number of scans and intensity for peak picking
S/N threshold (intensity)	The signal to noise ratio cut-off

Table 1. XCMS peak picking parameters

2.6 Chemometrics

Owing to the large complexity and size of metabonomics data, chemometric methods are typically applied to model these large datasets for multivariate visualisation and successful interpretation.¹¹⁰ Furthermore, these methods provide a means for data mining of significant or discriminant features which can be used for diagnostic purposes following successful validation. In addition, chemometrics serves an important purpose for modelling and analysing complex and challenging datasets that are noisy and collinearly structured.¹¹⁰ During multivariate analysis two main approaches are used to model metabonomics datasets, which include supervised partial least squares (PLS) analysis and unsupervised principal component analysis (PCA).¹¹¹

PCA is a technique that reduces the dimensionality of the data, where there is no *a priori* information on sample class.¹¹¹ This method allows inherent clustering of sample groups

to be visualised in the dataset as well as allowing the identification of outliers. PCA models are made up of principal components, these are described mathematically as eigenvectors and depict the amount of variance in the variance matrix of a dataset. The first principal component accounts for the largest source of variance, whilst the second principal component which is orthogonal to the first accounts for the second largest source of variance, and so on until the maximum number of principal components are computed.¹¹¹ PCA models are visualised through scores, which represent samples or patients, and loadings which represent variables such as metabolites.

Partial least squares (PLS) analysis is an example of a supervised approach, here the only variance in the data or matrix of observations (X-matrix) between groups or a continuous variable (Y-matrix) such as healthy controls vs liver disease or liver disease vs clinical score is modelled.¹¹² Consequently, PLS can identify variables (metabolites) that are characteristic per group. Additionally, PLS is often referred to as a discriminant analysis (PLS-DA) where variables that discriminate between classes being compared are identified.

An orthogonal partial least squares-discriminant analysis (OPLS-DA) is an extension of PLS-DA, which adds an orthogonal signal correction which rotates the model and allows better visualisation of discrimination between classes.¹¹³ A further type of PLS model is a PLS regression model which regresses the X-matrix against a continuous variable (Y).¹¹⁴ This type of modelling can be used to identify confounders in the data such as age, gender or height, or identify associations in the data with known clinical measures such as prognostic scores.

The loadings from the PLS or OPLS model can be observed in a number of ways and in this thesis are presented in the form of a S-plot and a VIP (variable influence on projection parameters) plot.¹¹⁵ The S-shaped distribution in an S-plot show the covariance and correlation and the loadings at the top and bottom extreme ends of this plot represent the loadings most influential on group membership (Y-variable).¹¹⁵ Whilst the VIP plot summarises the importance of variables that explain variance in X and its correlation to Y.¹¹⁵

The statistical parameters R^2 and Q^2 are calculated to check both the predictive ability and goodness of the fit of the model.¹¹⁰ The R^2 value quantifies the variance shown in the model between classes and the Q^2 value quantifies how good the model is at predicting variance in the model. An $R^2=1$ indicates perfect description of the data by the model and $Q^2=1$ indicates perfect predictability of the model. A large discrepancy between the R^2 and Q^2 values indicates the model is over fitted and doesn't resemble the sample population. Therefore, as well as using a model with the highest Q^2 value, this value needs to remain within a close range to the R^2

value.^{112,113} Outliers from PCA scores are identified using a Hotellings T^2 ellipse in scores plots for PCA modelling and uses a significance level of 0.05.¹¹⁶

The quality and reliability of the supervised models is further assessed by performing a seven-fold internal cross-validation, where the data is split into a test set and a validation set comparing how good the model for the test set is at predicting the variance in the validation set. Furthermore, a permutation test is also performed to further validate the PLS or OPLS models. This test compares the Q^2 value, obtained from the model for the original dataset, with a distribution of Q^2 values calculated when the original predicted (Y) values are randomly assigned to the individual samples in the classes. 1000 permutations are typically performed to test the validity of the O-PLS model with a p-value ≤ 0.05 , which is indicative of a valid PLS-DA model.¹¹³ A further test, is the cross-validated-analysis-of-variance (CV-ANOVA) statistic, which corresponds to a null hypothesis of equal predictive residuals between the models under investigation, a p < 0.05 suggests the model is superior to one chosen at random.

Chapter 3

^1H NMR SPECTROSCOPIC PROFILING

3.1 Introduction

Metabolic phenotyping by ^1H NMR spectroscopy has previously been applied to improve diagnostic accuracy or prognosticate patients and enhance mechanistic understanding of disease. ^1H NMR spectroscopic profiling of human plasma is a well-established technique and allows rapid detection of a range of metabolite classes, which include amino acids, organic acids, sugars and lipids.^{93,94} Furthermore, prior ^1H NMR spectroscopic profiling studies of liver disease have identified important metabolites that are perturbed in serum and plasma due to declining liver function, these include amino acids, carbohydrates, energy metabolites and lipids.^{86,117,118}

In this chapter a global untargeted ^1H NMR spectroscopic approach was employed to profile patient plasma and discriminate between acute and chronic liver disease, and also within these groups between the subgroups. Patients who suffer from chronic disease – encompassed a wide range of cirrhosis groups, which are grouped according to disease severity, ranging from stable cirrhosis (SC) to acute decompensation (AD) and acute-on-chronic liver failure (ACLF). Whilst, patients with acute liver disease included those with acute liver injury (ALI) and acute liver failure (ALF). Furthermore, classification of cirrhosis into subgroups was according to the CLIF-SOFA scoring system, whilst acute liver disease was classified according to the definition by O’Grady et al.²¹

3.2 Aims and Objectives

The main objective of this work was to identify metabolic changes per disease class, focusing on comparisons between different liver disease groups. These comparisons were primarily aimed at characterising the progression of cirrhosis to liver failure: SC vs AD, AD vs ACLF and SC vs ACLF, and also the progression to liver failure in acute liver disease: ALI vs ALF, as well as, metabolic distinctions between acute and chronic liver disease: ALF vs ACLF. An additional aim was to characterise metabolic perturbations further by identifying downstream metabolic pathways affected. Finally, a further aim was to correlate metabolic differences with clinical metadata to deduce any mechanistic links to known markers of liver disease.

3.3 Materials and Methods

3.3.1 Study design and patient recruitment

All human serum and plasma samples in this project were obtained from the Liver Unit and Liver Intensive Therapy Unit (LITU) at Kings College Hospital (KCH) between December 2012 and July 2015. The study was granted ethics approval by the local research ethics committee (12/LO/1417), and patients or their nominated representative (next of kin), in the event a patient was unconscious or had hepatic encephalopathy (HE), provided written and informed consent within 48 hours of presentation to hospital. Admission to the LITU was offered to eligible patients, as determined by the attending hepatologist or intensivist. A multidisciplinary team of specialists managed all patients according to standard evidence-based protocols.¹¹⁹ The inclusion criteria for all subjects were that they were 18-75 years of age, and the exclusion criteria for all subjects included any of the following: pregnancy, hepatocellular carcinoma, non-hepatic malignancy, pre-existing immunosuppressive states including Human immunodeficiency virus (HIV) infection and chronic granulomatous diseases and known inflammatory bowel disease. Although, patients with immunosuppressive diseases were excluded, some patients receiving immunosuppressive drugs were included, such as those with autoimmune hepatitis. All patients were monitored for both the duration of their admission and were regularly followed up until 1 year from their first admission date. Furthermore, healthy volunteers who were recruited into the study, were members of staff from the institute of liver studies at KCH and served as healthy controls (HC). See Figure 8 for an overview of patient recruitment.

Cirrhosis was diagnosed in patients from a previous liver biopsy or by clinical presentation (presence of hepatocellular jaundice/ascites/HE) or biochemical presentation (hyperbilirubinemia, prolonged prothrombin time and/or thrombocytopenia), and also by a typical ultrasound scan, computed tomography (CT) or magnetic resonance imaging (MRI) which showed scarring of the liver. Following diagnosis, cirrhosis was further classified into stable cirrhosis (SC), acute decompensation (AD) and acute-on-chronic liver failure (ACLF) according to the CANONIC criteria.¹²⁰ SC was classified according to the absence of ascites, variceal haemorrhage and hepatic encephalopathy (HE).¹²¹ The AD and ACLF groups were classified and discriminated according to the CANONIC criteria¹²⁰ and therefore presence of

decompensating events absent in SC. Whilst ACLF and AD patients were recruited from the liver unit at KCH, the SC patients were recruited from day clinics. Hence, SC patients were a greater challenge to enroll into the study, as they were present in hospital for much shorter period of time, explaining their smaller number compared to the other groups.

Acute liver disease was defined in individuals, who had no previous or current record of liver disease including liver fibrosis or cirrhosis, who, following an acute hepatic insult related to an adverse drug reaction or overdose, presented with symptoms of liver failure (jaundice/coagulopathy and encephalopathy) or liver injury within the previous 6 months.²¹ The classification of acute liver injury (ALI) and acute liver failure (ALF) was performed according to the definition by O’Grady et al.²¹

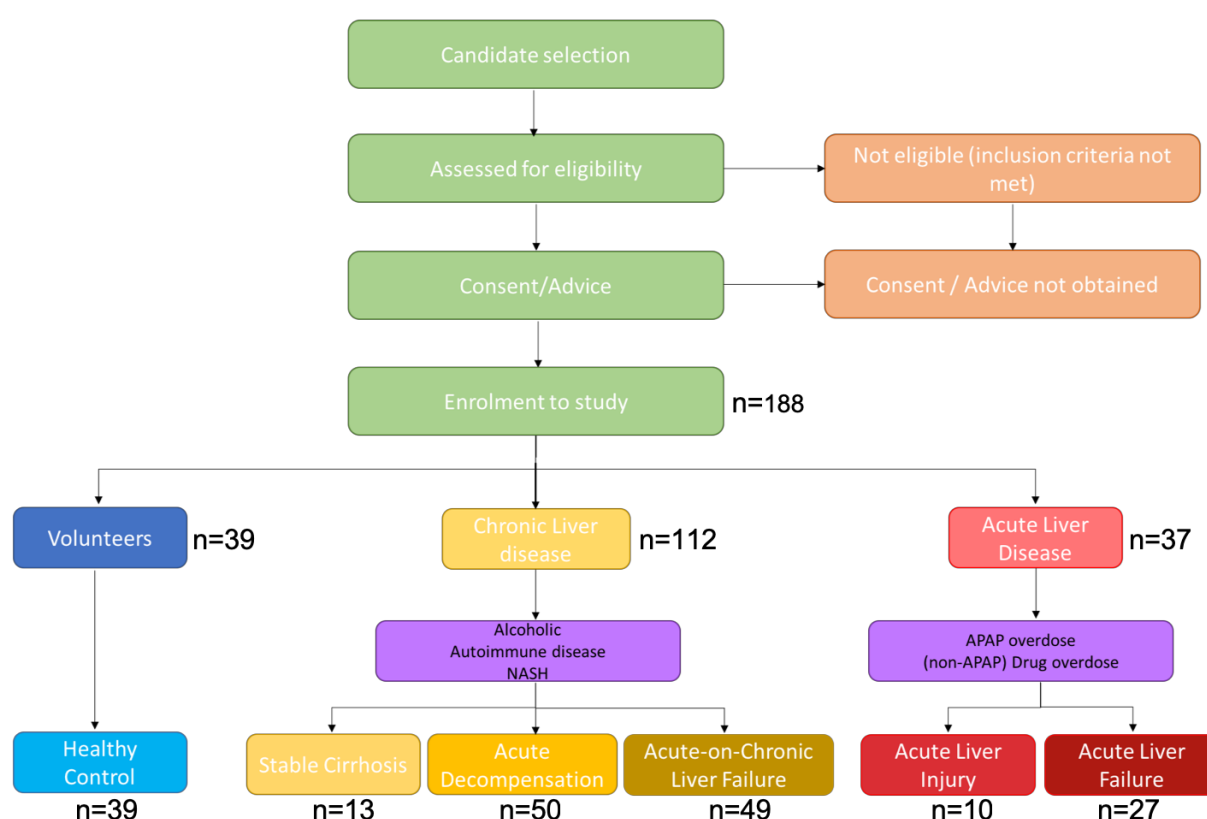


Figure 8. A summary of the study participant recruitment protocol

The flow chart depicts the strategy used for candidate selection and the major groups recruited: Healthy Controls (HC); Chronic Liver Disease: Stable Cirrhosis (SC), Acute Decompensation (AD) and Acute-on-Chronic Liver Failure (ACLF); Acute Liver Disease: Acute Liver Injury (ALI) and Acute Liver Failure (ALF). Numbers per group (n=);

Demographic information collected at the time of sample collection included age and gender. Whilst medical information collected included liver disease aetiology, past medical history, medications, bedside physiology, blood biochemistry, haematology and blood gas

results. Clinical scores calculated included the Child-Pugh Score (CPS), MELD, United Kingdom end-stage liver disease (UKELD), SOFA and CLIF-SOFA (see Table 2).

Plasma samples for metabolic profiling were obtained within 24 hours of admission to hospital. Blood was drawn into lithium heparin containing vacuum tubes and (BD Vacutainer, Franklin Lakes, NJ, USA) plasma fractions were collected, following incubation at room temperature, and centrifuged at 12,000g for 10 minutes at 4°C. The plasma aliquots were then stored immediately at -80°C until further analysis.

3.3.2 ^1H NMR spectroscopy

All plasma samples were prepared according to the previously published paper by Dona et al.⁹⁴ Sample preparation and analysis order was orthogonal to metadata variables including 90-day survival, disease group, age and gender. Following randomisation, plasma samples (350µl) were thawed, vortexed and centrifuged at 13,000g for 5 minutes at 4°C. Supernatant aliquots (300µl) were added to 96 x 5mm NMR tube Sample Jet boxes, using a Bruker Sample Track system and a Liquid Handler 215 preparation robot. Phosphate buffer was also added to the 5mm NMR tubes (300µl of 0.075M NaH_2PO_4 , 20% D_2O , pH 7.4, 2mM NaN_3 and 4.6mM TSP; 3-trimethyl-silyl-[2,2,3,3- $^2\text{H}_4$]-propionic acid, sodium salt 98 atom % D (Sigma-Aldrich, UK)). The tubes were sealed with POM balls (Bruker Biospin, Rheinstetten, Germany) and the samples thoroughly mixed by inverting the box and shaking multiple times.

^1H NMR spectra were acquired on a Bruker 600MHz (Avance III) spectrometer, operating at a ^1H frequency of 600.16 MHz (14.1 Tesla) at 300K using a 5mm BBI probe and an automated Sample Jet system (Bruker Biospin, Rheinstetten, Germany). A one dimensional Carr Purcell-Meiboom-Gill (CPMG) spin echo sequence (d_1 -90°-(τ - 180°- τ)_n-acquire) was used to attenuate the broader peaks arising from lipids and proteins.⁹³ For each sample, 32 transients were collected into 73k data points using a spectral width of 20ppm and a total spin echo time of 76.8ms (τ of 300µs, number of loops - 128). Suppression of the water signal was performed during the relaxation delay (d_1 - 4s) and a line broadening function of 0.3Hz was applied to all spectra prior to Fourier transformation.

3.3.3 NMR data processing

¹H NMR spectral preprocessing was performed manually in TopSpin 3.0 NMR software (Bruker Biospin, Rheinstetten), where all spectra were Fourier transformed, phased, baseline corrected, and subsequently referenced to the α -glucose anomeric proton resonance at 5.23ppm. The digitised spectra (33k data points) were imported into Matlab (R2014a Mathworks, Natick, USA) and spectral regions exhibiting unwanted resonances were removed, for example, the water resonance region (δ 4.32-4.84). Subsequently, the ¹H NMR spectroscopic data were mean centered and scaled to unit variance, prior to multivariate modelling which was performed using in-house scripts in Matlab.

3.3.4 Multiplex cytokine array

Plasma cytokines including IFN- γ , IL-1b, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13 and TNF- α were quantified simultaneously from 50 μ L of plasma using a Multiplex Cytokine CBA (Cytometric Bead Array) kit in a calibrated FACS Calibur flow cytometer (BD). Individual cytokine concentrations were quantified from their fluorescence using standard reference curves from CBA Software (BD). The kits and CBA software were provided by BD Biosciences (San Jose, CA).

3.3.5 Statistical analyses

The ¹H NMR spectroscopic data were subjected to principal component analysis (PCA) to visualise clustering of samples in the multivariate space and to identify outliers. This was followed by supervised modelling using OPLS-DA (orthogonal – projection to latent structures – discriminant analysis¹²²) to focus on variance related to class membership (see Chapter 2, section 2.6).

A univariate approach was also applied to analyse the ¹H NMR peak integral data using Prism 7.0 (Graphpad, California, USA) and Excel 2013 (Microsoft, Redmond, USA). Both datasets were logarithmically transformed (base 10) with an added offset of 1 ($\text{Log}_{10}(X) + 1$) and subjected to a 1-way ANOVA analysis with a post-hoc Sidak test to adjust for the number of class comparisons per metabolite and false discovery rate correction (FDR) using the Benjamini and Hochberg algorithm¹²³ to adjust for the number of metabolites. A statistical significance threshold was set for all p-values and FDR adjusted p values at $q < 0.05$.

Significant differences across classes in patient metadata were assessed using a Kruskal

Wallis test with a post-hoc Dunn's test to adjust for class comparisons for all continuous variables. A chi-squared (χ^2) test was used to assess differences between classes for categorical data.

3.3.6 Metabolite intercorrelation analysis

Metabolite-metabolite correlations were calculated from $\text{Log}_{10}(X)+1$ transformed ^1H NMR spectral peak integrals using Pearson correlation and were presented graphically in the form of heatmaps (Matlab 2014a). Metabolite orders were determined using the complete linkage method for hierarchical clustering (Euclidean distances, using a threshold of ≤ 2).

3.3.7 Metabolite Pathway Mapping

Metabolite pathway and quantitative metabolite enrichment analyses were performed in metaboanalyst¹²⁴ (<http://www.metaboanalyst.ca/>) and used metabolite information from the KEGG database¹²⁵ (<http://www.genome.jp/kegg>). The impact-value threshold (a threshold which determines if metabolic changes have a significant impact on a particular pathway) was calculated from pathway topology analysis and was set to 0.10, pathways below this threshold were filtered out.

3.4 Results

3.4.1 Patient demographics and clinical characteristics

The study included a total of 188 subjects of which 185 (due to availability of plasma samples) were subjected to ^1H NMR spectroscopic profiling (see Table 2). The subjects were split into the following classes: HC (n=40), SC (n=13), AD (n=50), ACLF (n=49), ALI (n=7) and ALF (n=27). Univariate analysis was performed on baseline clinical metadata from patients phenotyped by ^1H NMR spectroscopy (see Figure 9) and are summarised in Table 2. Data analysed include patient demographics, physiological observations, blood gas results, blood biochemistry, liver function tests, immune cell counts, cytokine array data and clinical scores. Metadata collected for the HC group were limited to age, gender and cytokine array data, in contrast to the SC, AD, ACLF, ALI and ALF patients.

The key demographic difference between classes was the median age of the chronic liver disease group, which was higher than the HC and acute liver disease groups. Furthermore, the

gender ratios were imbalanced for ALF and the chronic group, where the majority of the ALF group were female, whilst for the chronic groups, the majority of the groups were male. A breakdown of the main aetiologies for cirrhosis revealed that the most common cause was alcoholism for both groups. For acute disease groups, paracetamol overdose and non-paracetamol overdose were approximately well matched within ALI and ALF groups. Key differences in physiological observations were decreases in body mass index (BMI) and blood pressure from SC to ACLF, a rise in heart rate in ACLF relative to SC. Furthermore, there were also significant elevations in FiO_2 and pO_2 in ACLF and ALF, which are possibly a consequence of lung pathology, most likely acute lung injury. Changes in the Glasgow Comma Scale (GCS) and HE grade were indicative of HE development in ACLF and ALF patients, where GCS dropped notably, and HE grade rose significantly.

Key differences observed from the haematology test results, were a reduction in haemoglobin concentrations from SC to ACLF and a significant decrease in peripheral corpuscular volume (PCV) and haematocrit concentrations, indicating a reduction in red blood cell volume and therefore anaemia in ACLF. Furthermore, changes in immune cell counts were also observed; where there were significant rises in white blood cell counts from SC to ACLF and included neutrophils, monocytes and basophils, there was also a selective rise in eosinophils in AD. There were also significant changes in markers linked to blood clotting, where there was a marked reduction in platelets and rise in activated partial thromboplastin time (APTT) in ACLF, and a significant reduction in international normalised ratio (INR) in all chronic liver disease groups compared to acute liver disease.

The blood biochemistry data revealed raised calcium, magnesium, urea, creatinine and bilirubin concentrations in ACLF plasma. Whilst the liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were significantly elevated in ALI and ALF compared to chronic liver disease. Albumin and total protein were reduced from SC to ACLF, and total protein was also significantly reduced in acute liver disease compared to ACLF. Ammonia (NH_4^+) and C-reactive protein (CRP) concentrations increased in ACLF and ALF and there were selective elevations of alpha fetoprotein (AFP), ferritin and free T_4 in ALF. There were also significant reductions in triglyceride and cholesterol concentrations in AD and ACLF relative to SC, and also a reduction of these in the ALI and ALF groups compared to SC. Furthermore, there was a significant elevation of lactate in ALF compared to ACLF and a reduction in HCO_3^- in ACLF and ALF, which are both markers of acidosis.

The main findings from the cytokine array analysis were the significant rises in anti-inflammatory cytokines IL-2 and IL-10 and pro-inflammatory cytokines IL-1 β , IL-6, IL-8 and TNF- α in AD, ACLF, ALI and ALF patients relative to HCs.

Finally, survival at 30 and 90 days and 1-year post admission were monitored, and calculated survival percentages were the lowest for the ACLF group at 58%, 45% and 31% respectively. Whilst survival at 30 and 90 days and 1 year for ALF were 79%, 67% and 63% respectively. Analysis of the MELD and SOFA clinical scores, which were monitored in acute and chronic liver disease, showed that MELD scores were the highest in ACLF, ALI and ALF patients, whilst, SOFA scores were the highest in ALF and ACLF patients. The UKELD, Child-Pugh and CLIF-SOFA scores used to stratify patients with cirrhosis, increased with the severity of cirrhosis from SC to ACLF.

	Parameter	Cirrhosis Groups			Acute Liver Disease		HC	p-value
		SC	AD	ACLF	ALI	ALF		
		n=13	n=50	n=49	n=7	n=27	n=39	
Demographics	Age	60 (36-80)	56 (23-73)	49 (18-66)	35 (32-51)	42 (19-59)	36 (24-69)	<0.0001
	Sex (M:F)	9:4	28:22	33:16	3:4	6:21	19:19 n=38	0.006
Physiological Observations	BMI	27 (17-45)	27 (16-53) n=48	23 (17-33) n=48	22 (20-23)	21 (19-32)	-	<0.0001
	Systolic BP mmHg	132 (105-150)	110 (82-156)	113 (91-161)	115 (92-134)	118 (85-168)	-	0.012
	Diastolic BP mmHg	76 (54-95)	67 (42-92)	53 (41-77)	59 (39-69)	61 (39-90)	-	<0.0001
	MAP	95 (76-113)	80 (57-108)	74 (61-105)	81 (57-87)	79 (62-108)	-	<0.0001
	Heart Rate	74 (48-100)	80 (46-111)	88 (53-128)	89 (77-128)	95 (55-129)	-	0.0016
	Respiratory Rate	16 (14-26)	18 (11-30)	19 (10-29)	18 (14-26)	19 (14-35)	-	0.3069
	SaO ₂	98 (97-99)	98 (89-100)	97 (91-100)	98 (95-99)	98 (92-100)	-	0.1929
	FiO ₂	0.21 (0.21-0.21)	0.21 (0.20-0.40)	0.30 (0.21-0.70)	0.21 (0.21-0.35)	0.30 (0.21-0.80)	-	<0.0001
	Temperature	36.5 (36.2-37.3)	36.8 (35.6-38.1)	36.5 (33.9-38.2)	37.1 (36.7-37.8)	35.9 (32.9-38.5)	-	0.0008
Disease Aetiology	Aetiology (Cirrhosis)							
	Alcohol	7	29	28	N.A.	N.A.	-	0.8416
	Autoimmune	2	10	11	N.A.	N.A.	-	
	NASH	3	5	2	N.A.	N.A.	-	
	Other	1	6	8	N.A.	N.A.	-	
	Aetiology (ALF)							
	Paracetamol	N.A.	N.A.	N.A.	4	12	-	0.549
Clinical Scores	Non Paracetamol	N.A.	N.A.	N.A.	3	15	-	
	Child Pugh	6 (5-7)	9 (6-13)	12 (7-15)	N.A.	N.A.	-	<0.0001
	MELD	9 (7-13)	12 (4-28)	34 (12-50)	42 (23-55) n=5	42 (23-55)	-	<0.0001
	UKELD	49 (42-53)	55 (45-67)	63 (51-71)	N.A.	N.A.	-	<0.0001
	CLIF-SOFA	4 (2-8)	7 (3-15)	15 (8-25)	8 (5-12) n=5	15 (8-20)	-	<0.0001
	SOFA	4 (1-7)	5 (2-17)	12 (3-20)	6 (3-16) n=5	12 (5-18)	-	<0.0001
	GCS	15 (15-15)	15 (3-15)	8 (3-15)	15 (3-15)	3 (3-15)	-	<0.0001
Liver Enzymes	HE grade	0 (0-0)	0 (0-2)	3 (0-4) n=48	0 (0-4) n=6	3 (0-4)	-	<0.0001
	ALP IU/L	108 (63-229)	143 (58-765)	118 (29-1790)	134 (52-225) n=6	120 (16-112)	-	0.5
	ALT IU/L	20 (4-50) n=12	30 (13-2132) n=30	48 (8-2426) n=34	5860 (3912-14848) n=5	3734 (44-100660) n=25	-	<0.0001
	AST IU/L	29 (16-66)	55 (16-1670)	85 (17-8896)	4381 (283-16989) n=6	5008 (203-15560)	-	<0.0001
	GGT IU/L	75 (22-546)	90 (18-2423)	55 (13-1114)	259 (65-451) n=6	102 (22-382)	-	0.15

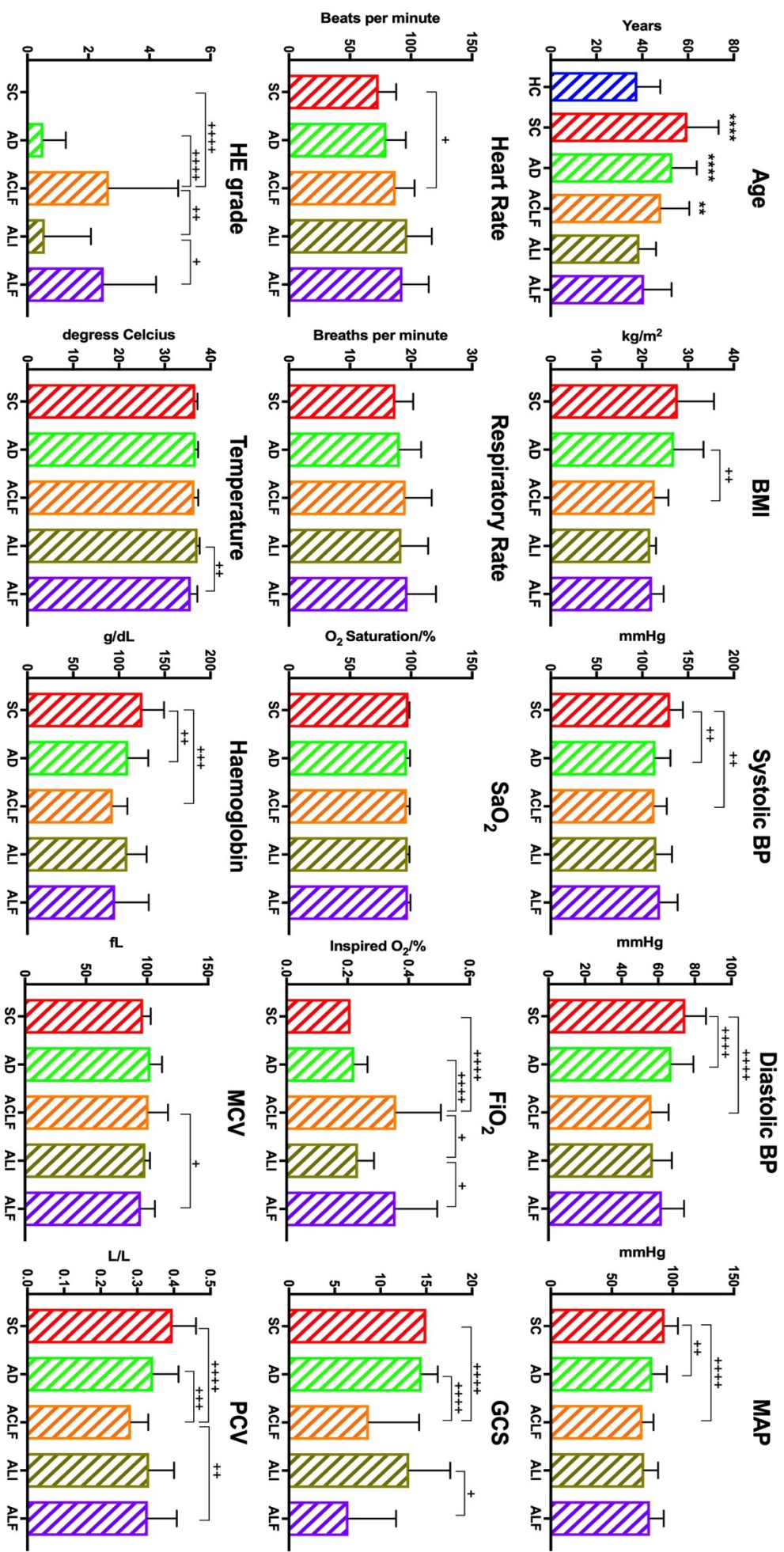
Haematology	Hb g/dL	133 (86-159)	110 (72-162)	91 (61-129)	111 (83-143) n=6	96 (7.4-154)	-	<0.0001
	INR	1.1 (1.0-1.6)	1.5 (1.1-2.9)	2.1 (1.3-7.7)	9.3 (1.1-15) n=6	5.8 (1.4-16.2)	-	<0.0001
	APTT	1.14 (1.06-1.41) n=4	1.33 (1.07-2.31) n=39	1.71 (1.29-3.08) n=45	1.69 (1.26-2.11) n=5	1.77 (1.11-3.42)	-	<0.0001
	Fibrinogen g/L	n=0	1.8 (1.1-4.1) n=9	1.6 (0.6-3.6) n=37	1.1 (0.6-1.9) n=5	1.4 (0.6-5.8)	-	0.3086
	MCV fL	98 (87-105)	102 (76-125) n=47	103 (12-133)	99 (93-103)	96 (63-119)	-	0.004
	PCV L/L	0.41 (0.27-0.47)	0.34 (0.22-0.5)	0.28 (0.19-0.40)	0.32 (0.26-0.45)	0.35 (0.20-0.49) n=26		<0.0001
	Haematocrit %	41 (27-47)	34 (22-50)	28 (19-40)	32 (26-45)	32 (20-49)	-	<0.0001
	WBC 10 ⁹ /L	4.5 (2.2-11.0)	6.2 (2.0-21.0)	10.6 (1.6-26.8)	8.3 (4.2-21.8)	11.3 (2.7-37.7)	-	<0.0001
	Neutrophils 10 ⁹ /L	2.8 (1.2-7.2)	3.9 (1.1-18.0)	8.5 (1.2-25.0)	7.7 (2.4-20.7)	9.6 (0.4-27.5)	-	<0.0001
	Lymphocytes 10 ⁹ /L	1.2 (0.6-3.4)	1.2 (0.37-3.1)	1.0 (0.2-4.9)	0.5 (0.3-1.9)	0.8 (0.2-0.6.8)	-	0.0265
	Monocytes 10 ⁹ /L	0.4 (0.1-0.8)	0.4 (0.1-1.5)	0.6 (0.03-2.5)	0.4 (0.04-0.7)	0.3 (0.1-1.5)	-	0.0007
	Eosinophils 10 ⁹ /L	0.2 (0.02-0.4)	0.2 (0.03-0.7)	0.1 (0.0-0.5)	0.04 (0.0-0.2)	0.03 (0.0-1.0)	-	<0.0001
	Basophils 10 ⁹ /L	0.02 (0.00-0.04)	0.03 (0.00-0.27)	0.04 (0.00-0.49)	0.02 (0.00-0.03)	0.02 (0.00-0.16)	-	0.0005
Biochemistry and Blood Gas	Platelets 10 ⁹ /L	111 (47-277)	107 (33-479)	82 (19-280)	125 (45-168)	110 (20-559)	-	0.0257
	Cj Bilirubin µmol/L	7 (2-11) n=12	25 (5-447) n=41	213 (12-688) n=38	64 (40-229) n=5	75 (13-864) n=24	-	<0.0001
	Bilirubin µmol/L	19 (5-75)	52 (10-466)	229 (23-714)	70 (15-274) n=6	96 (29-1010)	-	<0.0001
	Cj Bilirubin/Bilirubin %	40 (0-67) n=12	60 (0-96) n=41	88 (46-100) n=38	72 (62-88) n=5	78 (43-92) n=24	-	<0.0001
	Albumin g/L	41 (35-46)	31 (14-47)	26 (14-43)	26 (23-31) n=6	26 (13-34)	-	<0.0001
	Total Protein g/L	72 (61-84)	66 (39-87)	52 (30-82)	48 (41-52) n=6	47 (30-73)	-	<0.0001
	NH4+ µmol/L	45 (28-61) n=2	48 (20-107) n=15	73 (5-300) n=28	69 (35-75) n=3	77 (12-200) n=24	-	0.09
	CRP mg/L	4.9 (2.0-17.2) n=11	21.7 (2.0-102.9) n=30	53.4 (4.7-191.7)	6.5 (2.0-20.8)	17.3 (2.0-332.5)	-	<0.0001
	AFP kIU/L	2.0 (2.0-6.0) n=12	3.0 (2.0-17) n=42	3.0 (2.0-11) n=32	4.5 (2.0-6.0)	2.0 (2.0-228)	-	0.3737
	Ferritin µg/L	69 (13-475) n=12	260 (10-42315) n=46	990 (56-11521) n=35	5956 (1361-21727) n=4	6847 (97-300232) n=25	-	<0.0001
	TSH mIU/L	1.70 (0.03-4.10) n=11	1.80 (0.06-9.70) n=45	1.10 (0.05-8.40) n=36	0.31 (0.11-1.10) n=4	0.27 (0.03-5.10) n=21	-	<0.0001
	Free T4 pmol/L	13.4 (9.0-15.2) n=11	14.2 (9.6-23.0) n=45	11.0 (1.60-19.90) n=36	18.3 (8.0-24.0) n=4	12.5 (0.2-25) † n=21	-	0.0003
	Lactate mmol/L	1.4 (1.1-2.4)	1.7 (0.5-3.8) n=38	1.8 (0.7-11.2) n=46	3.3 (1.0-8.9) n=6	3.2 (1.5-16.1)	-	<0.0001
	Glucose mmol/L	6.1 (3.7-15.8)	7.2 (5.3-21.7) n=25	7 (3.5-14.3) n=45	5.7 (4.3-6.2) n=6	7 (3.7-14)	-	0.12
	Creatinine µmol/L	0.83 (0.51-1.35)	71.5 (20-311)	149 (26-693)	183 (57-462) n=6	164 (49-342)	-	<0.0001
	Urea mmol/L	5.3 (5.0-7.4) n=8	6.3 (1-41)	11 (1.1-28.7)	7.8 (3.1-18)	9.4 (2.0-24.0)	-	0.02
	Cholesterol mmol/L	4.1 (3.0-6.3) n=11	3.2 (1.5-6.6) n=39	2.4 (1.0-7.1) n=34	2.5 (1.8-2.6) n=5	2.2 (1.2-6.7) n=24	-	<0.0001

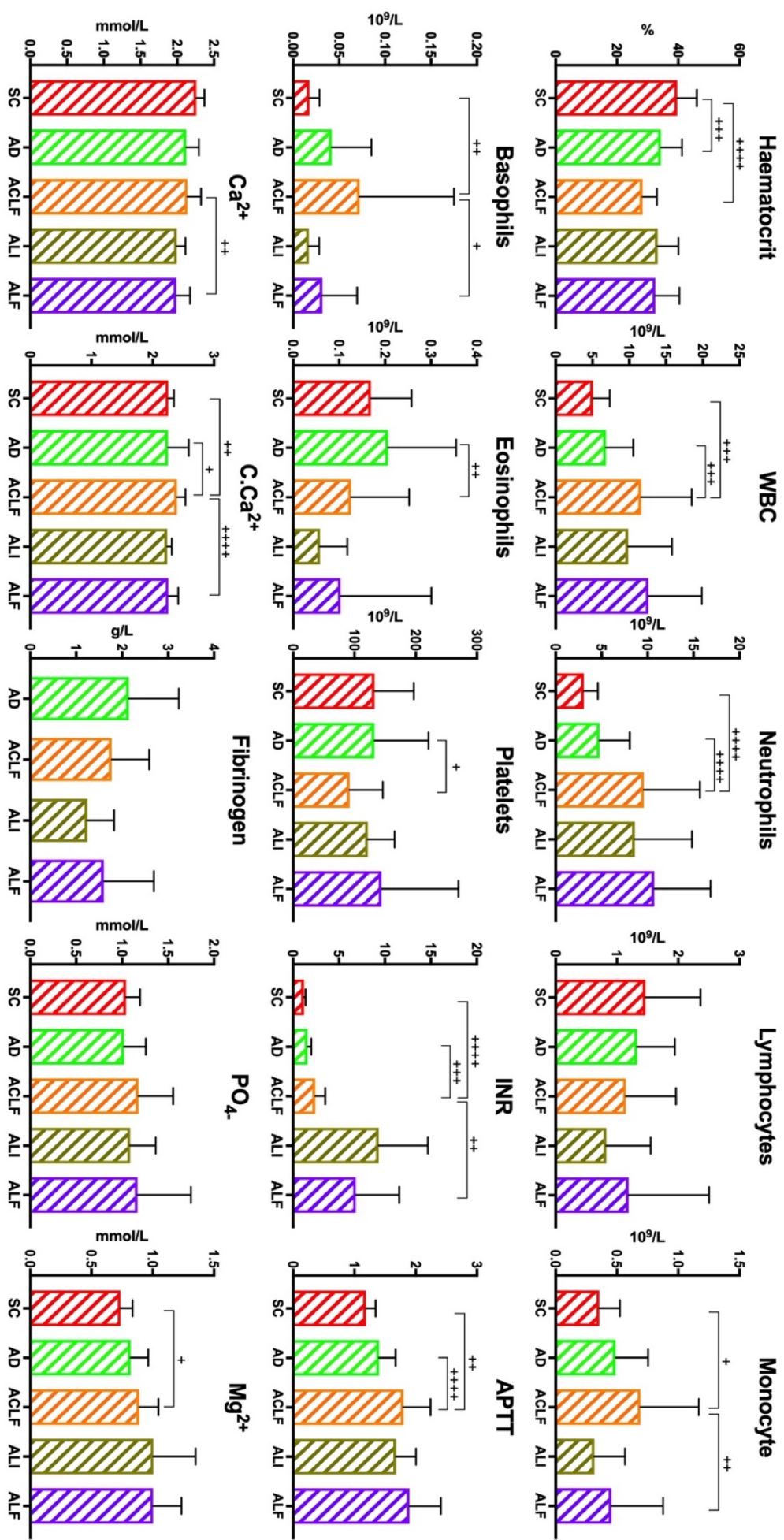
	Triglycerides mmol/L	1.0 (0.6-6.2) n=11	0.7 (0.4-2.9) n=39	1.2 (0.4-3.6) n=34	1.4 (1.1-6.2) n=5	1.4 (0.8-3.5) n=24	-	<0.0001
	Na ⁺	139 (134-142)	136 (123-149) n=26	135 (120-148) n=41	134 (133-137) n=5	136 (117-149)	-	0.1085
	K ⁺	4.2 (3.7-5.1)	4.2 (2.9-42.0)	4.4 (2.9-40.0) n=45	3.9 (3.6-4.5) n=5	4.3 (3.4-5.5)	-	0.3551
	Ca ²⁺	2.26 (2.03-2.48) n=12	2.16 (1.60-2.43)	2.14 (1.83-2.57) n=45	2.02 (1.80-2.10) n=5	1.94 (1.72-2.49)	-	<0.0001
	C.Ca ²⁺	2.24 (2.11-2.44) n=12	2.31 (0.08-2.51)	2.37 (2.16-2.79) n=45	2.24 (2.12-2.32) n=5	2.24 (1.99-2.63)	-	<0.0001
	PO ₄	1.04 (0.83-1.27) n=12	1.0 (0.45-1.72)	1.16 (0.39-2.25) n=45	1.01 (0.77-1.47) n=5	0.97 (0.26-2.81)	-	0.1085
	Mg ²⁺	0.73 (0.56-0.87) n=11	0.81 (0.50-1.15) n=30	0.88 (0.55-1.31) n=42	0.82 (0.73-1.54) n=5	0.98 (0.61-1.45)	-	0.0004
	Cl	107 (102-111)	105 (93-112) n=25	105 (96-139) n=41	102 (101-106) n=5	107 (86-120)	-	0.3892
	Blood pH	7.39 (7.30-7.45)	7.44 (7.21-7.53) n=39	7.41 (7.17-7.48) n=41	7.41 (7.36-7.48) n=5	7.39 (7.12-7.51)	-	0.0275
	pO ₂	5.69 (3.51-8.7)	10.60 (3.72-18.0) n=39	11.3 (3.0-27.3) n=42	13.6 (11.8-14.8) n=5	14.1 (8.81-32.7)	-	<0.0001
	pCO ₂	5.35 (2.89-6.97)	4.31 (3.0-6.38) n=39	4.90 (2.86-7.70) n=42	4.78 (2.92-4.83) n=5	4.5 (2.86-6.27)	-	0.0427
	HCO ₃	24.6 (21.9-30.1)	24.2 (18.2-29.4) n=39	22.45 (12.30-28.80) n=42	22.80 (12.30-26.10) n=5	22.60 (11.80-28.0)	-	0.0077
	P/F Ratio	203.2 (125.4-310.7)	328.9 (132.9-642.9) n=39	260.2 (56.3-539.3) n=42	485.7 (368.8-528.6) n=5	411.4 (136.4-678.0)	-	0.0002
Cytokine array data	IFN- γ	21.31 (4.68-577.70)	15.62 (0.08-108.7) n=47	29.51 (0.39-395.20) n=40	52.55 (1.67-251.20) n=5	20.39 (0.20-1315) n=24	14.99 (4.70-145.30)	0.08
	IL-10	0.52 (0.17-13.71)	0.85 (0.18-490.30) n=47	2.25 (0.36-115.2) n=40	26.41 (0.36-62.79) n=5	22.26 (1.53-477.3) n=24	0.25 (0.03-14.67)	<0.0001
	IL-12p70	2.30 (0.26-9.22)	2.30 (0.12-176.70) n=47	2.25 (0.04-261.9) n=40	1.46 (0.41-158.1) n=5	1.14 (0.10-223.8)	2.30 (0.05-2.3)	0.2259
	IL-13	1.8 (1.8-34.73)	6.78 (1.8-177.6) n=47	5.13 (0.99-239.40) n=40	5.38 (1.80-117.50) n=5	9.38 (1.80-84.75) n=24	1.8 (1.8-19.16)	0.0199
	IL-1 β	0.18 (0.16-0.68)	0.27 (0.07-122.6) n=47	0.18 (0.03-114.5) n=40	0.18 (0.10-112.3) n=5	0.32 (0.11-14.65)	0.18 (0.15-1.27)	0.0005
	IL-2	0.67 (0.63-1.04)	0.83 (0.21-38.21) n=47	1.39 (0.32-48.89) n=40	6.11 (1.17-14.4) n=5	3.52 (0.21-14.54) n=24	0.67 (0.67-1.13)	<0.0001
	IL-4	0.34 (0.05-1.40)	0.18 (0.01-0.98) n=47	0.26 (0.02-1.16) n=40	0.26 (0.18-0.433) n=5	0.34 (0.02-1.08) n=24	0.17 (0.02-0.34)	0.2632
	IL-6	3.61 (1.32-17.29)	8.06 (0.92-60.64) n=47	31.11 (2.47-9338) n=40	56.78 (4.21-87.68) n=5	65.93 (4.71-5745) n=24	0.84 (0.13-2.27)	<0.0001
	IL-8	20.28 (9.06-437.3)	31.18 (0.05-864.3) †† n=47	154.30 (0.11-5962) n=40	383.20 (0.76-934.5) n=5	219.8 (0.18-3239) n=24	7.65 (2.65-13.73)	<0.0001

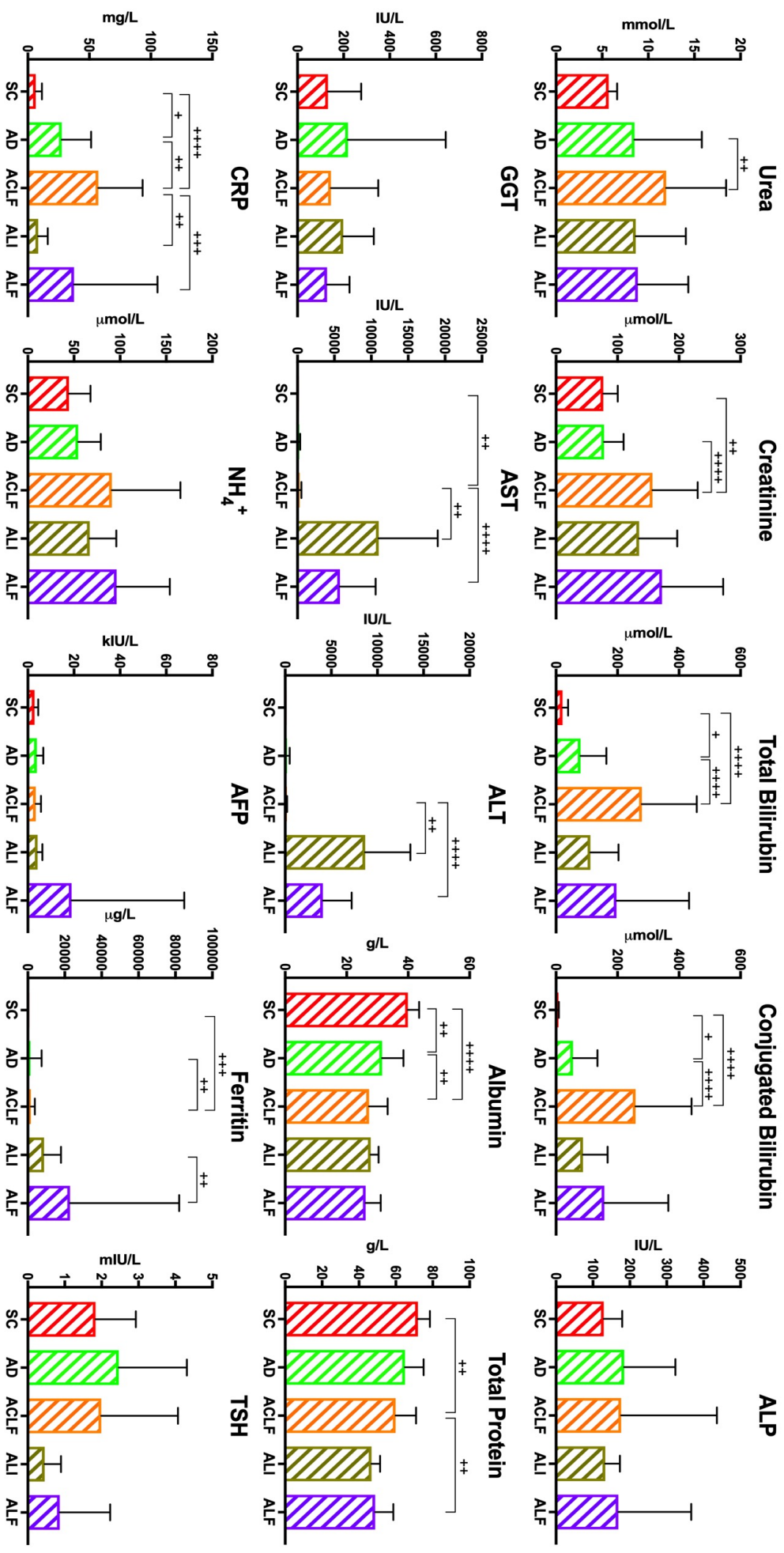
	TNF- α	3.90 (2.57-11.28)	3.95 (0.48-11.95) n=47	5.71 (1.39-118.10) n=40	6.92 (6.33-17.44) n=5	5.33 (0.76-21.75) n=24	1.56 (0.68-3.30)	<0.0001
Survival & Transplantation	Survival (%)							
	30 day	100%	100%	58% n=48	86%	79%	-	-
	90 day	100%	96% n=49	45% n=47	86%	67%	-	-
	1 year	100%	76% n=49	31% n=45	60%	63%	-	-
	Transplanted (n=)	0	27 n=41	17 n=36	0	11 n=26	-	-
	Transplanted patients - survival at 30-days (%)	-	100%	100%	-	81%	-	-
	Transplanted patients - survival at 90-days (%)	-	100%	83%	-	73%	-	-
	Transplanted patients - survival at 1 year (%)	-	100%	67%	-	64%	-	-

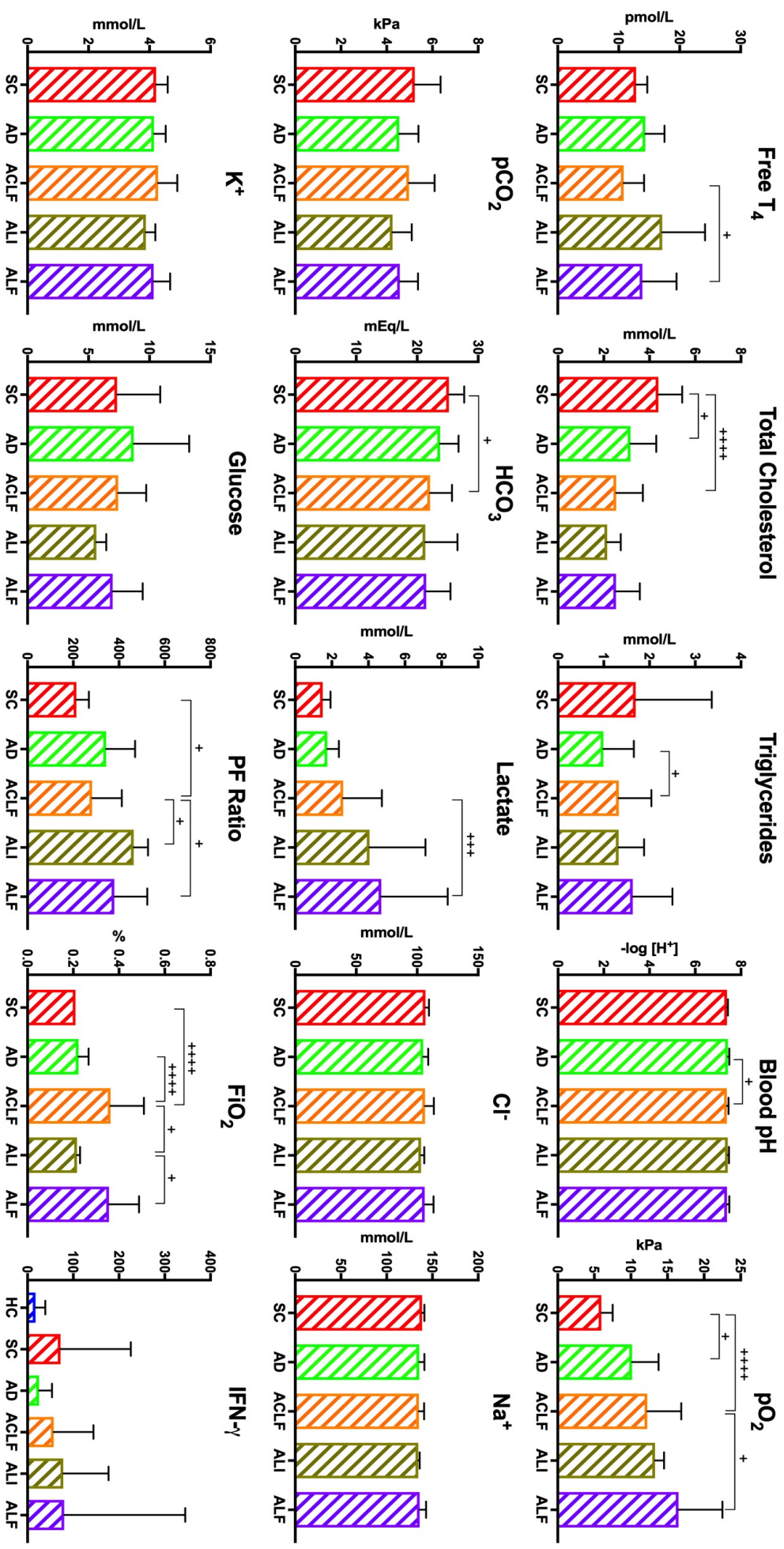
Table 2. Patient Demographics biochemical and physiological details from study population:

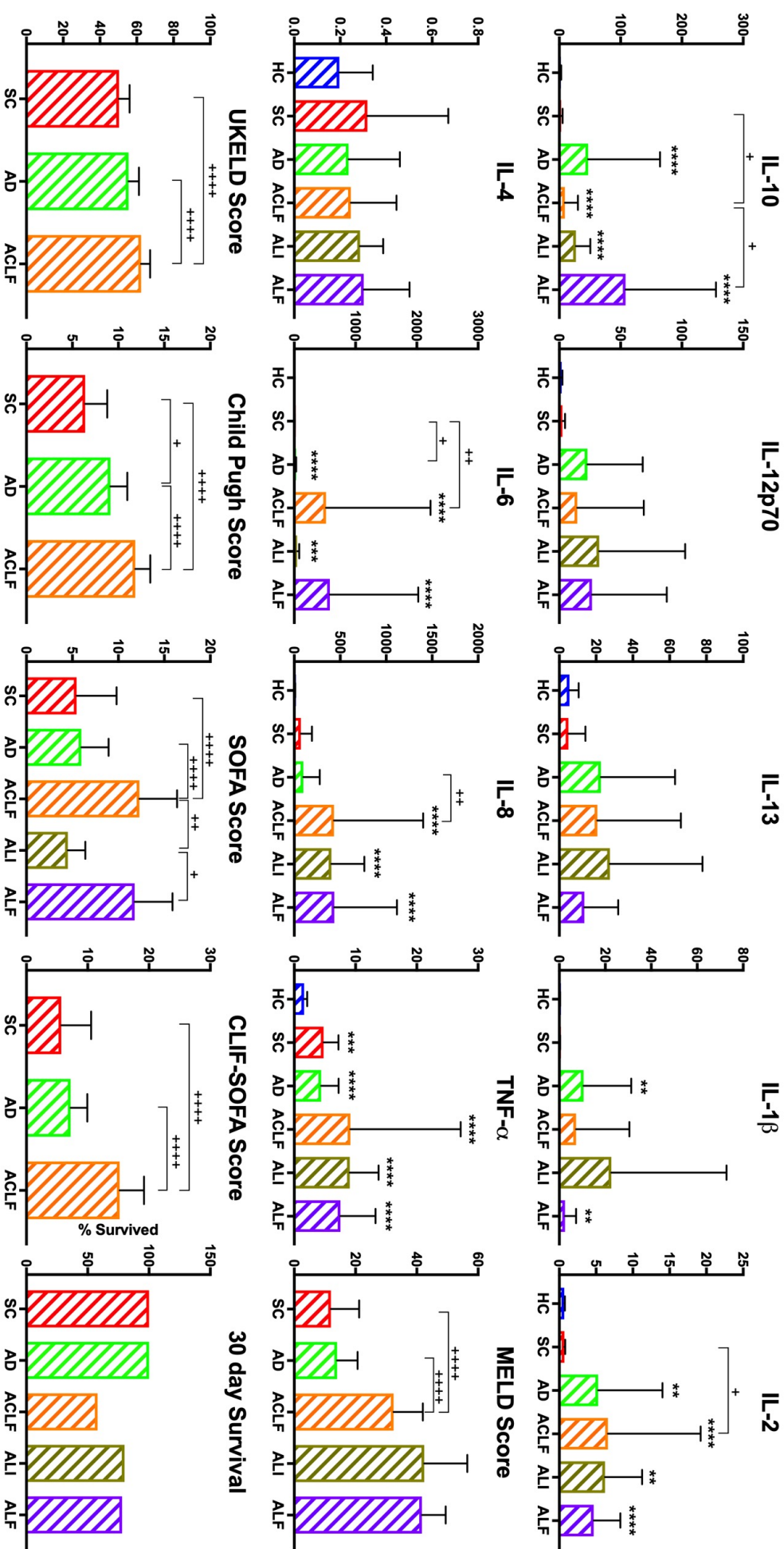
Data are presented as median values with full ranges in brackets for each parameter where applicable. A χ^2 (chi squared test) was performed for categorical variables. Sex and disease aetiology. A Kruskal Wallis test was performed for continuous variables with overall differences within each parameter reported. Metadata missing for some parameters is indicated by n-number next to median and range values. SC (stable cirrhotic); AD (acute decompensation); ACLF (acute-on-chronic liver failure); ALI (acute liver injury); ALF (acute liver failure); HC (healthy control); BMI (body mass index); MAP (mean arterial pressure); SaO₂ (oxygen saturation); FiO₂ (fraction of inspired oxygen); NASH (non-alcoholic steatohepatitis); MELD (model for end stage liver disease); UKELD (UK model for end stage liver disease); CLIF-SOFA (the chronic liver failure-sequential organ failure assessment); SOFA (sequential organ failure assessment); GCS (Glasgow coma scale); HE (hepatic encephalopathy grade); ALP (alkaline phosphatase); ALT (alanine aminotransferase); AST (aspartate aminotransferase); GGT (gamma-glutamyl transferase); Hb (haemoglobin); INR (international normalised ratio); APTT (activated partial thromboplastin time); MCV (mean corpuscular volume); PCV (peripheral corpuscular volume); WBC (white blood cell); Cj Bilirubin (conjugated bilirubin); NH₄⁺ (ammonia); CRP (C-reactive protein); AFP (alpha fetoprotein); TSH (thyroid stimulating hormone); Free T₄ (Thyroxine); Na⁺ (sodium); K⁺ (potassium); Ca²⁺ (Calcium); C-Ca²⁺ (Corrected Calcium); PO₄ (phosphate); Mg²⁺ (Magnesium); Cl⁻ (Chloride); pO₂ (partial pressure of oxygen); pCO₂ (partial pressure of carbon dioxide); HCO₃⁻ (Bicarbonate); P/F ratio (PaO₂:FiO₂); N.A. (not applicable);











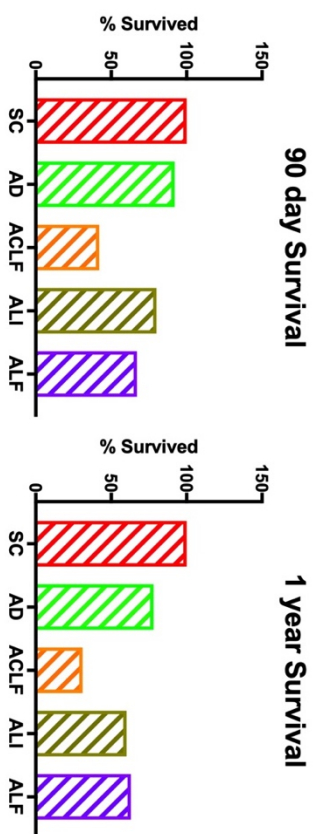


Figure 9. Univariate analysis of the cohort clinical metadata

These plots display changes in clinical metadata as summarised in Table one. Multiplicity adjusted p-values denoted by asterisks (*) show significance relative to HC and crosses (+) show significance within disease groups and are specified by brackets; ****<0.0001; ***<0.001; **<0.01; *<0.05

3.4.2 Assessment of QC data from ^1H NMR spectral data and study population

Pooled quality control (QC) samples were utilised to assess reproducibility of the analytical run, a PCA of all samples and QCs showed that all QCs clustered tightly as expected (data not shown). Following this, metabolites representing the full spectral width of 0-10ppm were integrated and analysed. The full list of ^1H NMR assignments for metabolites in human plasma in this study are listed in the Appendix see Table 36.

The stability of each metabolite was assessed across the run and no metabolites showed any significant changes consistent with run order (Figure 10), lactate showed some minor fluctuations across the run, but these were not consistent with run order. Coefficients of variation (CVs) per metabolite integral were also calculated (See Appendix see Table 36). All CVs were less than 5%, except for formate which has a CV of 9% but has the lowest signal to noise (S/N) ratio intensity and therefore a higher CV as the mean value is closer to zero so the CV is more sensitive to standard deviation.

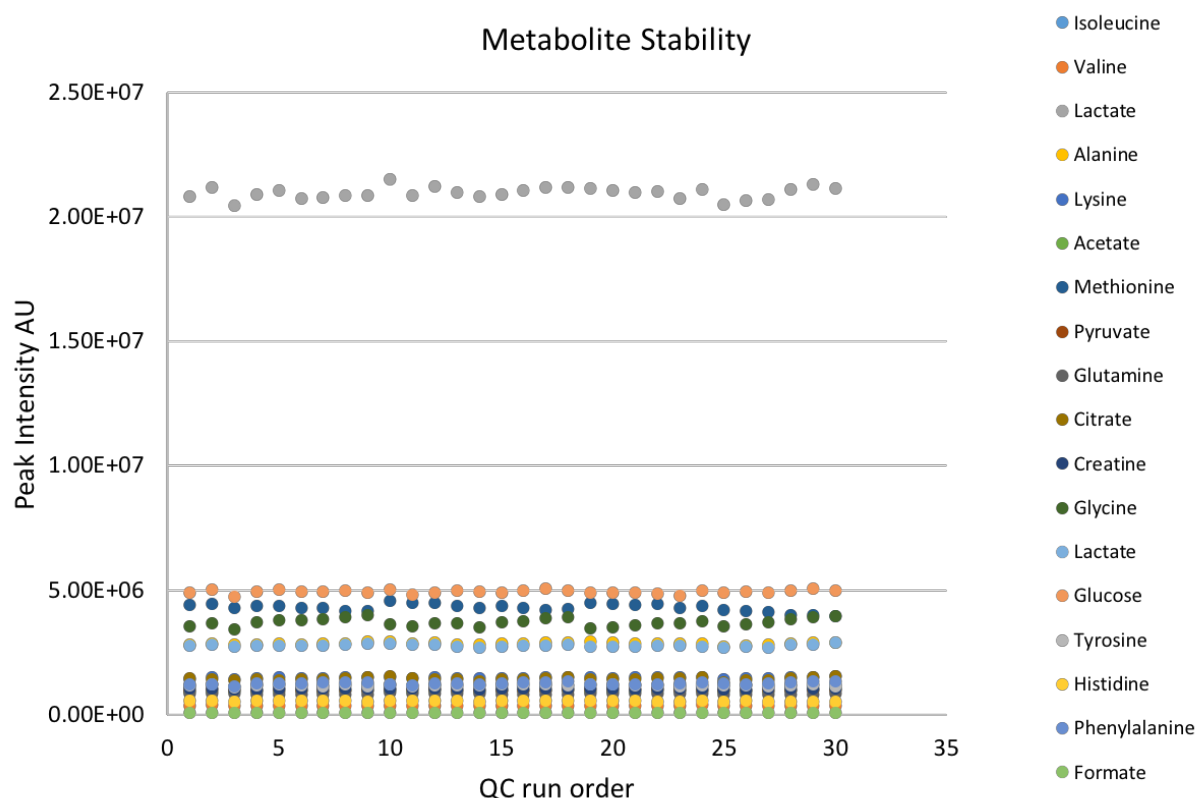


Figure 10. Stability of metabolites over ^1H NMR run in QC samples
Peak integrals per metabolite are plotted per QC sample in run order.

3.4.3 Drug related resonances in ^1H NMR spectra

Broad lipid-like resonances were visible in the ^1H NMR spectral data (Figure 11C) across the entire spectral region from 0.8-7.7 ppm. These were likely to be from drug related compounds (DRCs), such as sedative and anaesthetic agents, being administered to critically ill patients receiving intensive care.

A PCA model of all classes HC, SC, AD, ACLF, ALI & ALF (Figure 11; $R^2=0.51$; $Q^2=0.46$;) showed separation of samples in PC1 which accounted for resonances from DRCs. These resonances resemble lipid like peaks and appear at the highest intensity in three ACLF patients and one ALI patient (Figure 11A). The ^1H NMR plasma spectral profiles showed these DRC resonances were present in most ACLF plasma, and were also in some ALI, ALF and AD plasma (Figure 11C). This is understandable as these patients were on a number of drugs, particularly the ALF, AD and ACLF groups who were sedated at the time of sampling.

A statistical total correlation spectroscopic (STOCSY) analysis of one of the DRC resonances, confirms the other resonances observed across the spectra are all structurally correlated suggesting the resonances originate from the same or similar group of drugs compounds (Figure 11D). Four samples, which included three ACLF patients (GLA 53, 73 and 88) and one ALI patient (GLA 39), had extremely high concentrations of DRCs which distorted the spectra considerably and overlapped with most endogenous metabolites present in the spectra, hence, these spectra were excluded from subsequent multivariate analyses.

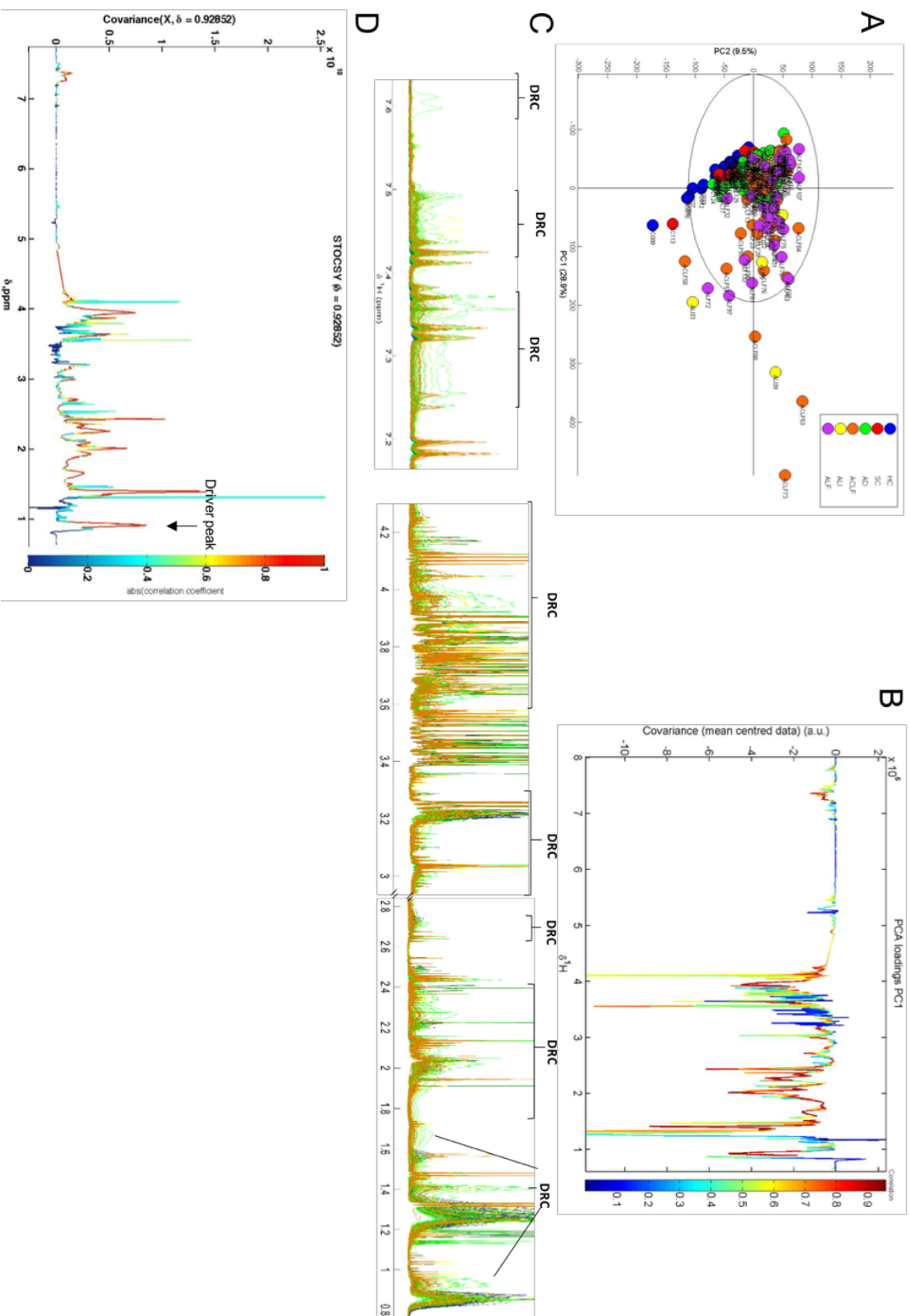


Figure 11. Drug related resonances present in plasma ^1H NMR spectroscopic data

A) PCA scores plot of all day 1 plasma samples from all classes (5 component model $R^2=0.51$; $Q^2=0.46$). B) PC1 loadings plot C) All spectra from Day 1 and HC patients D) STOCsy of DRC peak at 0.93 ppm.

3.4.4 Multivariate analysis of ^1H NMR spectroscopic data

Following the removal of outliers from the ACLF and ALI groups, further PCA modelling was employed to model variance in the ^1H NMR spectral data from all liver disease groups and HCs.

A three component PCA model of plasma ^1H NMR spectra from HC and cirrhotic groups: SC, AD & ACLF ($R^2=0.40$; $Q^2=0.36$) revealed a distinct separation in the scores in PC2 (22% of total variance) between HC and SC vs AD and ACLF, but no separation between AD and ACLF or HC and SC (Figure 12A & B). Similarly, a three component PCA model of acute liver disease and HCs ($R^2=0.48$; $Q^2=0.38$), also showed a clear separation between HC and ALF scores in PC2 which explains 29% of total variance for the model (Figure 12C & D).

The PCA loadings for both set of PCA models for acute and chronic liver disease, revealed a decrease in lipid resonances (LDL, vLDL and phosphatidylcholine), as well as, a decrease in the branched chain amino acids (BCAAs) isoleucine and valine and raised amounts of lactate and formate and the amino acids lysine, glycine, glutamine and the aromatic amino acids (AAAs) tyrosine and phenylalanine (Figure 12B and D) relative to HCs.

Two outliers were also observed in the scores plots which include a HC sample (Figure 12C) and a SC sample, the loadings for these scores revealed the presence of high lipidic resonances compared to the other samples in the group (data not shown). The ALF samples that lie outside the Hotelling's ellipse and separated from all other samples in PC1 also revealed the presence of DRCs in the plasma ^1H NMR spectra (data not shown).

Subsequent supervised O-PLS-DA pairwise modelling to discriminate AD vs ACLF and ALF vs ACLF and ALI vs ALF produced weakly predictive ($Q^2Y < 0.2$) or invalid models (data not shown).

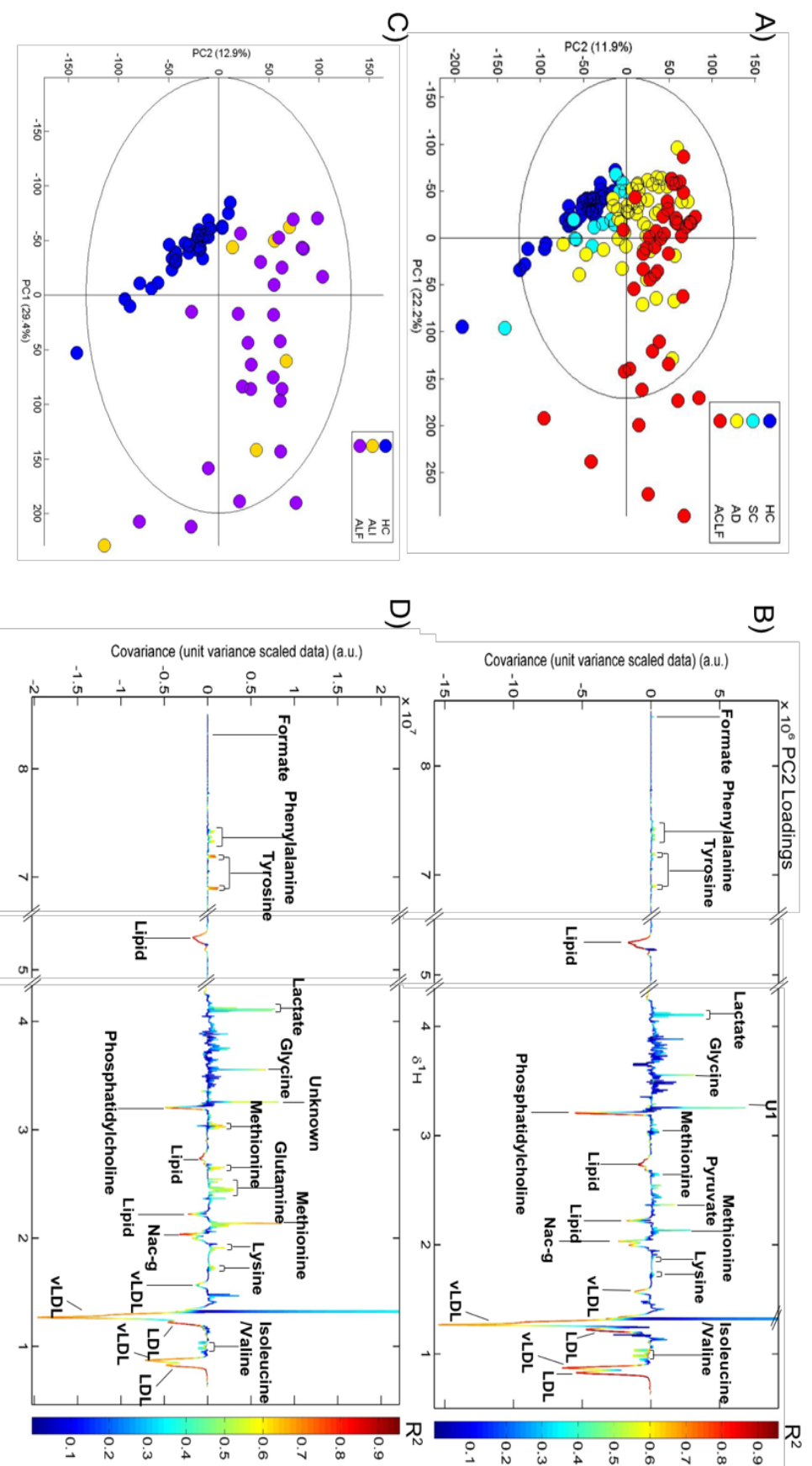


Figure 12. PCA analyses of plasma ^1H NMR spectral profiles from patients with acute and chronic liver disease and healthy volunteers

(A) A three component PCA model scores plot of HC, SC, AD and ACLF plasma sampled at Day 1 ($R^2=0.43$; $Q^2=0.31$) (B) Loadings plot for PC2 for scores plot in A, showing metabolic changes responsible for separation of scores in PC2 (C) A three component PCA model of HC and ALF plasma sampled at Day 1 ($R^2=0.43$; $Q^2=0.37$). (D) Corresponding loadings plot for PC2 in (C) shows changes in the metabolic resonances for PC2. Positive and negative signals denote metabolites that increase or decrease in intensity with worsening cirrhosis, scale is indicated by the coefficient of determination (R^2 , scale shown); U1 – unknown metabolite at 3.256ppm; LDL – low density lipoprotein; VLDL – very low-density lipoprotein; Nac-g- n-acetylglycoprotein;

3.4.5 Univariate analysis of ¹H NMR spectral peak integrals

Univariate analysis, using the same pairwise comparisons as for the multivariate analyses, revealed similar metabolic differences between, ALI, ALF, ACLF and AD and HCs. Individual representative metabolite peaks not overlapping with other peaks (n=19) were integrated and peak integrals were compared across all classes (Figure 13). For full reported p-values including overall ANOVA – p-values per metabolite with FDR correction and post hoc test p-values see appendix Table 37.

3.4.5.1 Metabolic differences in AD, ACLF, ALI and ALF relative to HC

Pairwise comparisons of AD vs HC, ACLF vs HC and ALF vs HC show a depletion in the BCAAs - isoleucine and valine by 0.6, 0.7 and 0.8-fold respectively. These comparisons also showed a rise in the aromatic amino acids (AAAs) including tyrosine in AD, ACLF, ALI and ALF by 1.7, 2.2, 3.2 and 3.6-fold respectively and phenylalanine in AD, ACLF, ALI and ALF by 1.5, 1.7, 2.6 and 2.3-fold respectively. Further amino acids significantly elevated in liver disease relative to HC included, lysine which was elevated in ACLF, ALI and ALF by 1.3, 2.1 and 1.9-fold respectively. Also, glycine which was elevated in ACLF, ALI and ALF by 1.5, 2.0 and 2.1-fold respectively and methionine was also elevated in ACLF, ALI and ALF by 2.0, 2.6 and 2.9-fold respectively.

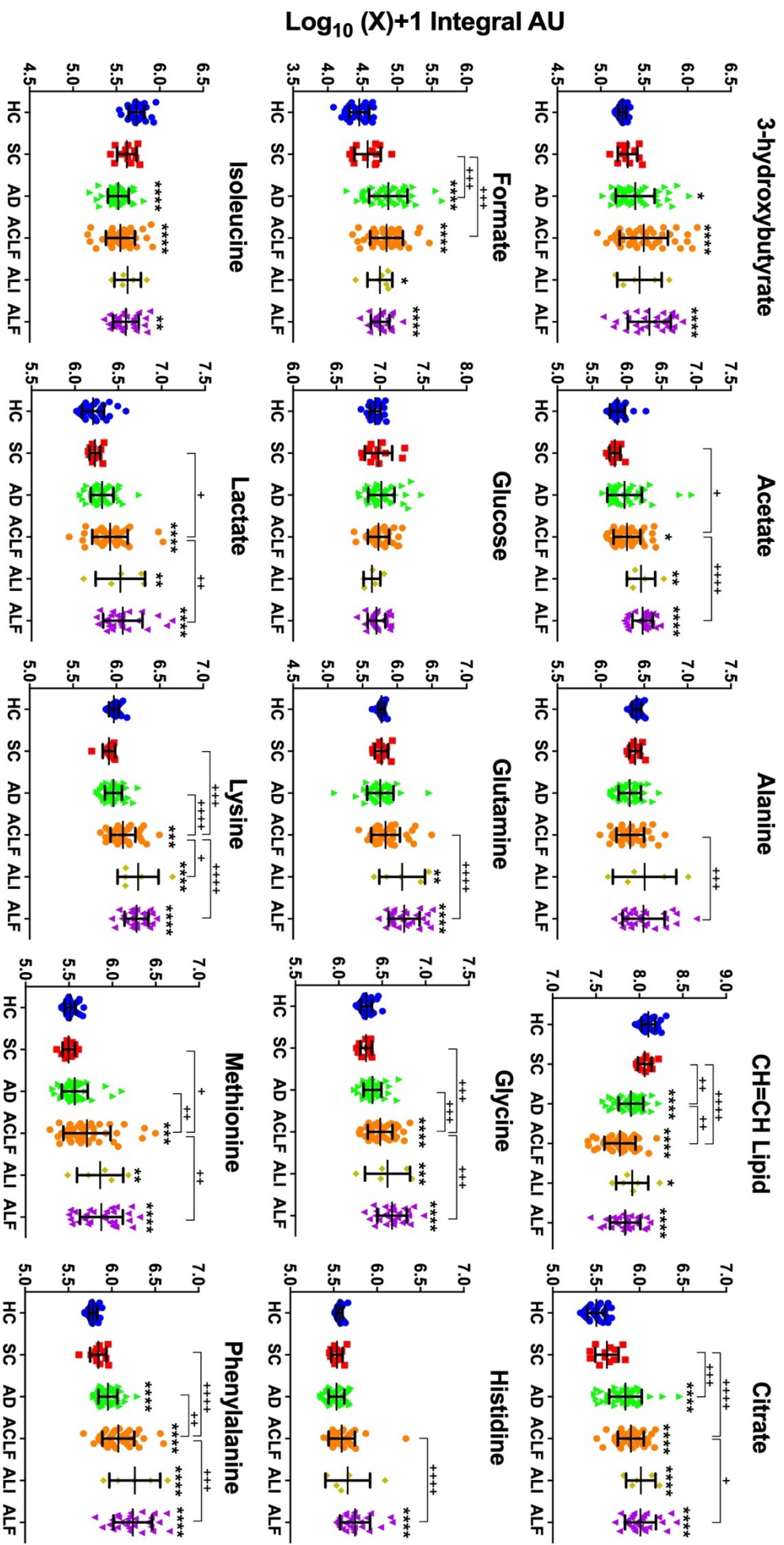
Furthermore, metabolites linked to energy metabolism also showed fold change differences relative to HC, this included acetate, which was raised in ACLF, ALI and ALF by 1.5, 2.3 and 2.3-fold respectively and lactate, which was also elevated in ACLF, ALI and ALF by 1.7, 2.3 and 2.4-fold respectively. Whilst, pyruvate was raised in ACLF and ALF by 1.8 and 2.2-fold respectively. In addition, 3-hydroxybutyrate was raised in AD, ACLF and ALF by 1.6, 2.2 and 2.3-fold respectively. Finally, an unknown metabolite observed at 7.663ppm was selectively raised in ACLF by 1.6-fold. See appendix Table 37 for all fold change differences between groups.

3.4.5.2 Metabolic differences discriminating between AD and ACLF

For pairwise comparisons focusing on differences between AD and ACLF, the main metabolic differences were a 0.8-fold depletion in lipid and a 1.3-1.6-fold increase in glycine, lysine, methionine, phenylalanine and pyruvate in ACLF compared to AD.

3.4.5.3 Metabolic differences discriminating between ACLF and ALF

The main metabolic changes observed in ACLF relative to ALF were a 0.6-fold decrease in lysine and a 1.8 increase in the unknown resonance (7.663pm). Further metabolic differences seen in ACLF relative to ALF was a 0.53-0.75-fold decrease in acetate, citrate, glutamine, glycine, lactate, methionine, phenylalanine and tyrosine.



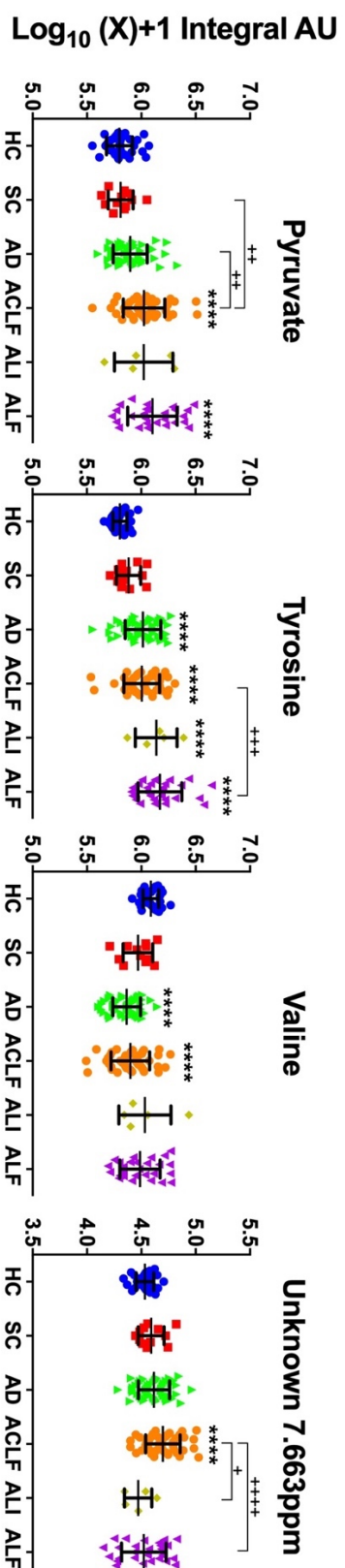


Figure 13 Univariate analysis of peak integrals from ^1H NMR spectroscopic data

These plots display metabolite changes relative to HC (*) and between different liver disease groups (+) for patients. A 1-way ANOVA was performed on log transformed data ($\text{log}_{10}(X) + 1$) followed by a post-hoc Sidak test. Error bars representing the standard deviation and indicating the mean are shown per group. Multiplicity adjusted p-values denoted by asterisks (*) show significant differences relative to HC and crosses (+) show significant differences between disease groups, specified by brackets; ****<0.0001, ***<0.001, **<0.01, *<0.05

3.4.6 Hierarchical cluster analysis of ^1H NMR integrals

The plasma ^1H NMR spectral integrals were assessed further to determine metabolite correlation patterns in acute and chronic liver disease. Metabolites that correlate strongly are often part of common metabolic pathways, hence this analysis was applied to characterise changes in metabolite correlations in HCs and acute and chronic liver disease groups. Based on this rationale, heat maps were computed from ^1H NMR spectral integral data showing metabolite-metabolite correlations (Pearson correlation with a r^2 colour scale).

Two main clusters of metabolites were determined from the HC ^1H NMR spectral data (Figure 14A). The main cluster which showed the strongest correlations, is indicated in the blue section of the dendrogram. The strongest correlations within this cluster were between lysine and isoleucine and valine ($r^2 > 0.8$). Furthermore, strong correlations were also seen between tyrosine, phenylalanine, methionine and lysine ($r^2 > 0.6$). The other main cluster was comprised of three sub-clusters of which the biggest sub-cluster (indicated in green), showed a strong correlation between lactate and pyruvate ($r^2 > 0.5$).

In the acute liver disease group (Figure 14B), two main metabolite clusters were determined, of which the biggest cluster (green nodes) showed strong correlations between pyruvate, lactate and alanine ($r^2 > 0.8$), as well as, strong correlations between glycine, glutamine, phenylalanine, acetate and lysine ($r^2 > 0.5$).

In cirrhosis (Figure 14C) two clusters were determined from the main cluster, the bigger cluster of these (red nodes) identified a group of amino acids showing strong correlations to each other, these included: phenylalanine, lysine, alanine, histidine, glutamine and glycine ($r^2 > 0.5$). Another strong correlation within the cluster was between valine and isoleucine ($r^2 > 0.8$), as well as, between lysine and alanine ($r^2 > 0.6$). In the other blue sub-cluster, a strong correlation was observed between pyruvate and lactate ($r^2 > 0.8$).

Overall, metabolite correlations intensify to a greater degree in the acute liver disease group. Furthermore, a greater number of metabolites show strong inter-correlation in acute liver disease compared to cirrhosis.

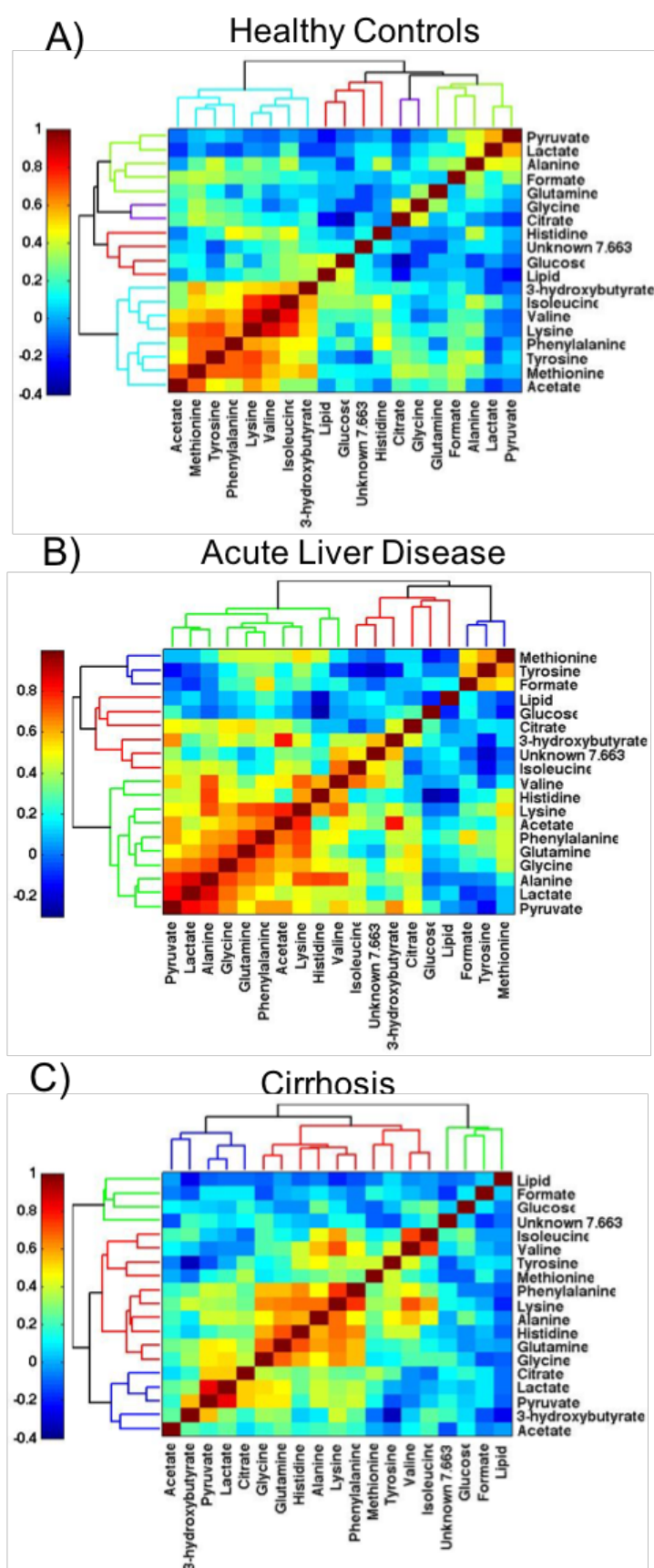


Figure 14. Heat map visualisation of metabolite-metabolite correlations

Correlations determined from ^1H NMR spectral integrals in plasma from HCs, chronic liver disease patients (SC, AD & ACLF) and acute liver disease patients (ALI and ALF) – Coloured squares represent Pearson's correlation coefficients (r^2) calculated from metabolite spectral integrals.

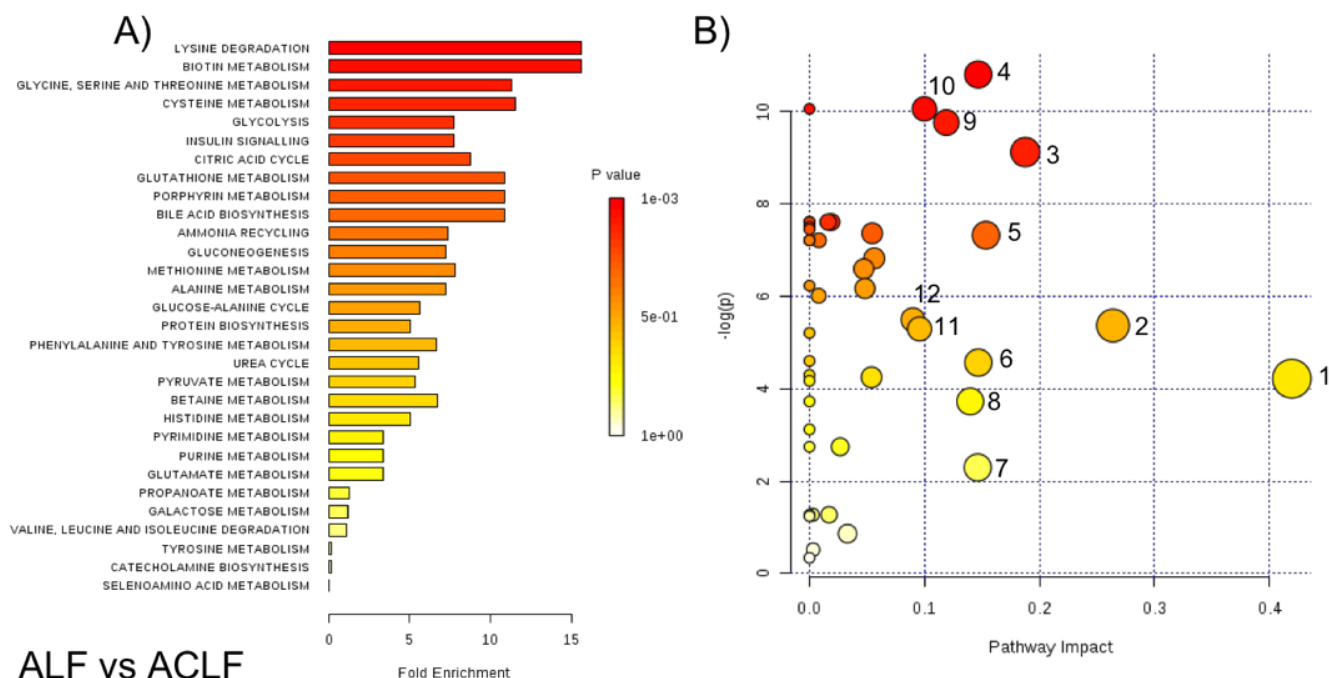
3.4.7 Metabolite set enrichment and pathway analysis

To identify the top pathways enriched in the data, the ^1H NMR integral data were analysed using metabolite set enrichment analysis (MSEA) a method employed to identify biologically meaningful patterns in metabolomics data.¹²⁴ Pathway analysis which combined results from MSEA with pathway topology analysis, was carried out to identify specific metabolic pathways upregulated, which explain metabolic distinction between groups of interest. Comparisons were made between ALF vs ACLF and AD vs ACLF, which generated statistically significant metabolic pathways enriched and upregulated by MSEA and pathway analyses respectively (Figure 15). Other comparisons were made between ALI vs ALF, SC vs AD and SC vs ACLF, these comparisons did not identify any statistically significant pathways perturbed (data not shown).

MSEA showed (Figure 15A & C) that perturbations in 17 metabolites identified by ^1H NMR spectroscopy, upregulated several metabolic pathways present in humans. The top pathways enriched for AD vs ACLF included: lysine degradation, biotin metabolism, glycine, serine and threonine metabolism, cysteine metabolism, glycolysis, insulin signalling and the citric acid (TCA) cycle ($p < 0.0001$). MSEA analysis for ALF vs ACLF revealed that the top pathways enriched included, pyrimidine metabolism, purine metabolism, glutamate metabolism, protein biosynthesis, ammonia recycling, lysine degradation and biotin metabolism ($p < 0.0001$).

Pathway analysis for both AD vs ACLF and ALF vs ACLF identified four top pathways that were common for both comparisons. The pathways identified were pyruvate metabolism, alanine, aspartate and glutamate metabolism, glycine, serine and threonine metabolism and lysine degradation (See Figure 15B & D, Table 3 & 4). The remaining 7 pathways identified in order of pathway impact differed in order between AD vs ACLF and ALF vs ACLF (Table 3 & 4). TCA metabolism was more significantly perturbed for AD vs ACLF compared to ALF vs ACLF, and histidine metabolism was more significant for ALF vs ACLF compared to AD vs ACLF (Table 3 & 4).

AD vs ACLF



ALF vs ACLF

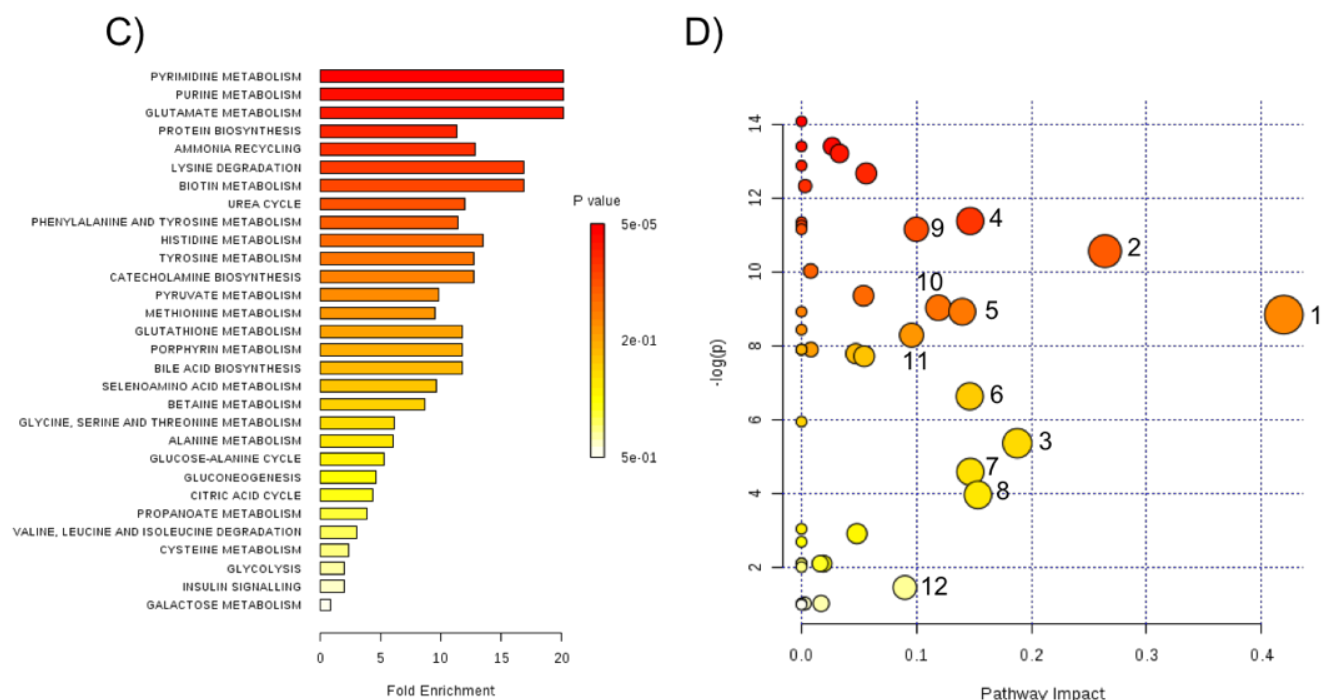


Figure 15. Metabolite set enrichment analysis and metabolite pathway analysis of changes in ^1H NMR spectral integrals in acute and chronic disease

Comparisons made include AD vs ACLF (A & B) and ALF vs ACLF (C & D); Metabolite set enrichment analysis (A & C) – X-axis refers to enrichment which was assessed using the MSEA Metabolic Pathway Library¹²⁴, pathways were considered enriched when $p < 0.05$; Summary of metabolite pathway analyses (B & D) - pathways are arranged according to the p-values (from quantitative enrichment analysis module; global test) on the y-axis and pathway impact values (from pathway topology analysis; node importance calculated from relative betweenness centrality)¹²⁶ on the X-axis. The nodes are coloured according to the p-value and node radius is determined by the pathway impact value.

AD vs ACLF				
No	Pathway Name	Total no. of Compounds	Hits	Pathway Impact
1	Pyruvate metabolism	32	4	0.42
2	Alanine, Aspartate and Glutamate metabolism	24	3	0.26
3	Glycine, Serine and Threonine metabolism	48	2	0.19
4	Lysine degradation	32	1	0.15
5	Citric acid cycle (TCA cycle)	20	2	0.15
6	Glyoxylate and dicarboxylate metabolism	50	3	0.15
7	Methane metabolism	34	2	0.15
8	Histidine metabolism	44	1	0.14
9	Phenylalanine metabolism	45	3	0.12
10	Lysine biosynthesis	32	1	0.10
11	Glycolysis and gluconeogenesis	31	4	0.10
12	Butanoate metabolism	32	1	0.09

Table 3. Pathway analysis summary of ^1H NMR data comparing AD vs ACLF

Total is the number of compounds in the pathway; the hits – the matched number from the user uploaded data; the impact – pathway impact value calculated from the pathway topology analysis (Figure 15B)

ALF vs ACLF				
No	Pathway Name	Total no. of Compounds	Hits	Impact
1	Pyruvate metabolism	32	4	0.42
2	Alanine, Aspartate and Glutamate metabolism	24	3	0.26
3	Glycine, Serine and Threonine metabolism	48	2	0.19
4	Lysine degradation	32	1	0.10
5	Histidine metabolism	44	1	0.14
6	Methane metabolism	34	2	0.15
7	Glyoxylate and dicarboxylate metabolism	50	3	0.15
8	Citric acid cycle (TCA cycle)	20	2	0.15
9	Phenylalanine metabolism	45	3	0.12
10	Lysine biosynthesis	32	1	0.10
11	Glycolysis and gluconeogenesis	31	4	0.10
12	Aminoacyl tRNA biosynthesis	75	10	0.06

Table 4. Pathway analysis summary of ^1H NMR data comparing ALF vs ACLF

Statistics are summarised from pathway analysis diagram shown in Figure 15D. For explanation of statistics reported see Table 3.

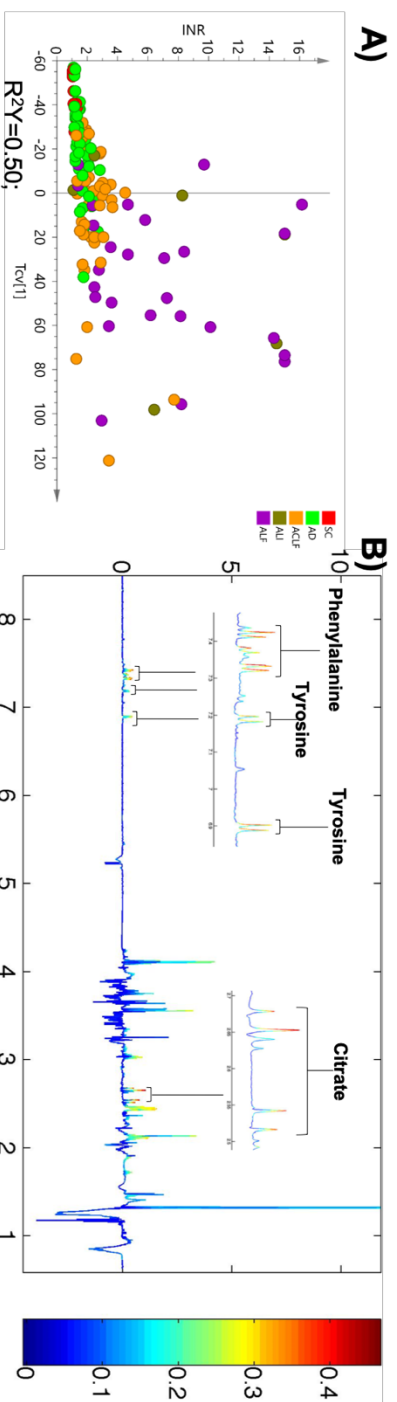
3.4.8 PLS regression with metadata

The ^1H NMR spectroscopic data for SC, AD, ACLF, ALF and ALI patients were regressed with the clinical metadata using a multivariate approach by PLS. These analyses were performed to identify any associations between the plasma ^1H NMR spectroscopic profiles and clinical

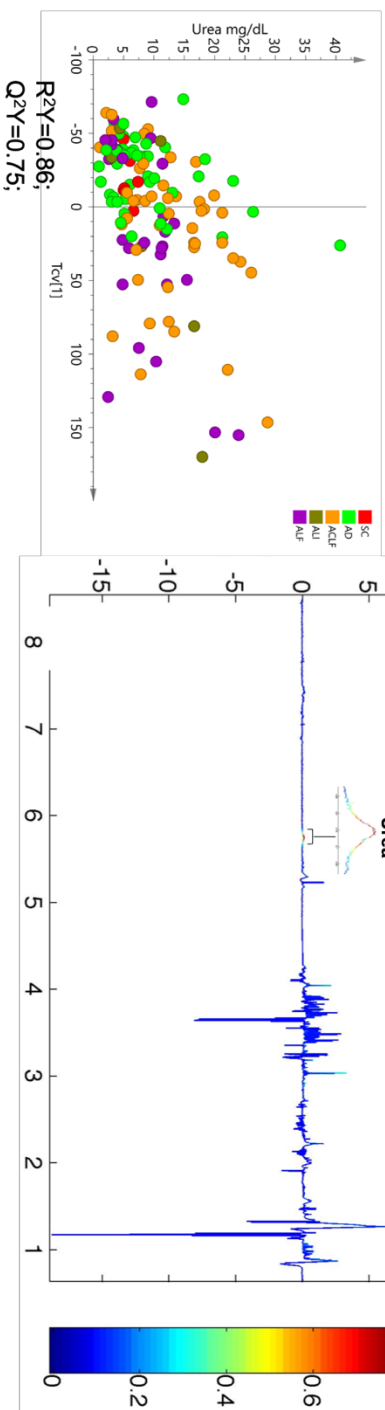
metadata and are summarised in Table 5. The ^1H NMR spectroscopic data were initially regressed against demographic data to check for confounders, these included age, gender, BMI and disease aetiology. All PLS regression models analysing associations between plasma ^1H NMR spectroscopic profiles and demographic data or disease aetiology, produced negative models (data not shown). Furthermore, the plasma ^1H NMR spectroscopic profiles were also regressed against survival data, including survival at 30 days, 90 days and 1 year which produced invalid models (data not shown).

Regression of the plasma ^1H NMR metabolic profiles against INR, revealed correlation with citrate and tyrosine ($r^2 > 0.4$) (Figure 16 A & B). Regression of the data against bilirubin and conjugated bilirubin revealed a strong correlation ($r^2 > 0.6$) with an unknown broad peak resonance at 4.97ppm (Figure 16G-J). Whilst, regression with albumin revealed correlation ($r^2 > 0.5$) to an unknown multiplet between 2.88-2.91ppm (Figure 16K & L). Regression against markers measured clinically, were also detectable by ^1H NMR spectroscopy and the ^1H NMR spectral peaks correlated with the clinical measurements, these included urea ($r^2 > 0.4$; Figure 16C & D), creatinine ($r^2 > 0.8$; Figure 16E & F), lactate ($r^2 > 0.8$; Figure 16M & N), triglyceride ($r^2 > 0.5$; Figure 16O & P) and cholesterol concentrations ($r^2 > 0.4$; Figure 16Q & R).

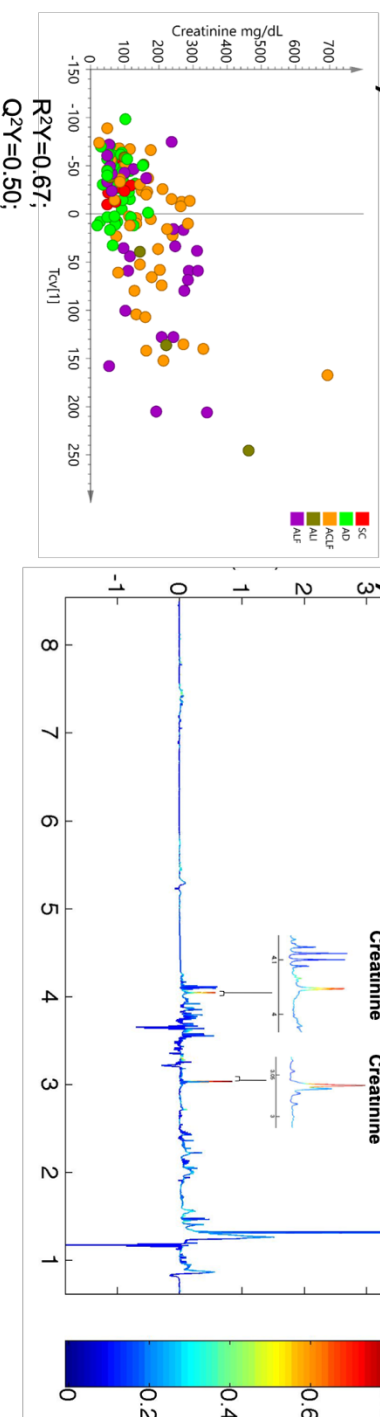
PLS regression with MELD score revealed strong correlations to lysine, methionine, phenylalanine and a DRC ($r^2 > 0.4$; Figure 16S & T). PLS regression was also performed separately for chronic liver disease (SC, AD, and ACLF) against UKELD score ($r^2 > 0.5$; Figure 16U & V) and also Child Pugh Score ($r^2 > 0.5$; Figure 16W & X), which showed strong correlations to lipid resonances in the plasma ^1H NMR spectra. Finally, PLS regression performed against ACLF grade (calculated from CLIF-SOFA scores) ($r^2 > 0.35$; Figure 16Y & Z) revealed a weak correlation to creatinine.

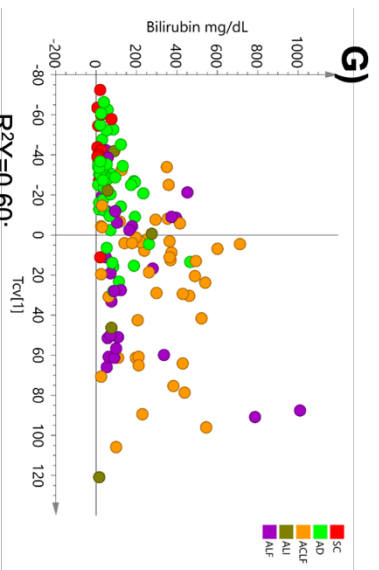


C)

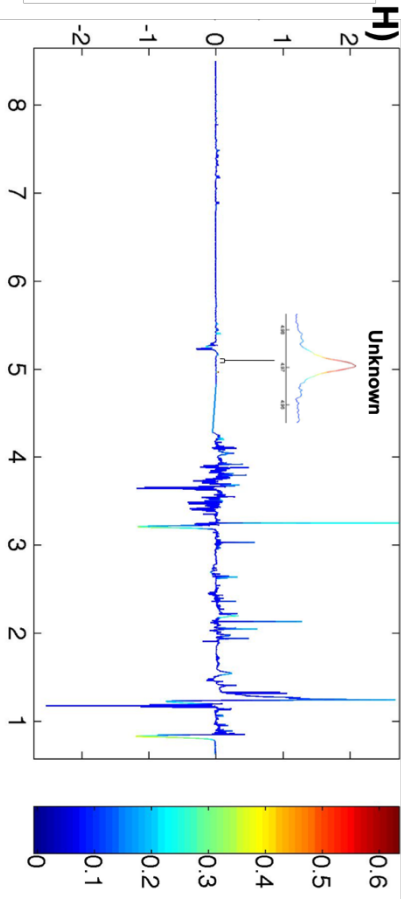
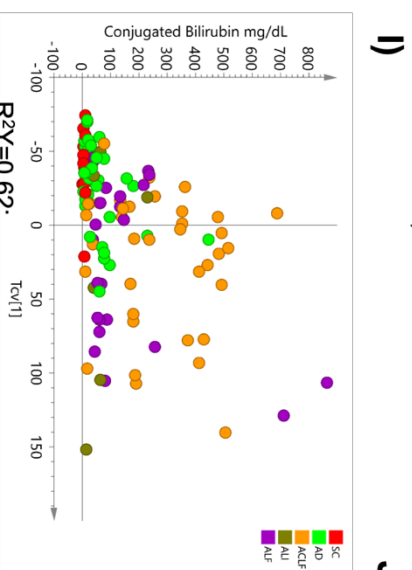


E)

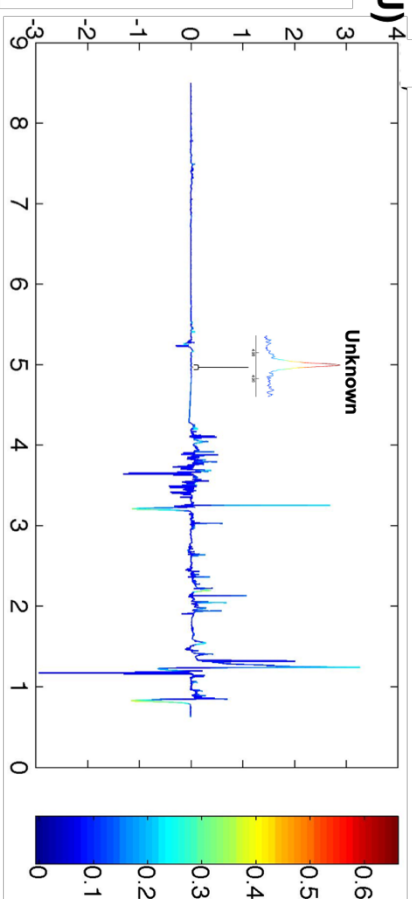
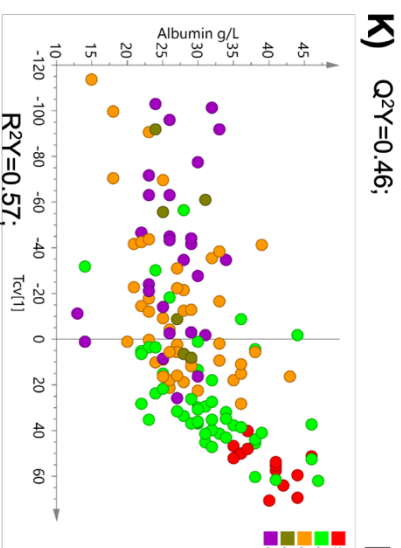




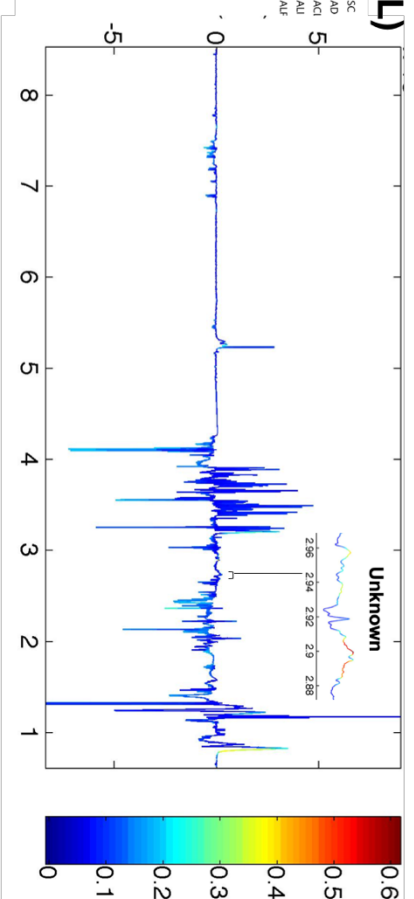
$R^2Y=0.60;$
 $Q^2Y=0.48;$

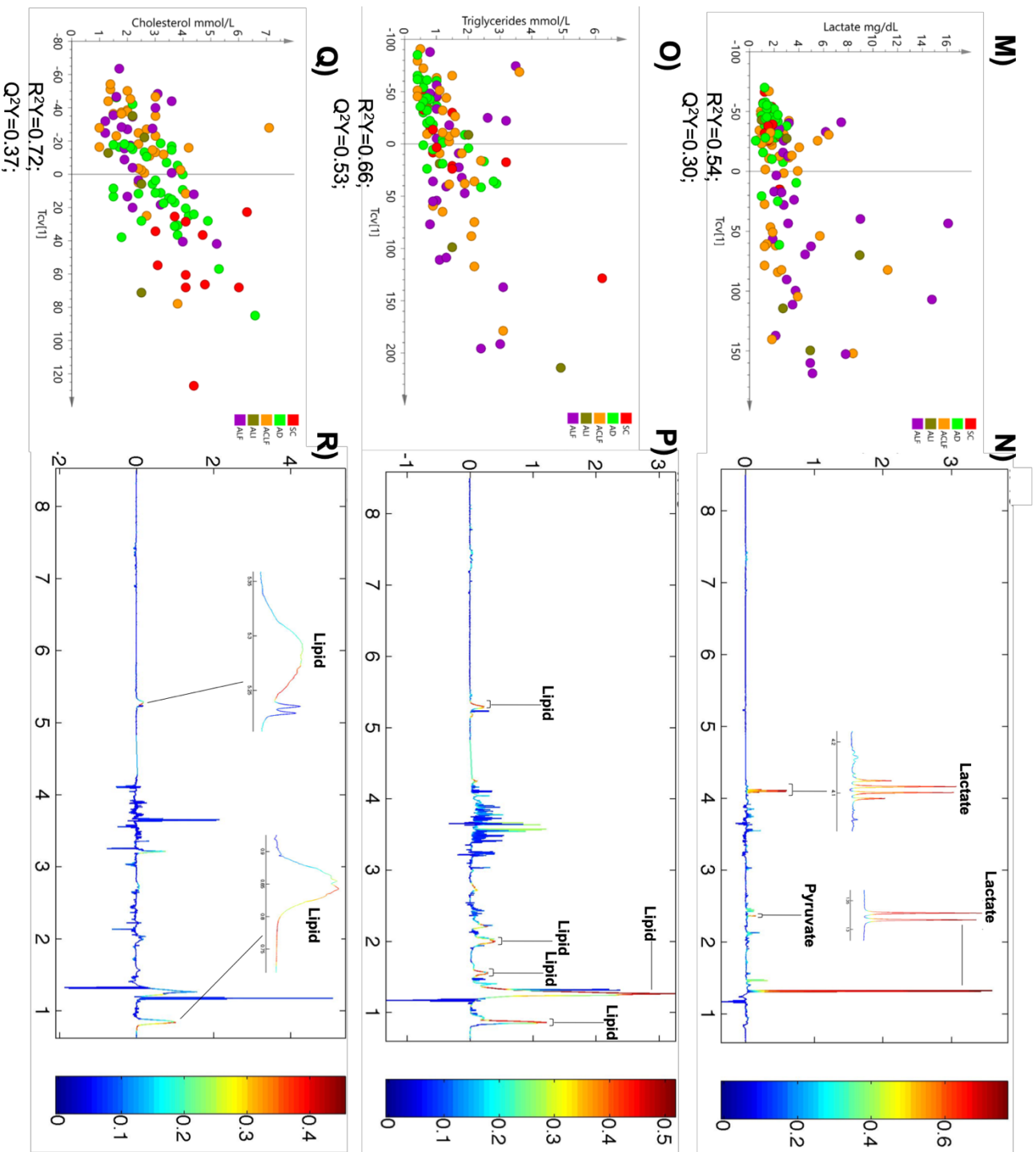


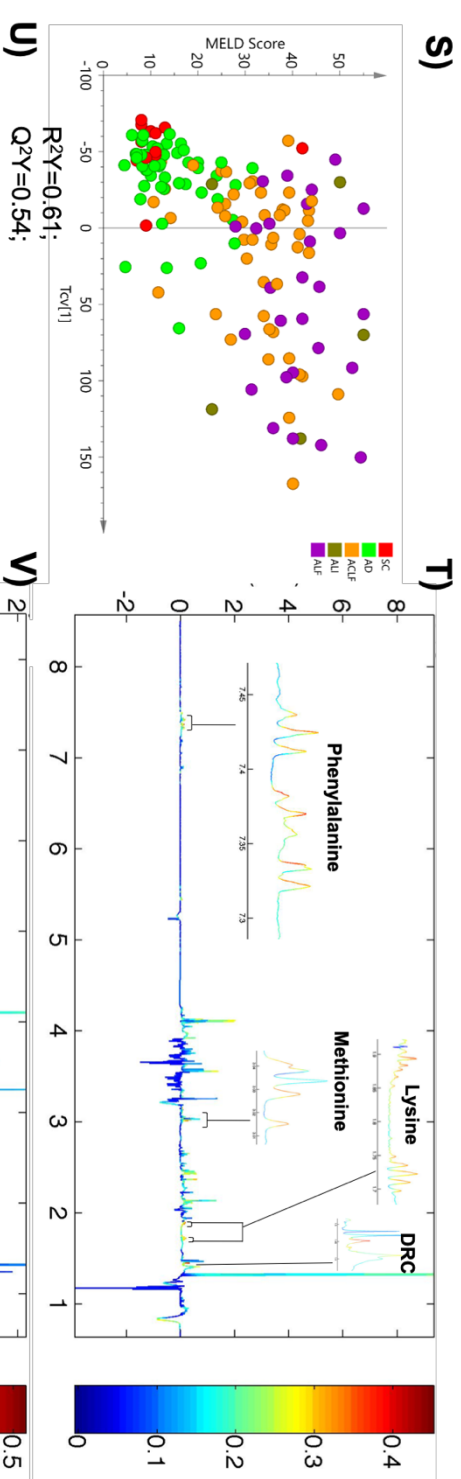
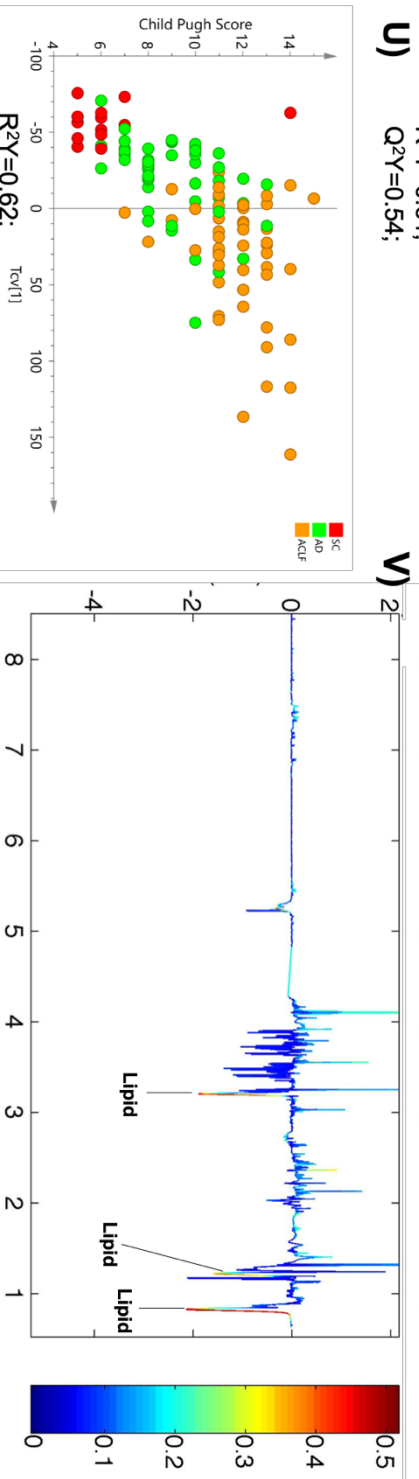
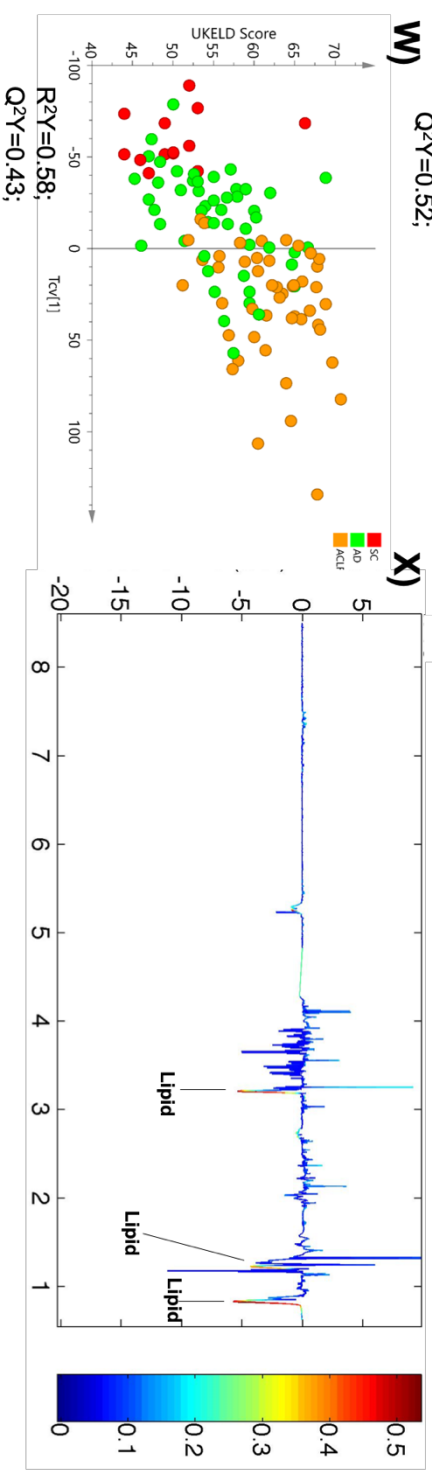
$R^2Y=0.62;$
 $Q^2Y=0.46;$



$R^2Y=0.57;$
 $Q^2Y=0.45;$







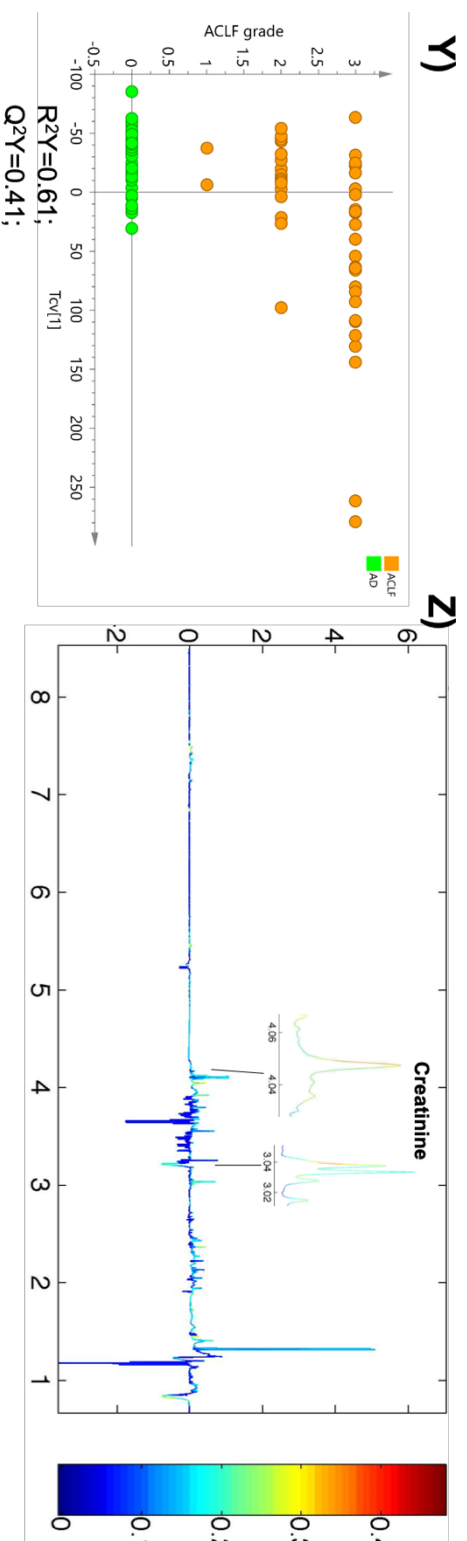


Figure 16. PLS regression of ^1H NMR plasma metabolic profiles with clinical metadata

^1H NMR spectroscopic data (X-variable) was compared to clinical metadata (Y-variable) by PLS-regression modelling. All models displayed were permuted ($n=999$; CV-ANOVA p-value <0.0001); For each model the cross-validated scores are plotted against the Y-variable and a corresponding PLS spectral loadings plot is displayed, correlation (r^2) is indicated by the colour scale on the right and the positive and negative signals denote metabolites that increase or decrease in intensity in the model. Cross-validated scores plots: A, C, E, G, I, K, M, O, Q, S, U, W & Y; PLS spectral loadings plots: B, D, F, H, J, L, N, P, R, T, V, X & Z; Y=INR – (A & B); Y=Urea – (C-D); Y=Creatinine – (E-F); Y=Bilirubin – (G-H); Y=Conjugated Bilirubin – (I-J); Y=Albumin – (K-L); Y=Lactate – (M-N); Y=Triglycerides – (O-P); Y=Cholesterol – (Q-R); Y=MELD Score – (S-T); Y=Child Pugh Score – (U-V); Y=UKELD Score – (W-X); Y=ACLF grade – (Y-Z);

Class	N=	Y- variable	No components	R ² Y	Q ² Y	Permuted R ² Y	Permuted Q ² Y	No. permutations	CV-ANOVA p-value
SC+AD+ACLF+ALI+ALF	143	INR	2	0.50	0.37	0.31	-0.04	999	5.03E-013
SC+AD+ACLF+ALI+ALF	137	Urea	2	0.86	0.75	0.59	0.09	999	9.19E-034
SC+AD+ACLF+ALI+ALF	137	Creatinine	2	0.67	0.50	0.31	-0.05	999	2.32E-019
SC+AD+ACLF+ALI+ALF	143	Bilirubin	2	0.60	0.48	0.31	-0.05	999	2.28E-018
SC+AD+ACLF+ALI+ALF	122	Conjugated Bilirubin	2	0.62	0.46	0.34	-0.03	999	5.37E-015
SC+AD+ACLF+ALI+ALF	143	Albumin	2	0.57	0.45	0.31	-0.04	999	8.81E-018
SC+AD+ACLF+ALI+ALF	112	Triglycerides	2	0.66	0.53	0.36	-0.04	999	4.59E-017
SC+AD+ACLF+ALI+ALF	112	Cholesterol	2	0.53	0.35	0.36	-0.026	999	8.51E-009
SC+AD+ACLF+ALI+ALF	128	Lactate	2	0.54	0.30	0.34	-0.04	999	1.31E-008
SC+AD+ACLF+ALI+ALF	142	MELD	2	0.61	0.54	0.31	-0.05	999	1.51E-022
SC+AD+ACLF	110	UKELD Score	2	0.58	0.43	0.38	-0.04	999	4.81E-012
SC+AD+ACLF	110	Child Pugh Score	2	0.62	0.52	0.38	-0.05	999	7.92E-016
AD+ACLF	99	ACLF Grade	2	0.61	0.41	0.37	-0.03	999	2.075E-10

Table 5. Summary of PLS regression models

PLS regression of ¹H NMR spectroscopic data with clinical metadata; N= number of patients; All models have 2-Y predictive components; INR – international normalised ratio; MELD -model for end stage liver disease; UKELD – United Kingdom model for end stage liver disease;

3.4.9 Analysis of sequential plasma samples from ALF and ACLF patients

Univariate analysis was also performed on the ^1H NMR spectral peak integrals from plasma metabolic profiles obtained from sequential plasma samples, which were collected at multiple time points between days 1-28 post admission from a selection of ALF and ACLF patients (see

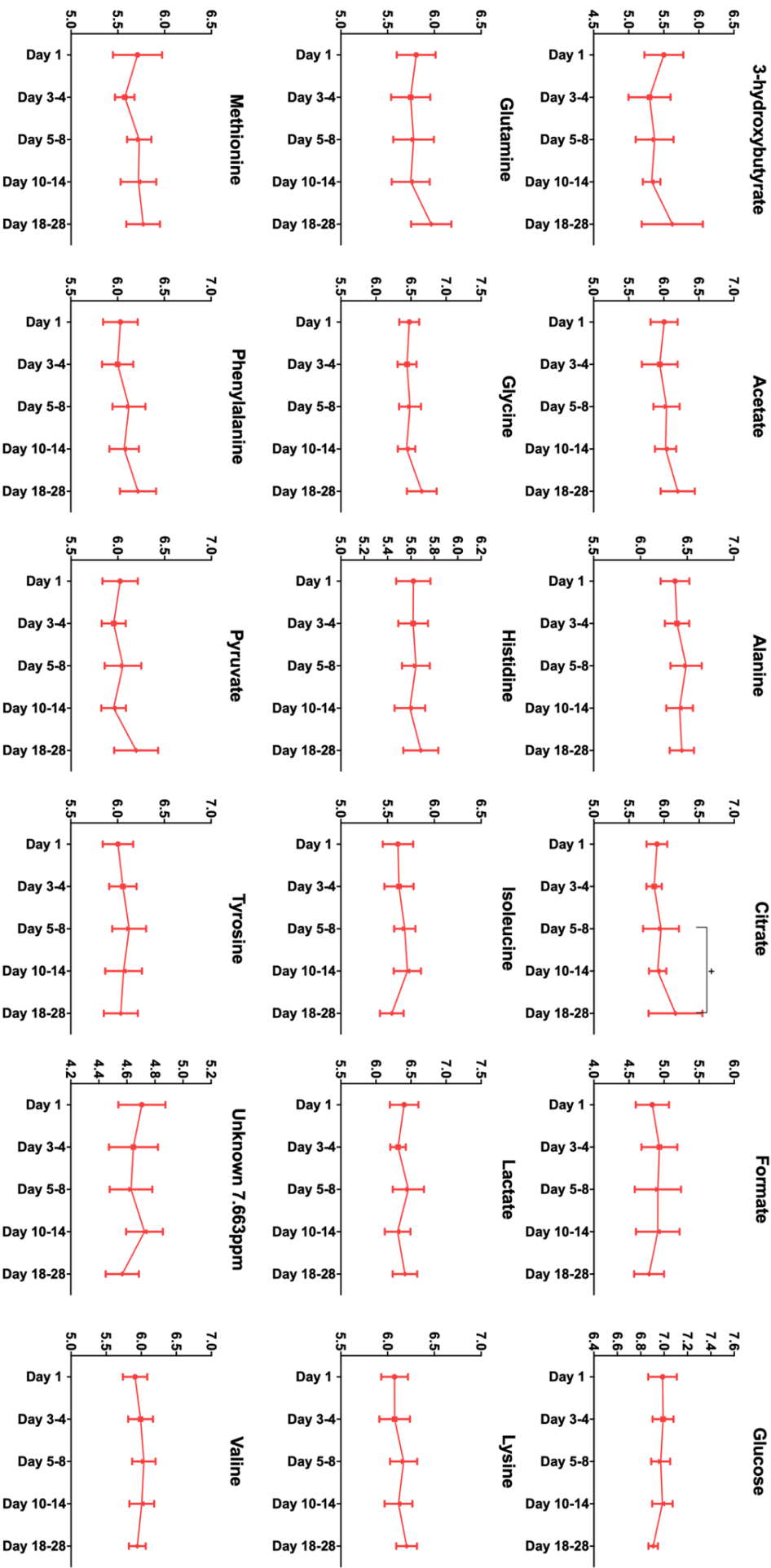
Table 6). Univariate analysis of metabolites from the ACLF patients showed no differences in intensity over time (Figure 17). In contrast, analysis of sequential plasma samples from ALF patients revealed changes in metabolite intensity over time in valine, alanine, lysine, glutamine, pyruvate, acetate, citrate, methionine, glycine, lactate, histidine, unknown metabolite (7.663 ppm), tyrosine and phenylalanine. The unknown metabolite increased in intensity from days 3-4 to days 10-14. The other metabolites showed an overall decrease in intensity at day 10-14 compared to earlier time points (Figure 17). Analysis comparing survivors and non-survivors at 30 days post admission in both the ALF and the ACLF groups was also performed and showed no differences at each timepoint (data not shown). Past day 8 post admission in the ALF group, all samples collected were from patients who were survivors at 30 days.

	ALF				ACLF			
	n=	30-day survival	Transplanted (n=)	T-30 day survival	n=	30-day survival	Transplanted (n=)	T-30 day survival
Day 1	27	79%	11	42%	48	58%	6	17%
Day 3-4	8	43%	2	50%	10	43%	0	-
Day 5-8	8	43%	1	0%	12	43%	0	-
Day 10-14	6	100%	0	-	10	43%	0	-
Day 18-28	1	100%	0	-	4	100%	0	-

Table 6. Sequential plasma samples from ALF and ACLF patients

The number of plasma samples per timepoint are summarised (n=) for ALF and ACLF; the proportion of survivors at 30 days post admission (%) are also indicated; T-30 day survival – survival at 30 days post admission for transplanted patients;

A)



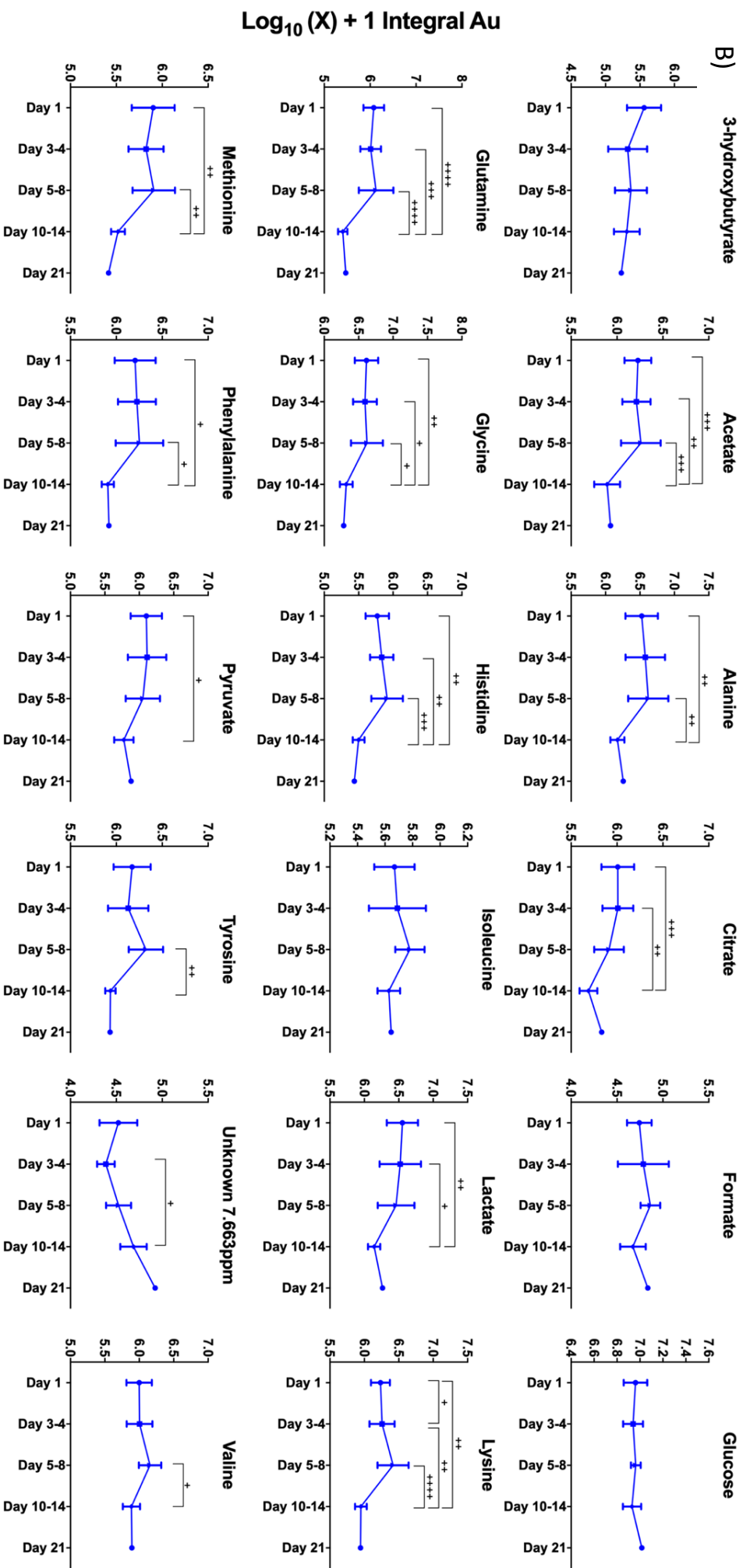


Figure 17. Time course analysis of metabolic changes in ALF and ACLF patients

These plots display metabolite changes over time between multiple timepoints from day 1 of admission to day 28 post admission. Analysis for ACLF patients is shown in part A and for ALF patients is shown in part B. Days post admission are grouped: Day 1, Days 3-4, Days 5-8, Days 10-14 and Day 21. A 1-way ANOVA was performed on log transformed data ($\log_{10}(X) + 1$) followed by a post-hoc Sidak test. Error bars representing the mean and standard deviation and mean are shown per timepoint. Multiplicity adjusted p-values denoted by asterisks (*) show significance between days' post admission by brackets; **** <0.0001; *** <0.001; ** <0.01; + <0.05.

3.5 Discussion

In this study, plasma metabolic profiles were generated by ^1H NMR spectroscopy from patients with early and advanced forms of acute and chronic liver disease and liver failure. Using an untargeted approach, ^1H NMR spectroscopy characterised discriminant differences between acute and chronic liver disease, identifying a panel of metabolites that show distinct perturbations both within and between the acute and chronic liver disease groups.

Initially, comparisons were made between plasma ^1H NMR metabolic profiles at day 1 of admission between patients with acute and chronic liver disease relative to HC. The main metabolic changes identified for acute and chronic liver disease, relative to HC, were a perturbation of lipid metabolism, glycolysis, the TCA cycle, the urea cycle, gluconeogenesis, and sulphur metabolism. These metabolic changes confirm findings from previous ^1H NMR spectroscopic profiling studies of ALF, ACLF and AD^{81,85,86} but also generate further novel understanding of these syndromes. Furthermore, a metabolic change unique to ACLF was also identified, where an unknown metabolite detected as a singlet peak at 7.663ppm was selectively upregulated in ACLF (Figure 13). Interestingly, this metabolite has been observed previously in human ^1H NMR spectroscopic profiling studies in hepatitis C induced fibrosis, NASH and alcoholic hepatitis,^{127,128} however it remains unidentified.

The Venn diagram shown in Figure 18 summarises the main metabolic findings for AD, ACLF and ALF relative to HC from univariate analysis of the ^1H NMR peak integrals. A core metabolic phenotype of AD, ACLF and ALF was revealed, in addition to, metabolic perturbations that were unique to ALF and ACLF. Subsequent analyses employing HCA, MSEA and pathway analysis of the data, identified specific metabolic pathways perturbed that explained the distinction for AD vs ACLF and ALF vs ACLF for the first time (see Figure 14 & 15).

Furthermore, PLS regression of the ^1H NMR spectroscopic data with the clinical metadata revealed valid models when the data were regressed with blood markers used in prognostic scoring systems such as MELD, CLIF-SOFA, KCC and UKELD. However, further analyses of these are required to understand the unique phenotypes associated with the different clinical scores. Finally, analysis of the sequential ^1H NMR data identified overall significant differences in metabolites between days 5-8 and 10-14 post admission in a subset of ALF patients (Figure 17) which are discussed further.

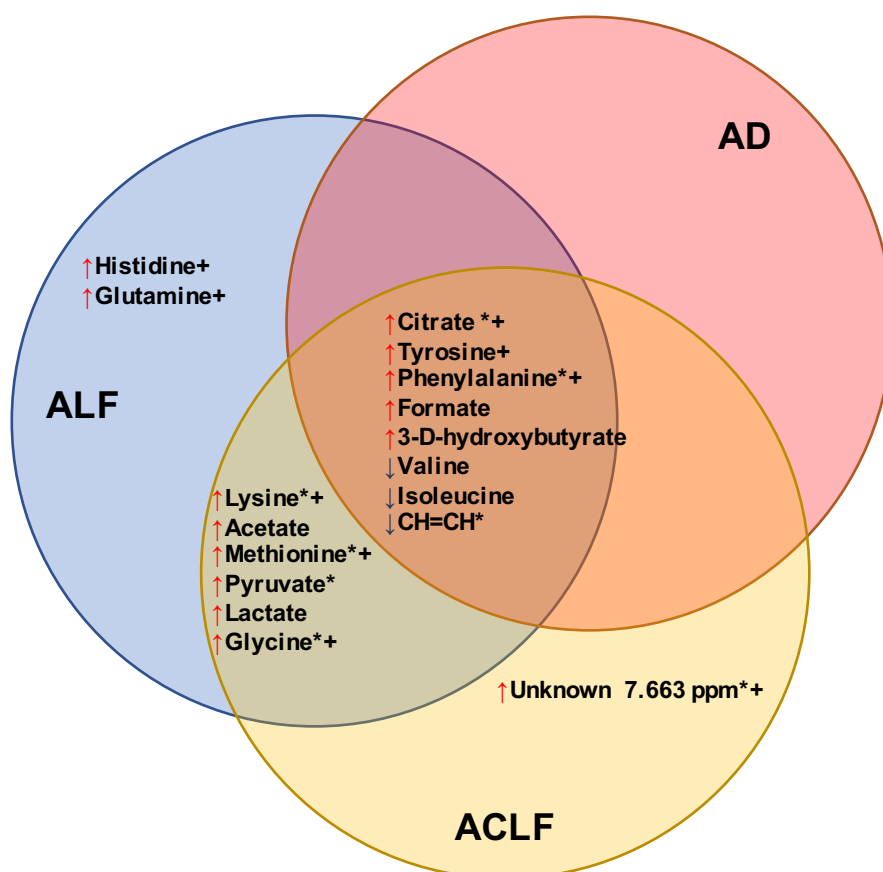


Figure 18. Venn diagram summarising metabolic differences in AD, ACLF and ALF

These diagrams summarise the metabolic changes observed in AD, ACLF and ALF relative to HC, as well as, changes between groups by ^1H NMR spectroscopy. Statistical significance was derived from earlier univariate analyses see Figure 13. Differences significant between AD vs ACLF are marked with an asterisk (*) and significant changes between ALF and ACLF are marked with a cross (+).

3.5.1 Disturbance of the TCA cycle is common to acute and chronic liver disease

Significantly elevated plasma resonances of acetate, lactate, pyruvate, citrate in acute and chronic disease (Figure 13) are implicated in the citric acid (TCA cycle) and linked to glycolysis and gluconeogenesis. A perturbation of these pathways explains the distinction between ALF and ACLF by pathway analysis and MSEA (Figure 15C), where a greater elevation of citrate and lactate in ALF is associated with increasingly poor hepatic reserve.¹²⁹ This trend and an increase in plasma pyruvate levels also explains the progression of AD to ACLF and is representative of an overall increase in hepatocellular damage. Furthermore, elevation of lactate and pyruvate also confirms the presence of lactic acidosis in AD, ACLF and ALF, where acids accumulate in the blood stream resulting in an excessively low blood pH.¹³⁰

Raised circulating lactate concentrations in liver failure are used as a marker of disease severity for prognostic purposes in several scoring systems, such as MELD, UKELD, CLIF-SOFA and KCC. However, the mechanism for high circulating levels of lactate is not clear but could

occur because of reduced hepatic capacity and therefore clearance of lactate via the hepatic enzyme lactate dehydrogenase to pyruvate is diminished in the failing liver. Or because of high circulating pyruvate levels which drive a rise in lactate, as in healthy individuals lactate and pyruvate are present in physiological equilibrium.¹³¹

Furthermore, a rise in lactate and no change in glucose suggests there is an impairment in the hepatic arm of the lactic acid cycle (hepatic gluconeogenesis) see Figure 19. An impairment of gluconeogenesis in cirrhosis and ALF is attributed to hypoglycaemia as a result of declining insulin tolerance and reduced hepatic glycogen storage.^{132–134} The PDK-1 signalling pathway, which regulates insulin signalling in the liver, has been shown to be inhibited in a previous study on liver failure¹³⁵ and therefore may be a downstream target defective in ALF, ACLF and AD. In addition, the impairment of hepatic gluconeogenesis has further implications for metabolic pathways involved in acid-base homeostasis and energy metabolism.¹³⁶

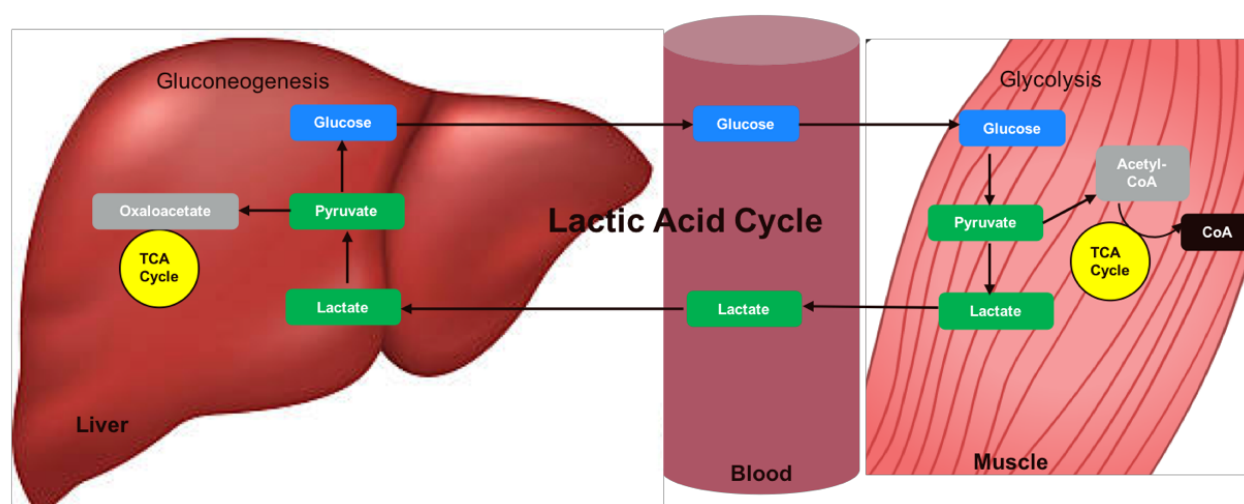


Figure 19. The Lactic Acid cycle in acute and chronic liver disease

Metabolites are coloured according to changes in ALF and ACLF relative to HC: green – increase in intensity; blue – no change; grey - metabolite not detected by ¹H NMR spectroscopy

A further key pathway linked to the TCA cycle is the glucose-alanine cycle (Figure 20) highlighted by MSEA analysis, where there was a greater perturbation in ACLF compared to AD and ALF compared to ACLF (Figure 15). This pathway removes excess extrahepatic ammonia and therefore may explain the presence of higher ammonia in these patients. Furthermore, the pathway also relies on hepatic transaminases which degrade alanine and other associated amino acids originating from muscles to produce pyruvate as an alternative pathway for energy metabolism, which also links it to increased muscle catabolism.

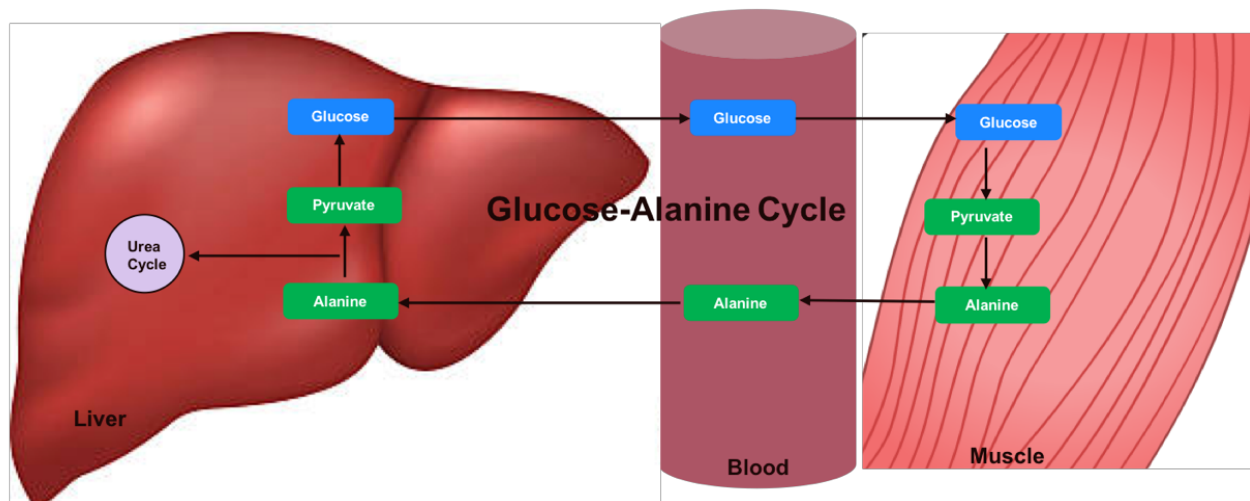


Figure 20. The Glucose-Alanine cycle in acute and chronic liver disease

Metabolites are coloured according to changes in ALF and ACLF relative to HC: green – increase in intensity; blue – no change;

Pyruvate, which feeds into the TCA cycle, is generated from the glycolysis pathway and is oxidised to Acetyl-CoA (Figure 21) which is catalysed by the enzyme pyruvate dehydrogenase (PDH). A recent study of a murine model of ALF, reported increased LDH and PDH activity which coincided with higher levels of acetyl-CoA and lactate and was linked to severity of disease, mortality and low hepatic reserve.¹³⁷ We hypothesise that PDH and LDH activity correlate with disease severity and decreased hepatic capacity and hence explains the trend seen in the associated metabolites in acute and chronic liver disease.

Furthermore, acetate which was significantly elevated in ACLF and ALF (Figure 13) is also linked to the TCA cycle, where it is oxidised by the enzyme acetyl-CoA synthetase to acetyl-CoA.¹²⁵ Previous studies on fatty liver disease and acute alcoholic hepatitis, also report high plasma levels of acetate and link this to a decrease in acetyl-CoA synthetase activity because of mitochondrial dysfunction or depletion of intracellular ATP and hepatocyte death.^{138,139}

An additional metabolic pathway linked to TCA cycle disturbance that is discriminant for ALF vs ACLF and AD vs ACLF are the glyoxylate and dicarboxylate metabolic pathways (see Tables 3 & 4). These pathways have been identified previously in studies of liver fibrosis, non-alcoholic fatty liver disease and hepatocellular carcinoma and have been linked to gut microbial metabolism.^{140–142} Furthermore, the breakdown of glyoxylates and dicarboxylates generates the TCA metabolites oxalate and pyruvate which feed into the TCA cycle,¹⁴³ however perturbation of this pathway in the context of cirrhosis and liver failure is not yet clear.

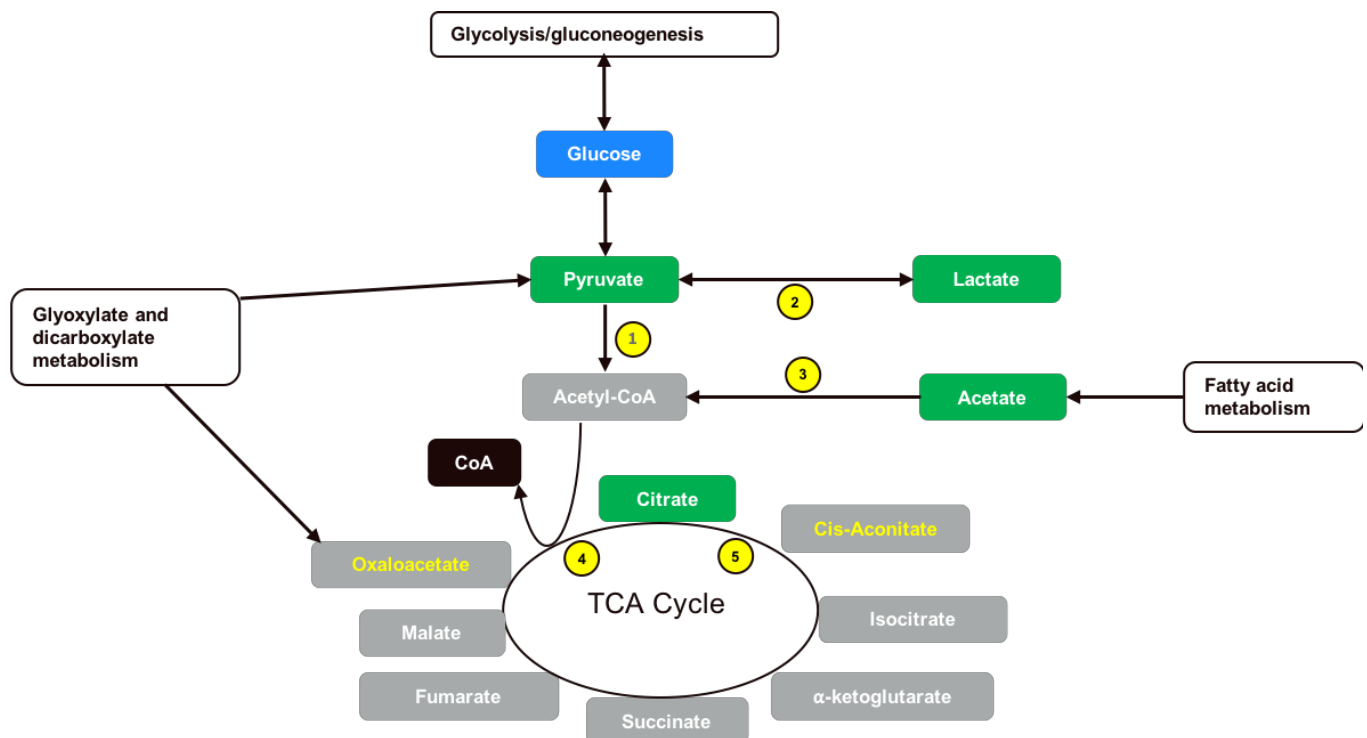


Figure 21. The TCA cycle in acute and chronic liver disease

Metabolites are coloured according to changes in ALF and ACLF relative to HC: green – increase in intensity; grey - metabolite not detected by ^1H NMR spectroscopy

3.5.2 Amino acid metabolism and impairment of the urea cycle in acute and chronic liver disease

Metabolic pathways linked to amino acid metabolism and protein biosynthesis which include the alanine, aspartate and glutamate pathway, the glycine, serine and threonine pathway and the glucose-alanine cycle were all discriminant for AD vs ACLF and ALF vs ACLF (see Figure 15). A disruption of these pathways is linked to the perturbation of the urea cycle, because of diminished hepatic reserve resulting in impaired nitrogen clearance leading to aminoacidemia and hyperammonemia.

In addition, histidine, which is an essential amino acid part of the histidine metabolic pathway, was significantly elevated in ALF compared to HC and ACLF (see Figure 13). This pathway is also linked to the TCA cycle, as degradation of histidine generates α -ketoglutarate via the intermediate metabolite glutamate (Figure 22).^{144,145} High plasma levels of histidine have been observed in studies on liver failure and cirrhosis and are thought to occur due to a loss in functioning hepatic tissue and therefore metabolic capacity of the liver to breakdown histidine.^{62,146,147}

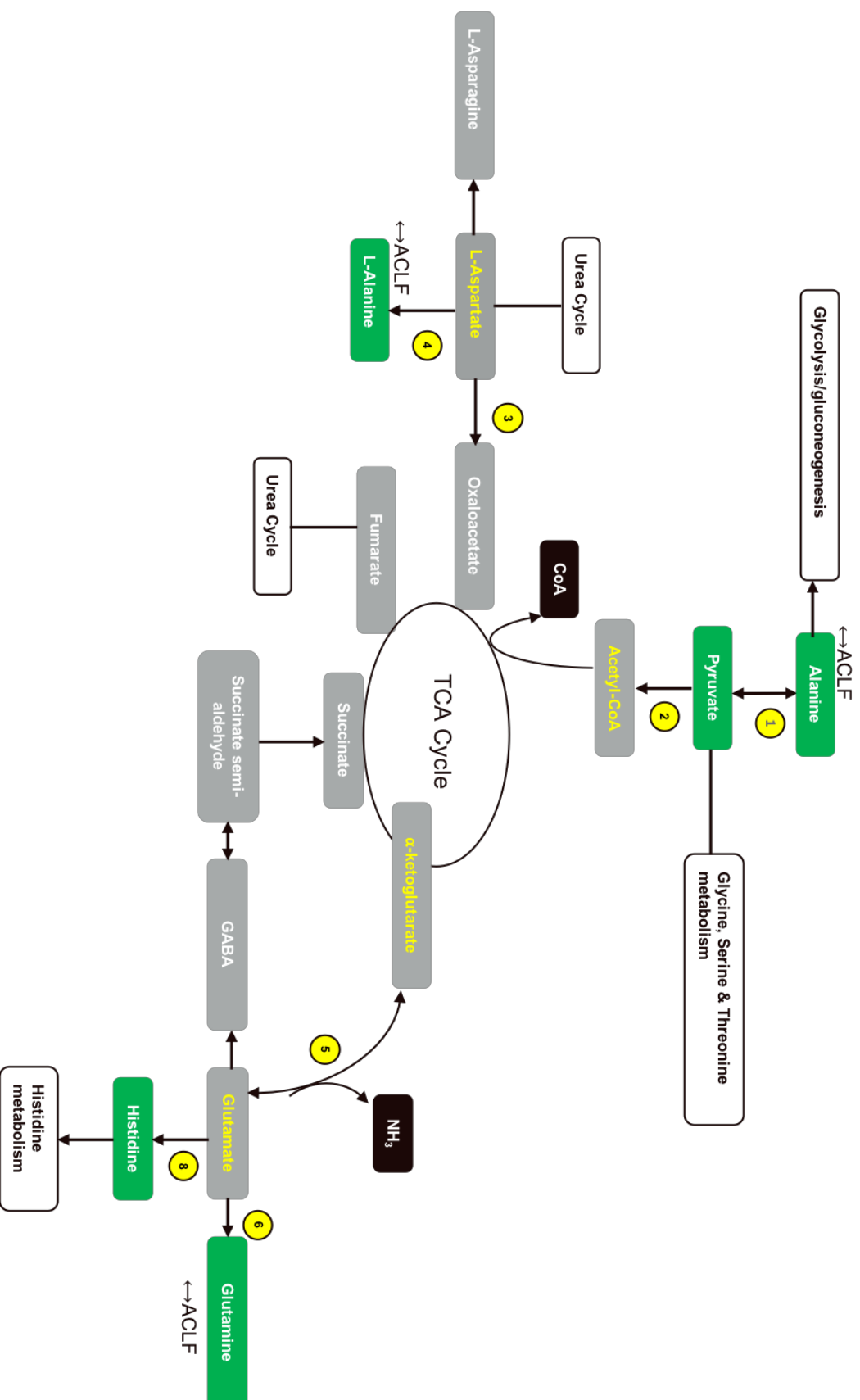


Figure 22. The Alanine, Aspartate and Glutamate pathway

Metabolites are coloured according to changes in ALF and ACLF relative to HC: green – increase in intensity; grey - metabolite not detected by ^1H NMR spectroscopy

Other pathways implicated included ammonia recycling and the urea cycle (Figure 23) these pathways have been widely observed to be perturbed in previous liver failure and cirrhosis studies (see Chapter 1, section 1.6.1). In this study, hyperammonemia is seen in both ALF and ACLF (see Figure 9), and ammonia levels were the highest in ALF and therefore explained a significant enrichment of the ammonia recycling pathway in ALF compared to ACLF (Figure 15C). The urea cycle, which is linked to this pathway and clears hepatic ammonia (see Figure 23) was also identified as discriminant for ALF vs ACLF and AD vs ACLF by MSEA. The ALF patients showed a greater metabolic perturbation, which can be explained by higher circulating amounts of alanine and glutamine (see Figure 13). These amino acids are linked to the urea cycle via the alanine, aspartate and glutamate pathway which was also discriminant for AD vs ACLF and ALF vs ACLF (see Figure 22) and a perturbation of these pathways results in impaired ammonia clearance.¹⁴⁸

In addition, hyperammonemia causes disruption of the TCA cycle and may also explain the distinction in perturbation of the TCA cycle between AD vs ACLF, as ammonia is known to deplete α -ketoglutarate, which is linked to the TCA cycle (Figure 23).¹⁴⁹ Hyperammonemia, is also linked to elevated glycine and glutamine concentrations, as excess ammonia is converted to glutamine and elevated glycine drives further ammonia generation (Figure 23).¹⁴⁸

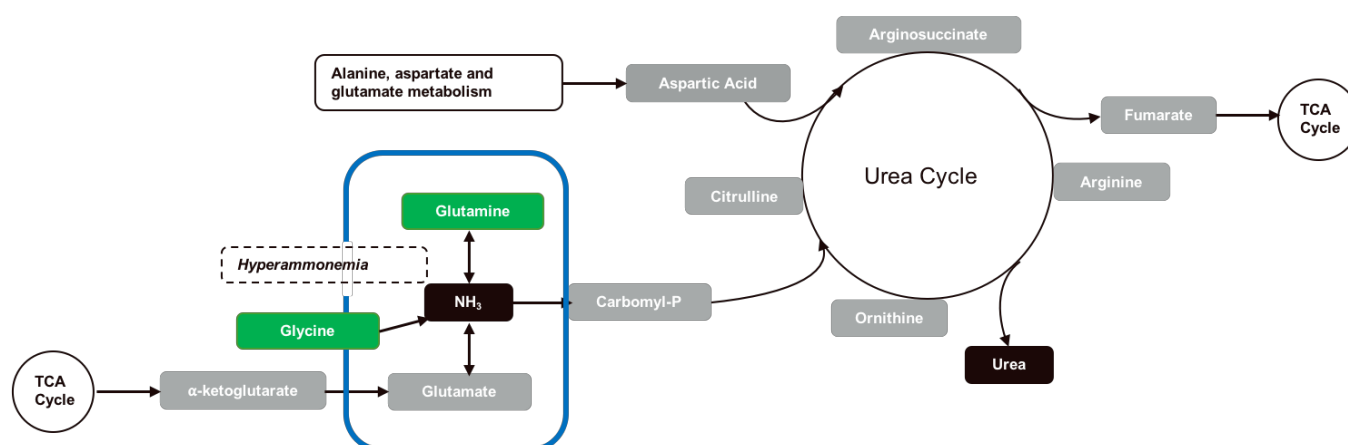


Figure 23. Hyperammonemia and the Urea Cycle

Metabolites are coloured according to changes in ALF and ACLF relative to HC: green – increase in intensity;

3.5.3 A perturbation of Purine and Pyrimidine metabolism is associated with liver injury

Pyrimidine metabolism and purine metabolism were the calculated as one of the top pathways discriminant between ALF vs ACLF (Figure 15D), both glutamine and glycine which

regulate these pathways are significantly more elevated in ALF compared to ACLF suggesting a greater perturbation of these pathways in ALF. The pyrimidine and purine pathways are involved in nucleic acid synthesis and are reportedly perturbed in previous studies on fatty liver disease and cirrhosis.^{128,150} A depletion of uridine, a precursor of nucleic acids, causes liver sensitisation to mitochondrial toxicity and an accumulation of lactate, as well as, a dysregulation of lipid metabolism.^{150,151} However, it is not clear if these mechanistic links to purine and pyrimidine metabolism are present in patients with ALF or cirrhosis.

3.5.4 Prognostic markers of liver damage correlate with plasma ¹H NMR profiles of acute and chronic liver disease

PLS regression of the clinical metadata (Table 5) with the ¹H NMR plasma metabolic profiles for SC, AD, ACLF, ALI and ALF confirmed results from the clinical data, which showed elevated markers indicative of liver dysfunction including lactate, urea and creatinine (Figure 16C-N).^{152–154} Furthermore, PLS regression with bilirubin and albumin, also produced valid models which showed a strong association with unknown metabolite peaks at 4.97ppm and 2.91ppm respectively, these require further investigation.

The ¹H NMR spectroscopic data was also regressed against clinical scores used for prognostication including the Child Pugh, MELD and UKELD scores and also ACLF grade – which was determined from the CLIF-SOFA scores (Figure 16S-Z). PLS regression with ACLF grade revealed a correlation to ¹H NMR spectral creatinine peak, this confirmed the prognostic importance of creatinine and that it is integral to the CLIF-SOFA scoring system.⁴⁵ Whereas, PLS regression with the MELD score (Figure 16T) identified the amino acids phenylalanine, lysine and methionine had the strongest correlation to the model, high levels of methionine and phenylalanine have been previously linked to survival.¹⁵⁶ In addition, PLS regression with the UKELD and Child Pugh scores (Figure 16 V & X) which are used for prognostication of chronic liver disease,¹⁵⁷ revealed strong correlations to lipid resonances in the ¹H NMR spectra. Hence, the ¹H NMR spectroscopic PLS models of UKELD and Child Pugh suggest that depleted plasma lipids correlate with the severity of chronic liver disease and therefore mortality risk, and has been reported in patients with AD previously.¹⁵⁶

3.5.5 ALF patients surviving past 10 days' post admission show signs of recovery

As the first metabonomics study to look at longitudinal sampling of ACLF and ALF in individuals considered unsuitable for ELT, 10 days' post admission was the point at which the

metabolic profile of ALF changes significantly. Longitudinal studies focusing on multiple time points are rare in clinical studies on ALF and no such studies are reported for ACLF as the majority focus on day 1 of admission.

Analysis of sequential plasma samples from ACLF patients revealed that there were no significant changes in metabolite intensity with respect to day or between survivors and non-survivors at all timepoints (Figure 17). This suggests that either the numbers per time point are too low or the metabolic differences are minute, or metabolism remains statically disturbed between days 1-14 post admission in ACLF.

Interestingly, sequential analysis of ALF patients revealed a reduction in alanine, lysine, glutamine, acetate, citrate, methionine, glycine, lactate, histidine, tyrosine and phenylalanine resonances after days 5-8 of admission (Figure 17). Prior to 10 days post admission, metabolic disturbance observed from day 1 to day 8 showed no change in intensity over time, and no differences between survivors and non-survivors at all timepoints (data not shown). This indicated that the initial metabolic perturbation caused by ALF in patients who go on to survive, appear to reverse from day 5. Other longitudinal sampling studies of ALF focused on paracetamol induced ALF and centre on APAP adducts and markers of hepatocellular death and injury.^{37,158,159} These studies show that peak rates of cell death occur at day 1 of admission and decline from day three-five, indicating metabolic recovery occurs after mechanisms of cell death and injury begin to reverse and the activity of liver enzymes is reversed.^{37,158,159} In this study, all patients sampled between 10-14 days and at day 21 post admission went on to survive at 30 days (Figure 17). However, the exact point at which recovery begins in ALF is not clear as no sampling was performed between 8-10 days. It is possible that the metabolic profile of survivor's vs non-survivors may be distinct between 8-10 days' post admission.

3.6 Study Limitations

One of the main limitations of this study already outlined above in section 3.4.2 was the presence of broad interfering drug related spectral resonances visible in AD, ACLF and ALF patients. This is due to the various medications the patients are receiving, the intensity of the DRC resonances was highest in patients with ALF and ACLF who are sedated as they are in intensive care, hence this was an unavoidable limitation. Furthermore, according to their disease aetiology and presence of other pre-existing medical conditions, the quantity and combination of medications including diuretics and antibiotics varied between patients.

Hence, it was not possible to account how each of these medications and the various combinations of these would affect the plasma ^1H NMR spectral profiles collectively per disease class. Furthermore, the interfering DRC resonances overlapped with resonances originating from endogenous metabolites of interest and hence limited analysis of the data.

3.7 Conclusions

Comparative analyses of plasma ^1H NMR plasma metabolic profiles generated from patients with acute and chronic liver disease revealed changes in metabolism which confirmed previous findings and extended our understanding of disturbance of pathways related to energy metabolism, gluconeogenesis, nitrogen clearance and protein catabolism. Whereby, the data presented in this chapter identified lipids and amino acids as the main metabolite groups perturbed in acute and chronic liver disease.

As the first metabonomics study to compare ALF and ACLF, a greater metabolic perturbation was determined for ALF compared to ACLF. In addition, pathway analysis and MSEA revealed that the same pathways were responsible for progression of AD to ACLF, as well as, the distinction between ALF and ACLF indicating the degree of metabolic perturbation was the main discriminatory factor. A greater metabolic perturbation, was indicative of the liver's diminished metabolic capacity, resulting in high plasma concentrations of metabolites. However, metabolic differences between acute and chronic disease determined from the ^1H NMR data are not strong enough to build multivariate discriminant models for either AD vs ACLF or ALF vs ACLF. This may be compounded by the sensitivity of the technique and also the presence of interfering drug metabolites. Hence, mass spectrometry, which has a greater sensitivity was then applied to phenotype the metabolic profiles of these patients further.



Chapter 4

TARGETED UPLC-MS AMINE ANALYSIS

4.1 Introduction

Imbalances in circulating amino acid and amino compound concentrations are a hall mark of liver disease, as the liver is the main organ that regulates protein biosynthesis and amino acid metabolism. Perturbation of major metabolic pathways such as the urea cycle and gluconeogenesis, which are linked to amino acid metabolism are widely documented in liver disease.^{62,64,146} These are linked to several pathophysiological mechanisms that are well characterised in acute and chronic liver disease, such as hyperammonemia, lactic acidosis and muscle wasting.^{130,160,161} However, not all of the metabolic changes observed in liver disease are well characterised. Furthermore, a clear understanding of the precise pathophysiological implications for a number of these changes remaining poorly elucidated.

Previously, a number of global metabolic changes have been partially characterised in acute and liver disease patients by ¹H NMR spectroscopy (see Chapter 3). In this chapter, changes in amino acid and amino compound profiles are more comprehensively analysed and interrogated further, using a targeted, quantitative UPLC-MS method, validated for analysis of amino acids and amino compounds in plasma and urine.¹⁰¹ This method has been applied to analyse the plasma of a subset of patients previously phenotyped by ¹H NMR spectroscopy in Chapter 3.

4.2 Aims and Objectives

The main aims of this work are the same as the aims covered in Chapter 3 which focused on the analysis of the metabonomics data (in this case targeted UPLC-MS data) generated from human plasma samples. The objectives were to characterise class specific metabolic changes between liver disease groups, as well as, correlating metabolic differences with clinical metadata to identify mechanistic links to known markers of liver disease.

4.3 Materials and Methods

4.3.1 Subset cohort characteristics

A subset of plasma samples, from subjects initially profiled by ¹H NMR spectroscopy, were analysed by targeted UPLC-MS for the detection and quantification of a range of amino compounds, including amino acids. These samples included representatives of all the groups previously profiled by ¹H NMR spectroscopy and comprised: HC (n=38); SC (n=5); AD (n=22);

ACLF (n=23); ALI (n=10) and ALF (n=27) see Table 7. However, a larger number of ALI plasma samples were analysed by targeted UPLC-MS in contrast to previous ¹H NMR spectroscopic analyses, as the initial aim of this analysis was to focus on global metabolic perturbations in acute liver disease samples and correlation to, HE. Therefore, the ALF and ALI samples were prioritised for this analysis, and fewer chronic liver disease samples were analysed as the number of samples that could be analysed were limited. For a summary of the full cohort see Table 2 in Chapter 3.

Parameter	Cirrhosis group			Acute Liver Disease		HC (n=38)	p-value
	SC (n=5)	AD (n=22)	ACLF (n=23)	ALI (n=10)	ALF (n=27)		
Age	55 (54-80)	57 (23-68)	51 (18-66)	36 (28-51)	39 (19-59)	35 (24-69)	<0.0001
BMI	28 (23-45)	27 (16-53)	23 (20-33)	22 (20-38)	21 (19-32)	-	0.0065
Sex (M:F)	3:2	14:8	16:7	3:7	6:21	19:19	<0.05
CLIF-SOFA	4 (3-21)	8 (3-15)	15 (8-20)	N.A.	N.A.	-	<0.0001
SOFA	4 (3-19)	6 (2-17)	12 (5-20)	5 (2-16)	12 (6-19)	-	<0.0001
HE grade	0 (0-0)	0 (0-1)	2 (0-4)	0 (0-4)	4 (0-4)		<0.0001
AST IU/L	28 (16-50)	77 (16-1670)	86 (20-1613)	6196 (283-21560)	5199 (203-15560)	-	<0.0001
Hb g/dl	128 (97-159)	107 (78-148)	92 (61-128)	105 (83-147)	96 (7.4-154)	-	0.0422
INR	1.06 (1.01-1.36)	1.53 (1.15-2.85)	1.84 (1.25-3.66)	4.47 (1.08-15.00)	7.10 (1.40-16.15)	-	<0.0001
Bilirubin μ mol/L	11 (5-75)	69 (10-234)	205 (23-714)	70 (15-274)	89 (29-786)	-	0.0025
Albumin g/L	41 (35-44)	30 (14-47)	26 (15-39)	28 (23-42)	26 (13-33)	-	0.0059
Total Protein g/L	70 (61-84)	62 (39-87)	56 (41-82)	48 (41-72)	47 (30-67)	-	<0.0001
Urea mmol/L	5.1 (5.0-5.3)	7.95 (1.2-26.4)	12.4 (4.1-28.7)	7.8 (3.1-41.8)	8.4 (2.0-24.0)	-	0.0061
Survival							
30 day	100%	100%	57%	81%	81%	-	-
90 day	100%	86% n=21	52%	80%	70%	-	-
Transplanted (n=)	0	5 (n=16)	6 (n=17)	0	12	-	-
Transplanted -30 day survival	-	100%	100%	-	83%	-	-

Table 7. Demographic, biochemical and physiological details of study population subset for UPLC-MS amine analysis

Data are presented as median values with full range in brackets for each parameter where applicable. A Kruskal Wallis test was performed for continuous variables with overall differences within each parameter reported. Categorical variables such as aetiology for SC, AD, ACLF groups and gender were subjected to a Chi squared test with a significance threshold set at p<0.05. Metadata missing for some parameters is indicated by the n-number next to median and range values. BMI (body mass index); CLIF-SOFA (the chronic liver failure-sequential organ failure assessment); SOFA (sequential organ failure assessment); AST (aspartate aminotransferase); Hb (haemoglobin); INR (international normalised ratio); N.A. (not applicable);

4.3.2 Quantitative UPLC-MS analysis of Amino compounds

4.3.2.1 Sample preparation and analysis

A detailed description of the methodology used is published by Gray et al.¹⁰¹ Briefly, the study plasma samples were randomised and left to thaw at 4 °C. Once thawed 10 µL per sample was added to 10 µL of Optima grade water and 5 µL of a 10 µg/mL internal standard mixture in Eppendorf tubes. Proteins were precipitated by the addition of 40 µL of cold isopropanol containing 1 % formic acid (v/v) and vortex mixed. After 20 minutes samples were centrifuged at $\geq 10,000g$ for 10 minutes at -20 °C and then 10 µL of the supernatant was transferred to a 96-well plate for derivatisation.

For derivatisation, 1 mL of acetonitrile was added to the AccQTag Ultra™ reagent powder, vortex mixed for several seconds dissolved by heating at 55 °C for 15 minutes. Then, 70 µL of borate buffer was added to the 10 µL of standards previously placed in the glass HPLC vials and study samples in 96-well plates and vortex mixed. To the buffered mixtures 20 µL of the AccQTag Ultra™ derivatising reagent solution was added with further vortex mixing. The reaction mixtures were then heated at 55 °C for 10 minutes and then diluted 1 in 100 with Optima grade water prior to analysis.

UPLC-MS analysis was performed using an Acquity UPLC binary solvent manager, sampler manager and column manager (Waters, Milford, MA, USA) interfaced with a Xevo TQ-S tandem quadrupole mass spectrometer (Waters, Wilmslow, UK). MS/MS detection was via electrospray ionisation (ESI) in positive ion mode using multiple reaction monitoring (MRM) for the quantification of each compound with instrument settings as described.¹⁰¹

4.3.2.2 Acceptance Criteria for reporting UPLC-MS Amino Compound data

All acceptance criteria were determined based on the FDA “Guidance for Industry” on Bioanalytical methods.¹⁶² In summary, the correlation coefficient (R^2) of each amino compound calibration curve must be >0.99 , a minimum of 67% of the calibration points must be included in the range including the lower limit of quantification (LLOQ) and the highest concentration standard and have a coefficient of variation (CV) of $<15\%$ and $<20\%$ for LLOQ. Furthermore, the quality controls (QC) for each amino compound must have a CV $<15\%$ and a minimum of 67% of QC injections must meet these acceptance criteria. At least 50% of all QC injections at each concentration should meet the acceptance criteria. Analytes that did not fulfil these criteria were deemed as semi-quantitative. Table 8 provides a full summary of

the results for QCs and standard curves for each analyte. Additional amino compounds which did not have an internal standard, were monitored by the method and peak areas and fold changes between classes for these were calculated by comparing median values.

Amino Compound	Valid concentration range (µM)	R ²	Standard curve pass	No. of QCs passed?	Valid QCs (µM)	Valid for quantification?	Sample concentration ranges (µM)
4-hydroxyproline	1-400	0.9953	Yes	20/20	3, 10, 30, 150, 400	Yes	1.9-106.6
Alanine†	2-800	0.9949	Yes	16/20	10, 30, 150, 400	Yes	78.4-1841.0*
AABA	3-400	0.9925	Yes	20/20	3, 10, 30, 150, 400	Yes	1.1-57.3*
Asparagine	1-400	0.9943	Yes	19/20	3, 10, 30, 150, 400	Yes	9.0-193.4
Aspartic acid	1-400	0.9973	Yes	19/20	3, 10, 30, 150, 400	Yes	0.5-8.8*
BAIBA	3-400	0.9942	Yes	17/20	3, 10, 30, 150, 400	Yes	0.2-73.8*
Citrulline	3-400	0.9949	Yes	20/20	3, 10, 30, 150, 400	Yes	3.2-93.7
Cystathionine	1-400	0.9943	Yes	20/20	3, 10, 30, 150, 400	Yes	0.1-38.6*
Ethanolamine	1-400	0.9953	Yes	20/20	3, 10, 30, 150, 400	Yes	2.1-56.6
Glutamic acid	1-400	0.9971	Yes	20/20	3, 10, 30, 150, 400	Yes	6.6-110.4
Glutamine†	2-800	0.9942	Yes	16/16	10, 30, 150, 400	Yes	80.8-1677.4*
Glycine†	20-800	0.9938	Yes	14/16	10, 30, 150, 400	Yes	65.4-626.0
Histidine	4-400	0.9912	Yes	14/16	10, 30, 150, 400	Yes	18.2-329.0
Isoleucine	1-400	0.9959	Yes	20/20	3, 10, 30, 150, 400	Yes	6.1-84.1
Leucine	1-400	0.9955	Yes	20/20	3, 10, 30, 150, 400	Yes	13.8-192.8
Lysine†	2-800	0.9948	Yes	19/20	3, 10, 30, 150, 400	Yes	34.4-695.0
Methionine	1-400	0.9954	Yes	20/20	3, 10, 30, 150, 400	Yes	4.3-315.2
Ornithine	10-400	0.9954	Yes	12/16	10, 30, 150, 400	Yes	10.2-379.0
Phenylalanine	1-400	0.9964	Yes	20/20	3, 10, 30, 150, 400	Yes	17.8-363.3
Proline†	3-400	0.9961	Yes	16/20	10, 30, 150, 400	Yes	29.0-787.0
Sarcosine	1-400	0.9906	Yes	13/20	3, 10, 30, 150	Yes	0.9-11.8
Serine	3-400	0.9959	Yes	18/20	3, 10, 30, 150, 400	Yes	19.6-159.5
Threonine†	1-400	0.9966	Yes	20/20	3, 10, 30, 150, 400	Yes	25.0-238.5
Tryptophan	1-400	0.9929	Yes	20/20	3, 10, 30, 150, 400	Yes	2.1-73.5
Tyrosine	1-400	0.9934	Yes	20/20	3, 10, 30, 150, 400	Yes	6.9-387.8
Valine	1-400	0.9937	Yes	20/20	3, 10, 30, 150, 400	Yes	23.6-260.7
1-methylhistidine	10-400	0.7688	No	6/20	10	No	N/A
3-methylhistidine	10-400	0.9376	No	6/20	3, 10	No	N/A
Aminoadipic acid	1-400	0.9914	Yes	5/16	150	No	N/A
Arginine	10-400	0.9838	Yes	9/16	10, 30, 150, 400	No	0-340.0*
Beta-alanine	N/Q	0.9963	N/A	-	N/A	No	N/A
Carnosine	10-400	0.9924	Yes	13/16	10, 30, 150, 400	Yes	No response in samples
Cystine	N/Q	0.9914	N/A	-	N/A	No	N/A
GABA	3-400	0.9933	Yes	7/20	10, 30, 150	No	N/A
Homoserine	N/Q	0.5728	N/A	-	N/A	No	N/A
Hydroxylysine	10-400	0.9838	Yes	9/20	10, 150, 400	No	N/A
Phosphoserine	N/Q	0	N/A	-	N/A	No	No response in samples
Taurine	N/Q	0.9455	No	-	N/A	No	N/A

Table 8. Summary of the acceptance criteria used to determine appropriate reporting for all amino compounds with internal standards

Standard curve pass criteria, determined from R² (>0.99), the coefficients of variation (CV) - <15% (<20% LLOQ) for each point on the standard curve (data not shown). Also, a minimum of 67% of standard curve points had to pass. The criteria for the spiked in QCs -the CVs <15% (<20% lowest concentration QC) of spiked in QC's and the total number of QCs passing per compound analysed - minimum of 50%. The concentration range was determined from the QCs and the range of points on

the standard curve with acceptable CVs. In the amino compound column, † indicates the compound was re-analysed following a 1:1 dilution of the sample plasma as concentrations in many of the samples were significantly above the limits of quantification; Within the sample concentration ranges column, * indicates concentration ranges which fall outside the limits of quantification. For arginine CVs of <20% (<30% for lowest concentration) for the QCs, were used these are indicated in *italic*. N/A – not applicable; N/Q – not valid for quantification by the method; AABA – Alpha-amino-isobutyric acid; BAIBA – beta-amino-isobutyric acid; GABA – gamma-amino-butyric acid.

4.3.3 Statistical Analyses

UPLC-MS amino compound data were $\log_{10}$ transformed, with an added offset of 1 ($\log_{10}(X) + 1$) and mean centred before being analysed using both multivariate and univariate statistical methods. Multivariate modelling of the data was performed in SIMCA P+ v14.2 (Umetrics, Umea, Sweden) using the same approach as previously for the ^1H NMR spectroscopic data when comparing classes of interest: PCA modelling was followed by OPLS-DA. All OPLS-DA models computed were subjected to a cross validated analysis of variance (CV-ANOVA), which corresponds to a null hypothesis of equal predictive residuals between the models under investigation ($p < 0.05$ suggests the model is superior to one chosen at random). The S-plot loadings, which displayed correlation vs covariance of the quantitative amino compound concentrations, were used to determine the amino compounds contributing to class separation. PLS regression modelling was also performed, where the UPLC-MS data (X) were modelled to find any associations in the data with the clinical metadata (Y). A variable importance in projection (VIP) plot showed amino compounds, ordered according to how well they explain variance in the model, where a threshold of VIP score > 1.5 was used to select the most important variables for the model.

For univariate analysis of the UPLC-MS amino compound data, the same univariate approach was applied as for the ^1H NMR spectral data (see Chapter three section 3.4.5). Fold change differences for all class comparisons were calculated by comparing median values and were reported for all amino compounds detected in the plasma samples (see Appendix Tables 38 & 39). Furthermore, the clinical metadata was assessed for differences across classes using the same univariate methodology as in Chapter 3 (data not shown).

4.3.4 Hierarchical Cluster Analysis

Unsupervised hierarchical cluster analysis was performed on quantitative amino compound concentrations in Matlab 2014a using the same method used for ^1H NMR spectral integral data (See Chapter 3, section 3.4.6).

4.4 Results

A total of 26 amino compounds passed the acceptance criteria for quantification. Whilst, a further 9 compounds were deemed to be semi-quantitative as they failed to pass some of these criteria (despite the use of internal standards for their determination) and were analysed for fold change differences only (See Appendix Table 39). In addition, 10 compounds, for which no internal standards were available, were monitored by the method and were also analysed for fold change differences (See Appendix Table 40). Multivariate modelling was performed on all amino compounds which passed the acceptance criteria for quantification, aside from sarcosine which was only detectable in a small subset of plasma samples (HC; $n=15$, SC; $n=2$, AD; $n=11$, ACLF; $n=10$, ALI; $n=3$, ALF; $n=10$). Furthermore, multivariate analysis was not performed on semi-quantitative and monitored compounds, as there were a number of missing data, as compounds were not detectable in all samples.

Multivariate and univariate analyses were performed on the quantified compounds (Figure 24 & 25), whilst univariate analysis and calculation of fold change was performed on semi-quantified and monitored amino-compounds (See Figure 26 & 27 for the semi quantified and monitored compounds respectively).

4.4.1 Multivariate analysis of quantitative amino compound data

Multivariate analysis was performed using an unsupervised approach, followed by a supervised approach: PCA followed by OPLS-DA. A PCA model of the plasma amino compound profiles from HC and cirrhotic groups: SC, AD and ACLF (2 component model, cumulative $R^2=0.57$; $Q^2=0.41$) revealed a separation in the scores plot between HC and the AD and ACLF groups in principal component 1 (PC1) which accounted for 36% of the total variance in the model (Figure 24A). The corresponding loadings scores plot (Figure 24B) showed the amino compounds driving the separation seen in the scores and revealed that separation in PC1 was due to an elevation of ethanolamine, β -isoaminobutyric acid (BAIBA),

cystathionine and 4-hydroxyproline concentrations and a depletion of isoleucine, leucine, tryptophan and valine concentrations.

For acute liver disease (ALF and ALI), a PCA model (2 component model, cumulative $R^2=0.76$; $Q^2=0.68$) of HC and also ALF and ALI patients, revealed a clear separation between HC and acute liver disease in the score scatter plot in PC1, which accounted for 68% of the total variance in the model (Figure 24C). The corresponding loadings plot (Figure 24D) revealed elevated concentrations of ethanolamine, cystathionine, methionine and tyrosine and a depletion of isoleucine, leucine, tryptophan and valine concentrations in ALI and ALF plasma.

OPLS-DA modelling of AD vs ACLF and ALI vs ALF resulted in invalid models (data not shown). However, a valid OPLS-DA model of ALF vs ACLF was computed ($R^2Y=0.73$, $Q^2Y=0.55$, CV-ANOVA p-value = $1.35e-05$), which revealed a separation between the two groups in the scores plot (Figure 24E). Discriminating amino acids identified from the OPLSDA model, were determined from the S-plot loadings by using the following cut-offs: $p(\text{corr}) > 0.5$ and $p(1) > 0.2$ (Figure 24F) and a VIP score > 1.00 (Figure 24G). Elevated concentrations of alanine asparagine, cystathionine, glutamic acid, glutamine, glycine, histidine, lysine, methionine, proline, threonine and tyrosine in ALF were discriminatory to ACLF.

OPLS-DA modelling comparing AD vs ACLF produced an invalid model and numbers in SC group were very small so comparisons between SC and AD or ACLF also produced invalid models (data not shown). Furthermore, OPLS-DA analysis of ALI vs ALF also produced an invalid model (data not shown).

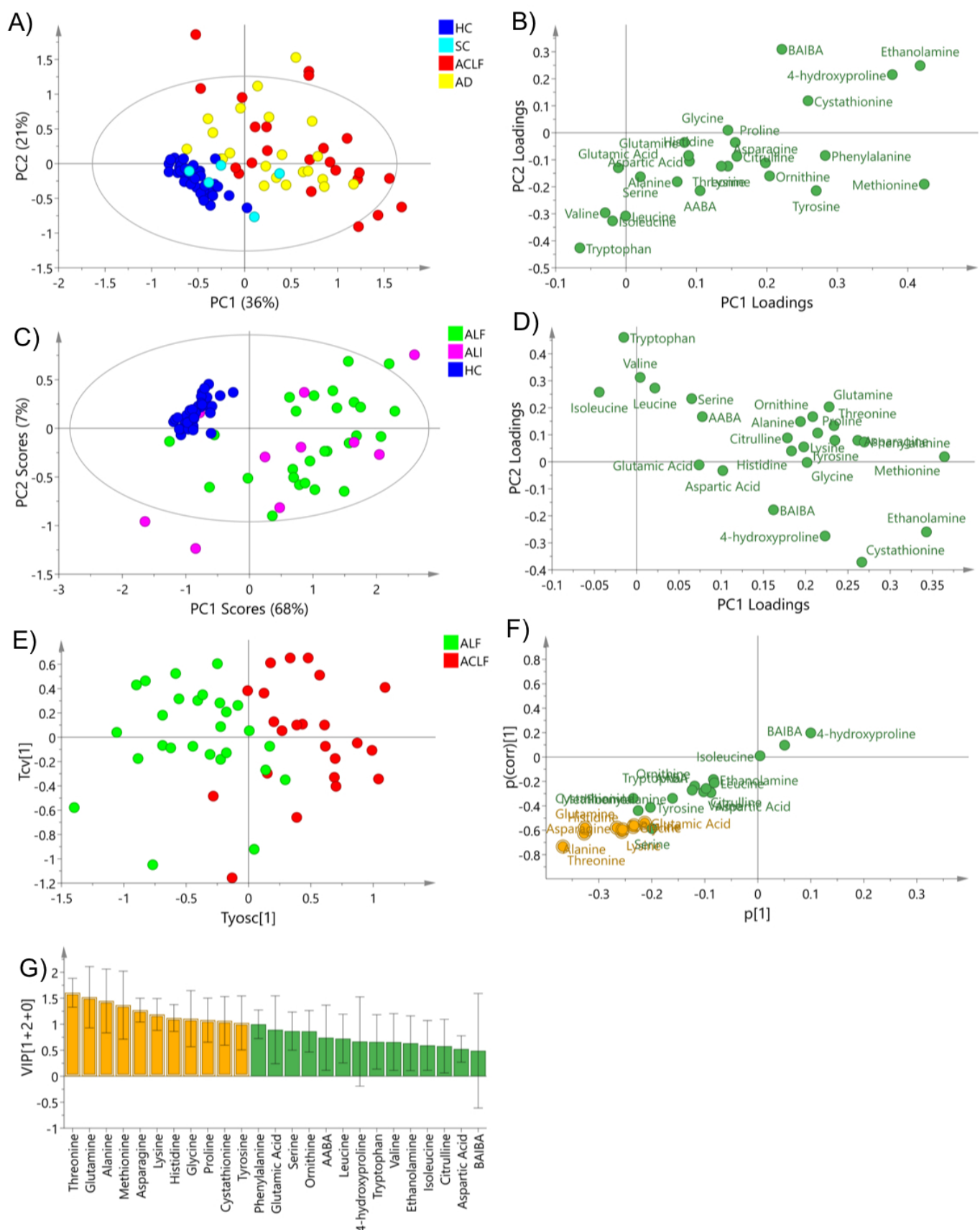


Figure 24. Multivariate analysis of UPLC-MS amino compound data

Unsupervised multivariate modelling of amino compound data reveals class specific changes in patients with cirrhosis and acute liver diseases (A-D). An OPLS-DA model demonstrated discrimination between patients with ALF and ACLF (E-G). (A) The PCA scores plot -2 component model of HC and

cirrhosis groups SC, AD and ACLF (Cumulative $R^2=0.57$ and $Q^2=0.41$) shows separation between the groups in PC1. (B) the corresponding loadings scores plot (p1 vs p2) for PCA model (A). (C) PCA scores plot - 2 component model of HC, ALF and ALI groups (Cumulative $R^2=0.7$ and $Q^2=0.68$) (D) a corresponding loadings plot (p1 vs p2). (E) Orthogonal partial least squares - discriminant analysis (OPLS-DA) scores plot (2-Y orthogonal component and 1-Y predictive component $R^2Y=0.73$ $Q^2Y=0.55$; permutations test $n=999$ $p\text{-value} < 0.0001$). (F) OPLS-DA S-plot loadings (p1 vs p(corr)). (G) OPLSDA VIP plot; Discriminant features shown in F and G are highlighted in orange. AABA-alpha-amino-n-butyric acid; BAIBA-beta-iso-amino-butyric acid.

4.4.2 Univariate analysis of quantitative amino compounds

Pairwise comparisons of quantified amino compounds in the HC, SC, AD, ACLF and ALF groups revealed significant changes relative to HC and between disease groups (see Figure 25). These confirmed results from the univariate analysis of the ^1H NMR spectroscopic data, as well as, identifying novel differences between the groups. The main findings common to acute and chronic disease included a depletion in plasma concentrations of the branched chain amino acids (BCAAs) – isoleucine, leucine and valine and a rise in the aromatic amino acids (AAAs) - phenylalanine and tyrosine. Furthermore, a rise in the amounts of circulating amino acids linked to, or part of, the urea cycle or transsulfuration pathway was also seen in patients with ALF and ACLF. All significant differences in plasma concentrations between groups ($p < 0.05$) are reported below (see appendix Table 38 for fold change differences). The amino compounds AABA, arginine and glutamic acid did not show any differences in concentration across groups.

4.4.2.1 Metabolic differences in AD, ACLF, ALI and ALF relative to HC

Pairwise comparisons of AD vs HC, ACLF vs HC and ALF vs HC showed decreases in the BCAA's. This included isoleucine which decreased in AD, ACLF and ALF by 0.6, 0.8 and 0.7-fold respectively, leucine which decreased in AD by 0.6-fold and valine which decreased in AD and ACLF by 0.6 and 0.7-fold respectively. Furthermore, the aromatic amino acids phenylalanine, tyrosine and tryptophan also showed fold change differences in AD, ACLF, ALI and ALF. Phenylalanine showed increases in AD, ACLF, ALI and ALF by 1.8, 2.5, 3.4 and 3.7-fold relative to HC respectively. In addition, tyrosine also showed a fold change increase in AD, ACLF and ALF by 2.0, 2.0 and 3.5-fold relative to HC respectively. In contrast, tryptophan showed a decrease relative to HC in AD, ACLF, ALI and ALF by 0.7, 0.6, 0.6 and 0.8-fold respectively.

The sulphur containing amino-compounds cystathionine and methionine also showed fold change differences in AD, ACLF, ALI and ALF. Cystathionine concentrations significantly increased by 8.4, 10.4, 21.8 and 25.8-fold in AD, ACLF, ALI and ALF respectively. Whilst, methionine concentrations significantly increased by 4.6, 5.1 and 6.9-fold in ACLF, ALI and ALF respectively.

Further amino compounds which showed differences common to AD, ACLF, ALI and ALF relative to HC included 4-hydroxyproline and ethanolamine. 4-hydroxyproline increased in concentration by 2.9, 4.0, 3.4 and 3.6-fold and ethanolamine increased by 4.1, 5.7, 7.7 and 7.4-fold in AD, ACLF, ALI and ALF respectively. The amino compounds asparagine and BAIBA also showed differences common to ACLF, ALI and ALF with asparagine increasing in concentration by 1.7, 2.8 and 3.4-fold and BAIBA increasing by 8.6, 4.3 and 4.3-fold in ACLF, ALI and ALF respectively.

Amino compounds showing concentration differences common to both acute liver disease groups (ALI and ALF) relative to HC, included alanine, glycine, histidine, lysine, proline and threonine. Alanine increased by 2.4 and 2.5-fold, glycine by 2.2 and 2.5-fold, histidine by 1.9 and 2.6-fold, lysine by 2.4 and 2.5-fold, proline by 2.4 and 2.3-fold and threonine by 2.4 and 2.8-fold in ALI and ALF respectively.

The urea cycle amino acids citrulline and ornithine showed concentration differences common to the liver failure groups ACLF and ALF relative to HC. Citrulline increased by 1.7 and 2.1-fold and ornithine increased by 2.2 and 2.7-fold in ACLF and ALF respectively.

Furthermore, concentration changes unique to ACLF and ALF relative to HC were also determined. Serine concentrations decreased selectively in the ACLF group by 0.8-fold relative to HC. Concentrations of aspartic acid and glutamine increased in ALF relative to HC, with the aspartic acid concentration rising by 2.0-fold and glutamine concentration by 2.9-fold.

4.4.2.2 Metabolic differences discriminant between AD vs ACLF

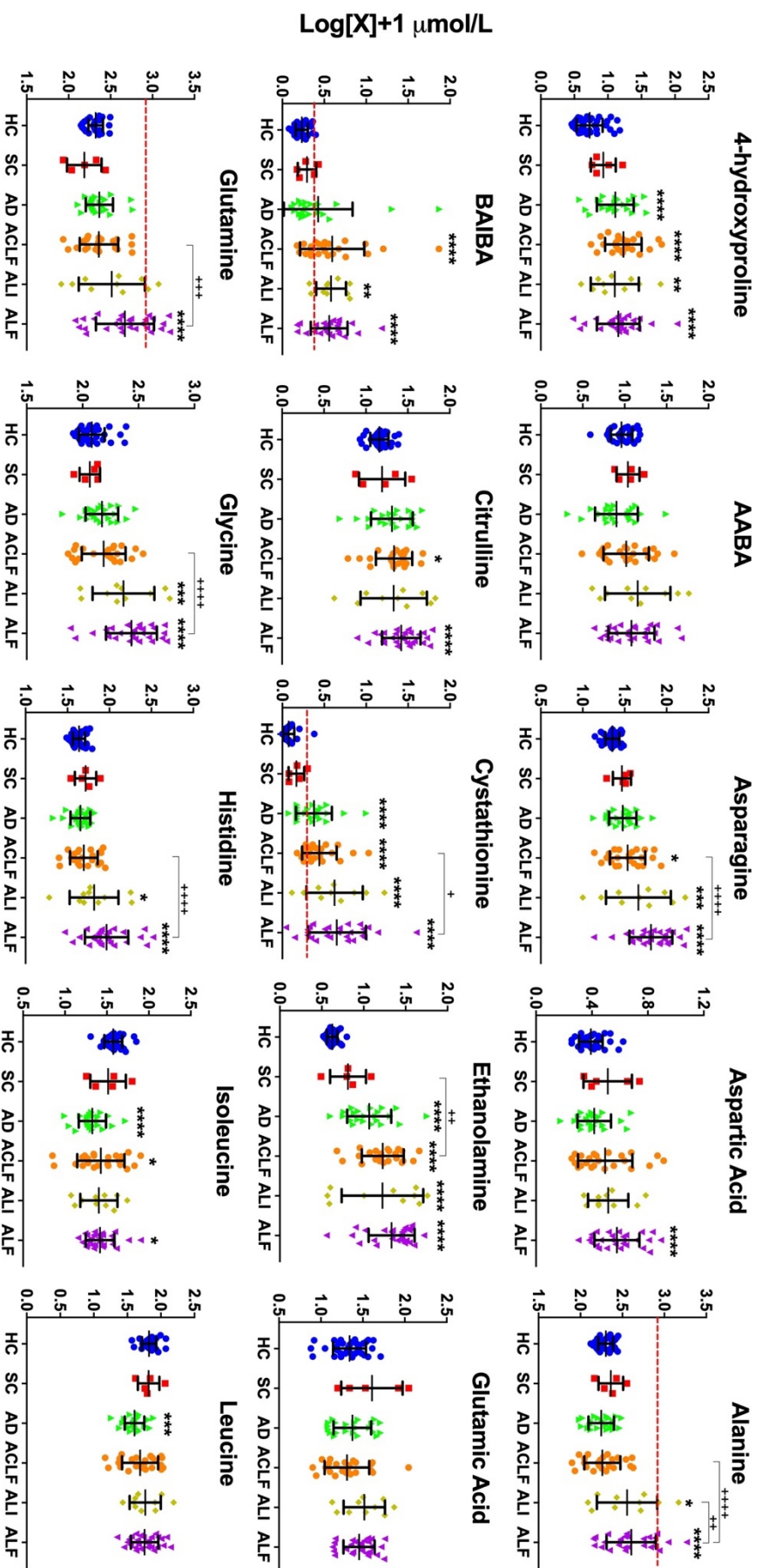
Pairwise comparisons of concentration differences between the chronic liver disease groups AD and ACLF revealed differences in serine concentrations, where serine decreased in ACLF plasma by 0.7-fold relative to AD.

4.4.2.3 Metabolic differences discriminant between ALI vs ALF

Pairwise comparisons of concentration differences between the acute liver disease groups ALI and ALF revealed differences in alanine which was increased in ALF plasma by 0.7-fold relative to ALI.

4.4.2.4 Metabolic differences discriminant between ALF vs ACLF

Pairwise comparisons of concentration differences in amino compounds between ACLF and ALF. Fold change decreases of 0.5, 0.4, 0.4, 0.4, 0.6, 0.5, 0.5, 0.7, 0.6, 0.6, 0.4 and 0.6 were seen for asparagine, alanine, cystathionine, glutamine, glycine, histidine, lysine, methionine, proline, serine, threonine and tyrosine respectively in ACLF compared to ALF.



Metabolite	[μmol/L]					
	HC (n=38)	SC (n=5)	AD (n=22)	ACLF (n=23)	ALI (n=10)	ALF (n=27)
4-hydroxyproline	3.7 (1.9-14.1)	5.8 (4.8-15.6)	12.0 (2.8-37.3)	15.6 (4.6-62.7)	9.2 (3.0-60.3)	13.6 (2.0-106.6)
Alanine	204.4 (131.8-280.4)	240.4 (145.4-356.2)	190.4 (96.2-308.2)	178.2 (78.4-452.2)	387.1 (122.0-1483.0)	354.2 (141.4-1841.0) *
AABA	8.3 (2.9-14.3)	10.8 (6.6-16.1)	6.4 (1.1-30.3)	9.5 (2.1-38.2)	12.8 (3.1-57.3)	10.6 (3.3-46.4)
Arginine	40.0 (20-80)	30.0 (20.0-50.0)	30.0 (10.0-80.0)	40.0 (0-110.0)	40.0 (20.0-100.0)	40.0 (10.0-100.0)
Asparagine	21.5 (13.0-29.7)	30.4 (18.3-36)	30.6 (12.5-68.1)	34.7 (12.8-85.1)	53.1 (9.0-165.7)	63.7 (12.5-170.3)
Aspartic Acid	1.4 (0.8-3.2) *	1.7 (1.2-4.5)	1.5 (0.5-3.8)	1.7 (0.8-7.2)	2.0 (1.2-5.1)	2.7 (1.0-6.8)
BAIBA	0.7 (0.2-1.5) *	0.9 (0.5-1.7) *	0.9 (0.3-73.8) *	2.2 (0.5-72.6) *	2.5 (1.1-5.4)	2.6 (0.5-14.2) *
Citrulline	13.6 (7.5-23.7)	16.0 (6.5-34.2)	20.6 (3.9-46.8)	23.4 (5.1-46.8)	24.9 (3.2-66.2)	27.6 (6.8-60.5)
Cystathionine	0.1 (0-1.4) *	0.5 (0.2-1) *	1.3 (0.2-9) *	1.4 (0.5-9.7) *	3.0 (0.3-15.5)	3.6 (0.1-38.6) *
Ethanolamine	3.2 (2.3-5.3)	5.5 (2.1-11.3)	10.2 (3.0-56.0)	16.3 (3.8-44.9)	25.0 (2.7-56.6)	27.3 (2.6-51.1)
Glutamic Acid	22.4 (6.6-50.6)	32.9 (14.9-110.4)	23.7 (11.1-50.4)	18.8 (7.0-110.3)	32.1 (12.6-73.8)	29.2 (12.8-56.7)
Glutamine	215.9 (139.2-312.6)	154.2 (85.4-275.0)	209.4 (128.4-584.8)	225.4 (85.2-571)	386.0 (80.8-1182.4)	523.2 (118.0-1607.0) *
Glycine	112.4 (81.4-243.8)	126.8 (82.6-135.8)	145.4 (65.4-297.6)	169.6 (73.2-346.8)	212.4 (94.4-600.4)	294.6 (76.8-563.0)
Histidine	42.7 (29.8-61.3)	51.0 (33.6-76.8)	45.6 20.4-65.7	43.3 (24.1-88.4)	57.0 (18.2-183.2)	79.8 (28.1-329.0)
Isoleucine	36.3 (19.2-69.9)	36.0 (17.0-62.2)	19.8 (8.9-51.5)	23.5 (6.1-78.8)	25.7 (10.8-54.3)	23.8 (13.3-78.7)
Leucine	65.1 (35.9-118.3)	61.6 (40.9-114.9)	39.8 (17.3-76.6)	54.7 (13.8-107.3)	47.6 (26.0-154.1)	54.0 (21.2-138.9)
Lysine	94.0 (60.4-169.4)	79.0 (50.8-93.4)	99.5 (38.0-186.0)	120.8 (34.4-276.0)	198.4 (79.4-274.2)	243.2 (71.8-512.2)
Methionine	12.4 (9.0-24.2)	17.6 (9.7-22.0)	18.8 (7.1-161.5)	33.0 (6.5-315.2)	59.3 (4.3-123.3)	73.9 (6.5-284.9)
Ornithine	29.2 (17.8-49.8)	27.6 (26.6-81.7)	48.4 (17.1-83.8)	46.1 (10.2-379.0)	51.3 (10.9-234.2)	65.7 (13.0-237.3)
Phenylalanine	32.3 (23.0-52.1)	49.6 (32.9-76.8)	50.0 (25.4-114.2)	58.3 (17.8-308.9)	82.0 (23.8-295.2)	93.1 (22.4-363.3)
Proline	108.7 (66.0-192.2)	121.4 (74.8-171.2)	135.3 (56.6-344.4)	136.8 (64.6-385.0)	211.9 (29.0-485.4)	212.6 (69.0-537.6)
Serine	58.0 (41.4-105.2)	67.1 (56.6-90.4)	62.6 (38.7-103.6)	42.5 (19.6-89.9)	49.5 (31.9-137.8)	61.2 (31.5-154.7)
Sarcosine	2.6 (0.9-6.8) n=15 *	1.9 (1.8-1.9) n=2	2.1 (1.1-4.1) n=11	2.0 (1.2-5.7) n=10	2.2 (1.0-2.6) n=3	3.4 (1.4-11.8) n=10
Theonine	66.5 (35.0-106.4)	64.6 (41.4-104.2)	73.4 (38.2-118.6)	65.8 (28.8-189.6)	145.5 (25.0-428.2)	191.8 (52.0-477.0)
Tryptophan	33.0 (21.3-48.0)	32.3 (22.7-50.8)	24.4 (3.3-64.7)	17.2 (2.1-49.3)	21.1 (6.6-42.1)	21.2 (8.9-73.5)
Tyrosine	33.4 (21.4-52.3)	50.4 (23.4-86.0)	65.9 (10.8-136.2)	71.9 (6.9-119.6)	67.9 (18.8-155.5)	95.5 (30.9-340.8)
Valine	112.2 (72.2-174.8)	117.9 (72.2-222.1)	71.0 (31.6-137.3)	82.2 (23.6-175.6)	72.4 (48.4-260.7)	84.6 (38.7-185.1)

Table 9. Amino compound median concentrations and ranges per class

– for each amino compound the median is reported in bold and range in brackets. Values with * next to the range indicate values that fall outside the limits of quantification. Note: arginine concentrations CVs of <20% (<30% for lowest concentration) for the QCs, were used these are indicated in italics;

4.4.3 Univariate analysis of semi-quantitative amino compounds

Where accurate quantification was not considered possible due to a failure of an analyte to pass one or more of the assay acceptance criteria, peak areas representative of relative amounts of amino compounds in the plasma were used as a measure of intensity and the compounds were termed “semi-quantitative”. This approach enabled fold change differences to be determined without attempting to assign an accurate concentration to a particular sample (Figure 26). The sulphur containing compound homoserine showed a fold change increase relative to HC in AD, ACLF, ALI and ALF by 2.3, 3.1, 2.8 and 3.3-fold respectively. Cystine, another sulphur containing amino compound, also showed a fold change increase in the AD, ALI and ALF groups by 2.1, 2.0, and 2.5-fold relative to HC.

The methylhistidines: 1-methylhistidine and 3-methylhistidine, also showed small fold change differences. 3-methylhistidine was 5.2-fold higher in ACLF plasma compared to ALF. In contrast, 1-methylhistidine was increased in AD, ACLF and ALI relative to HC by 3.0, 4.0 and 3.2-fold respectively. Furthermore, there was a relative increase in 1-methylhistidine by 2.4-fold in ACLF relative to ALF. The remaining amino compounds that were termed “semi quantitative” group of analytes (taurine, beta-alanine, aminoadipic acid, hydroxylysine and GABA) did not show any fold change differences compared to HC.

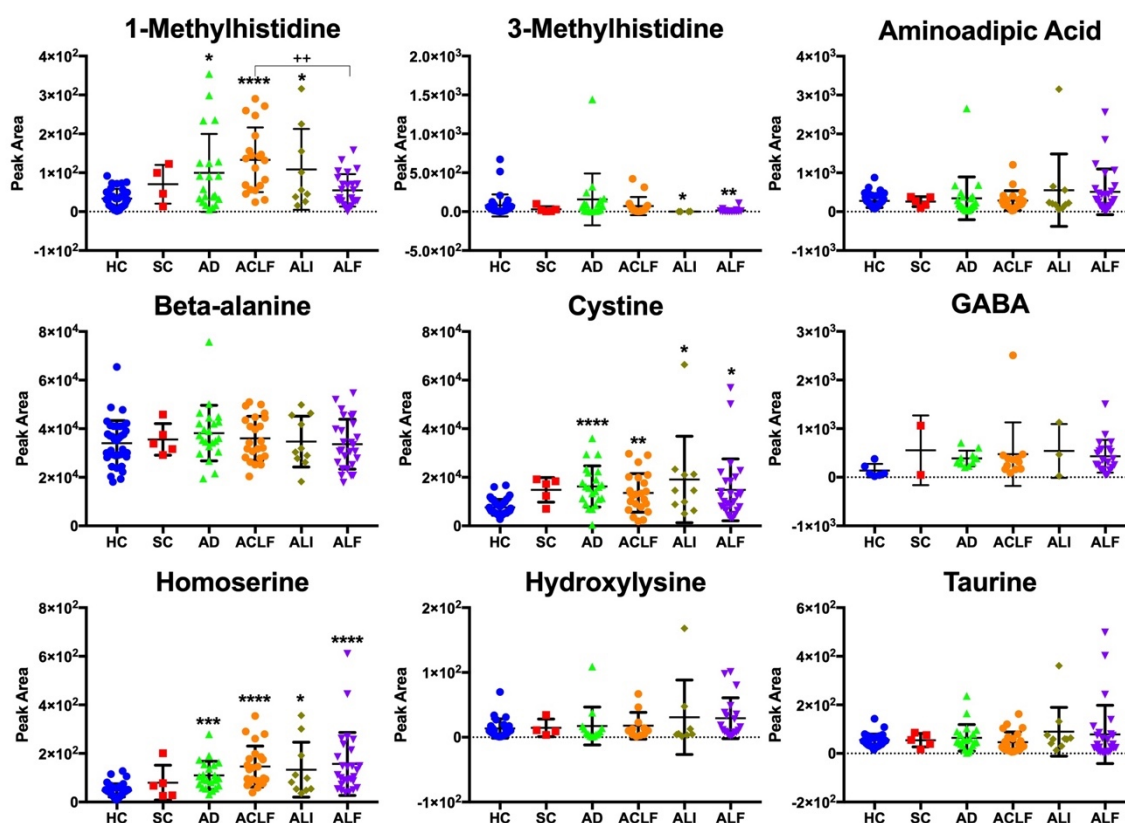


Figure 26. Univariate analysis of semi-quantified amino compounds

A 1-way ANOVA was performed on log transformed chromatogram peak areas ($\log_{10}(X) + 1$), followed by a post-hoc Sidak test. The mean is shown, and error bars represent the standard deviation for each group. Multiplicity adjusted p-values denoted by asterisks (*) are relative to HC and by crosses (+) are comparisons within disease groups, ****<0.0001; ***<0.001; **<0.01; *<0.05;

4.4.4 Univariate analysis of monitored amino compounds

Analysis of the monitored compounds (those for which no internal standard was available to facilitate quantification) compared the chromatographic peak area between all groups (Figure 27). 5-Aminovaleric acid showed a significant increase in AD and ACLF plasma relative to HC, increasing by 10.2 and 24.7-fold respectively. Glycylglycine also showed an increase in AD, ACLF and ALF relative to HC, where it increased by 2.7, 2.5 and 2.5-fold respectively. Allantoin increased in AD relative to HC by 2.3-fold, and also appears to show a trend for increase in ACLF plasma by 3.3-fold, however, this fold change was not statistically significant. The remaining amino compounds in the monitored group (creatinine, creatine, o-phosphoethanolamine, tryptamine, n-methyl-phenylalanine, cysteine and homocystine) did not show any significant differences between disease groups or relative to HC.

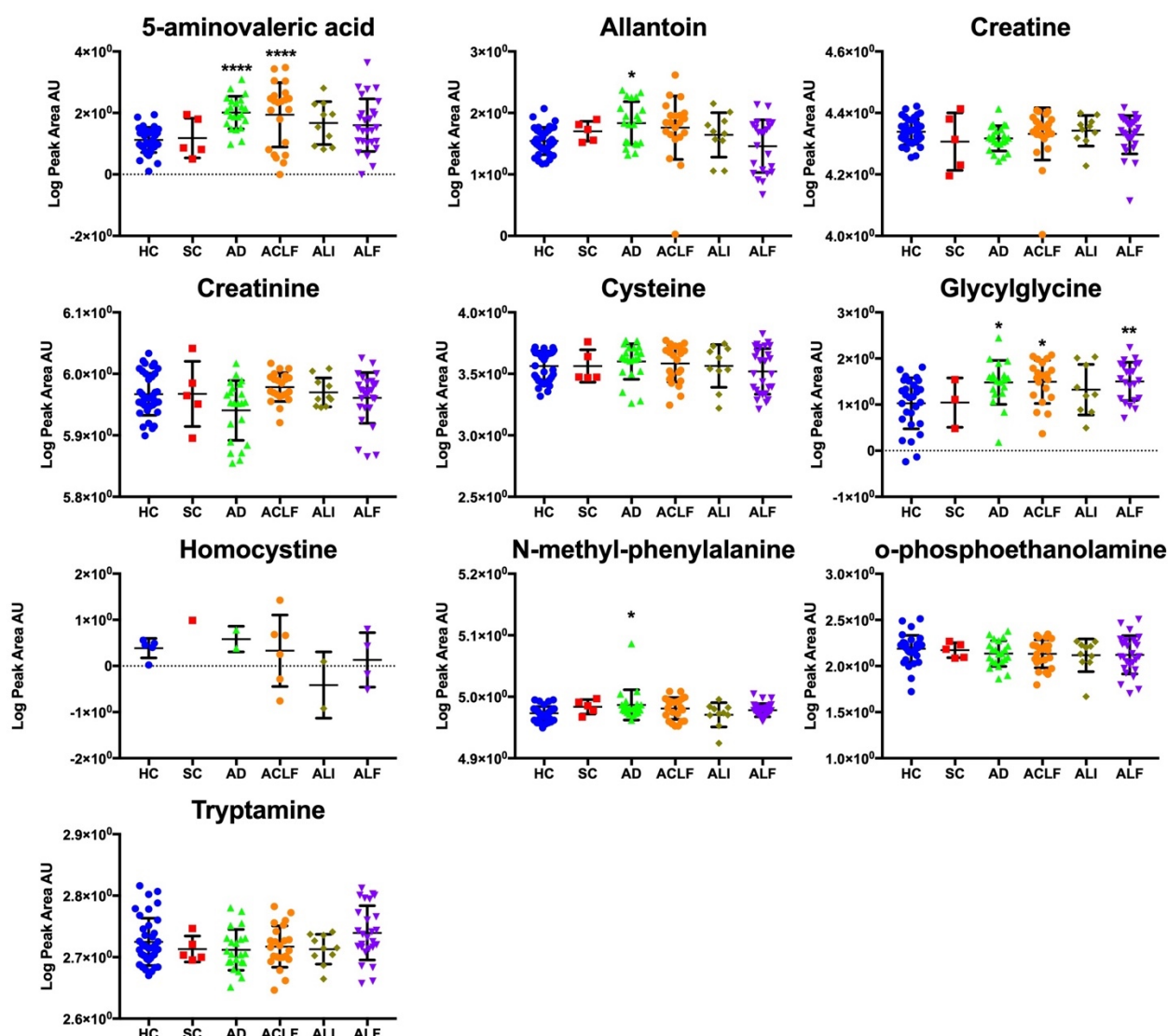


Figure 27. Univariate analysis of monitored amino compounds

A 1-way ANOVA was performed on log transformed chromatogram peak areas ($\log_{10}(X) + 1$), followed by a post-hoc Sidak test. The mean is shown, and error bars represent the standard deviation for each group. Multiplicity adjusted p-values denoted by asterisks (*) are relative to HC and by crosses (+) comparisons within disease groups, ****<0.0001; ***<0.001; **<0.01; *<0.05.

4.4.5 Hierarchical cluster analysis of quantitative amino compounds

The quantitative amino compound concentration data were assessed further to determine metabolite correlation patterns in acute and chronic liver disease, and heat maps were computed see Figure 28. Analysis was performed separately for HC, acute liver disease groups (ALI and ALF) and cirrhosis groups (SC, AD and ACLF).

Two main clusters of metabolites were determined for all analyses (Figure 28A, B and C). In HC, the main cluster, indicated in the blue section, was formed of two subclusters. The first cluster showed the strongest correlations overall between lysine, methionine, tyrosine, phenylalanine, tryptophan, leucine, isoleucine, valine, glutamic acid and aspartic acid. Within

this cluster, isoleucine, leucine, valine, aspartic acid and glutamic acid were the highest correlated ($r^2>0.5$) of these amino compounds. The BCAAs isoleucine, leucine and valine were the highest positively inter-correlated ($r^2>0.8$).

In the second subcluster, the most distinct strong correlation was between serine and threonine ($r^2>0.7$). Furthermore, asparagine from the first subcluster was strongly correlated with methionine and phenylalanine from subcluster two ($r^2>0.6$). The second main subcluster of the dendrogram, indicated in red showed no clear correlation patterns.

In the acute liver disease group (Figure 28), the strongest correlations are seen in the second main cluster of the dendrogram indicated in blue which is made up of two main subclusters. The larger subcluster (subcluster one) within the cluster is made up of amino compounds which were correlated to each other ($r^2>0.7$) and include tyrosine, ethanolamine, citrulline, methionine, ornithine, glutamine, threonine, lysine, phenylalanine, asparagine, proline, glycine, alanine and histidine. In the second subcluster serine, tyrosine and tryptophan are correlated to each other and with citrulline, methionine, threonine glutamine and ornithine ($r^2>0.7$) from subcluster one. Tyrosine was also correlated to a number of additional subcluster one metabolites including lysine, phenylalanine and asparagine ($r^2>0.7$). In the second cluster, indicated in red in the dendrogram, 4-hydroxyproline, cystathionine, BAIBA and aspartic acid were positively correlated ($r^2>0.4$).

In the cirrhosis group Figure 28C, there were two main clusters, the largest cluster indicated in red is distinctly formed of two main subclusters. Where, strong correlations were seen between AABA, isoleucine, leucine and valine ($r^2>0.7$) and between tyrosine, phenylalanine, and methionine ($r^2>0.7$). In the second subcluster of the red cluster, strong correlations were seen between lysine, alanine, asparagine, threonine and glycine ($r^2>0.7$) and between glycine and proline and 4-hydroxyproline ($r^2>0.7$). Metabolites correlated between the subclusters included asparagine which was highly correlated with histidine, methionine and phenylalanine ($r^2>0.7$) and serine and threonine which were highly correlated with each other. Cluster two, coloured in blue, did not show any strong correlations.

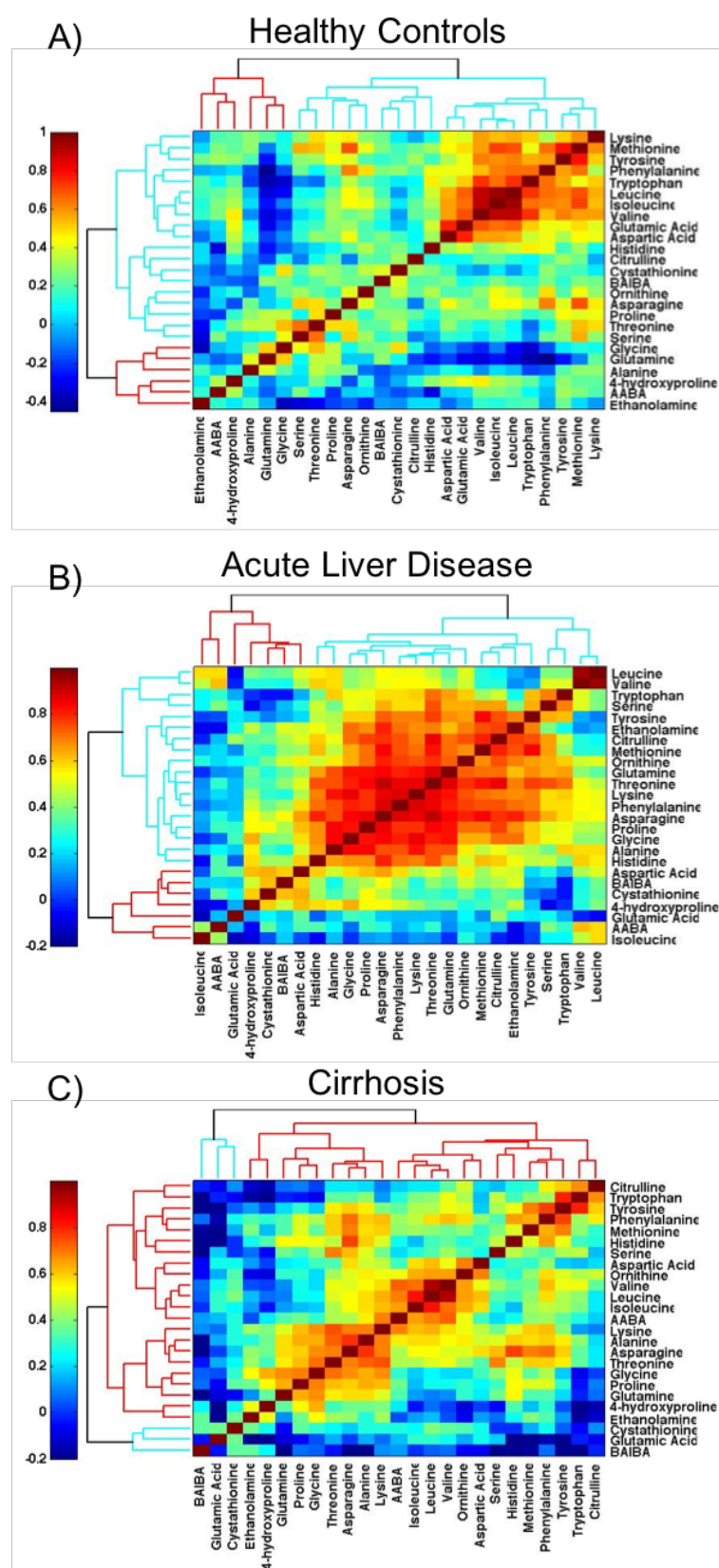


Figure 28. Heat map visualisation of metabolite-metabolite correlations from amino compound concentrations

Determined from quantitative amino compound concentrations by UPLC-MS of plasma from healthy controls, chronic liver disease patients (SC, AD & ACLF) and acute liver disease patients (ALI and ALF) – Coloured squares represent Pearson's correlation coefficients (r) calculated using metabolite integral data also reported in section 3.4.

4.4.5 Metabolite set enrichment analysis and quantitative pathway analysis

To identify pathways discriminately perturbed between ALF and ACLF, UPLC-MS data were analysed using metabolite set enrichment analysis (MSEA), a method employed to identify biologically meaningful patterns that are significantly enriched in metabolomic data.¹⁶³ MSEA showed that the 25-quantified amino-compounds were present in 27 pathways. The most significant pathways included: the glucose-alanine cycle, lysine degradation and the biotin, histidine, alanine, and porphyrin metabolic pathways (Figure 29A).

Subsequent pathway analysis, combining results from MSEA with pathway topology analysis, were performed to identify relevant pathways perturbed discriminately across ALF and ACLF. The most significant pathways were identified by metaboanalyst,¹²⁴ which utilises the KEGG database,¹²⁵ and included the alanine, aspartate, and glutamate pathways, the glycine, serine and threonine pathways, the arginine and proline pathway and the lysine degradation pathway (see Figure 29A and Table 10). Other comparisons made between ALI vs ALF, AD vs ACLF, SC vs AD and SC vs ACLF did not identify pathways with confidence (data not shown).

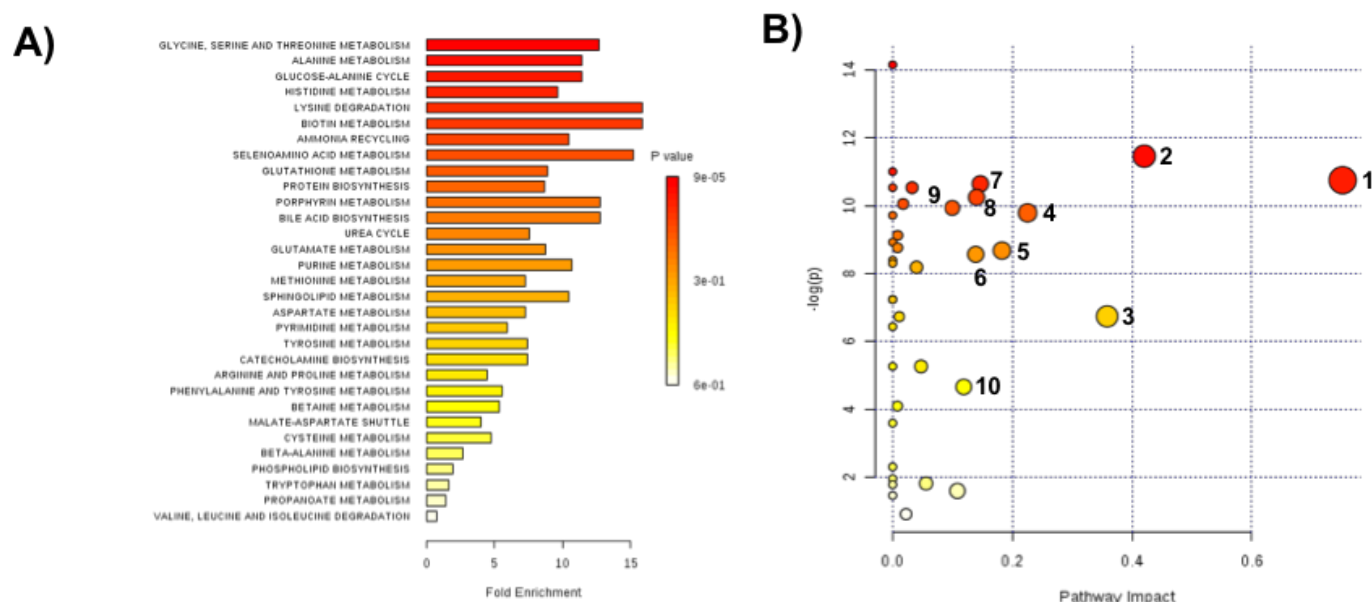


Figure 29. Metabolite set enrichment analysis and pathway analysis of quantified amino acid changes in ALF and ACLF

(A) Metabolite set enrichment analysis (A) – X-axis refers to enrichment which was assessed using the MSEA Metabolic Pathway Library¹²⁴ and the y-axis and colour of the bars correspond to the p-value, pathways were considered enriched when $p < 0.05$; (B) Metabolite pathway analysis - pathways are arranged according to the p-values (from quantitative enrichment analysis module; global test) on the y-axis and pathway impact values (from pathway topology analysis; node importance

calculated from relative betweenness centrality)¹²⁶ on the X-axis. The nodes are coloured according to the p-value and node radius is determined by the pathway impact value.

ALF vs ACLF				
No	Pathway Name	Total no. of Compounds	Hits	Impact
1	Alanine, aspartate and glutamate metabolism	24	5	0.75
2	Glycine, serine and threonine metabolism	48	6	0.42
3	Arginine and proline metabolism	77	7	0.36
4	Aminoacyl-tRNA biosynthesis	75	18	0.23
5	Cysteine and methionine metabolism	56	5	0.18
6	Lysine degradation	47	2	0.15
7	Histidine metabolism	44	3	0.14
8	D-Glutamine and D-glutamate metabolism	11	2	0.14
9	Phenylalanine metabolism	45	2	0.12
10	Tryptophan metabolism	79	1	0.11

Table 10. Pathway analysis summary

Pathway numbers correspond to circles in Figure 29B. Total is the number of compounds in the pathway; the hits - the matched number from the user uploaded data; the impact - pathway impact value calculated from the pathway topology analysis (Figure 29B).

4.4.5 Comparison to clinical metadata

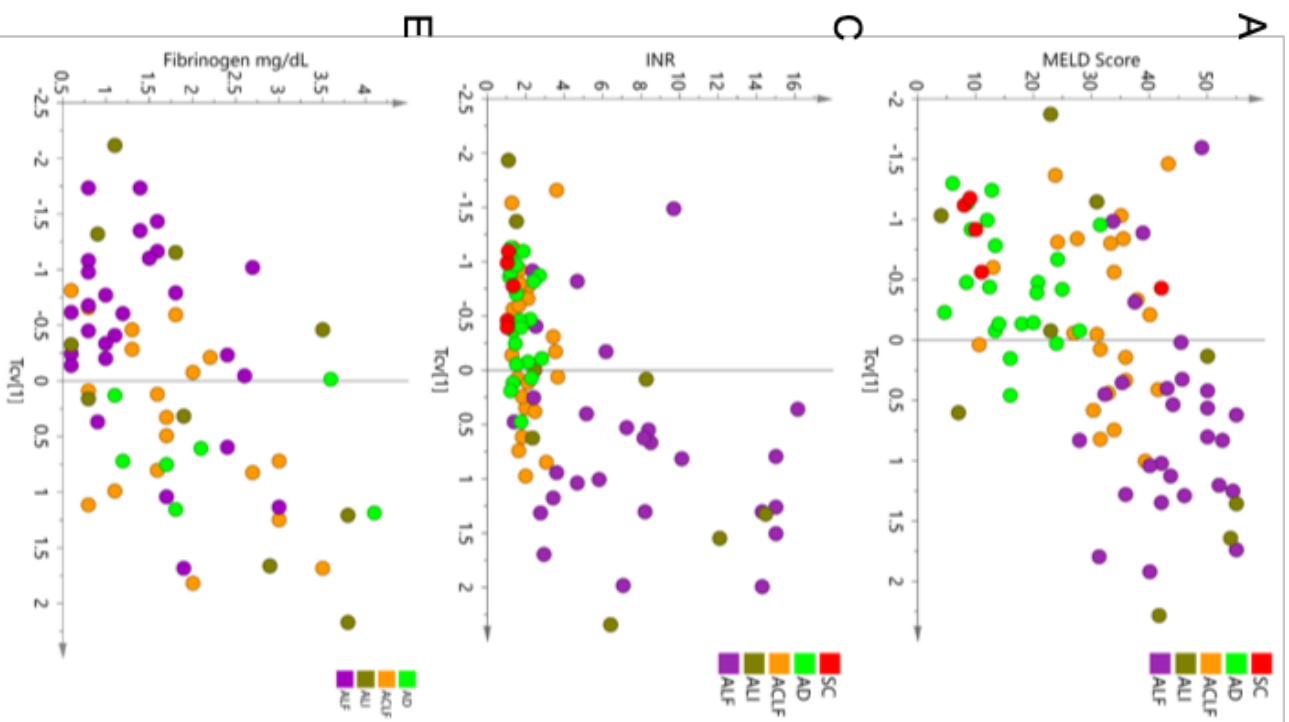
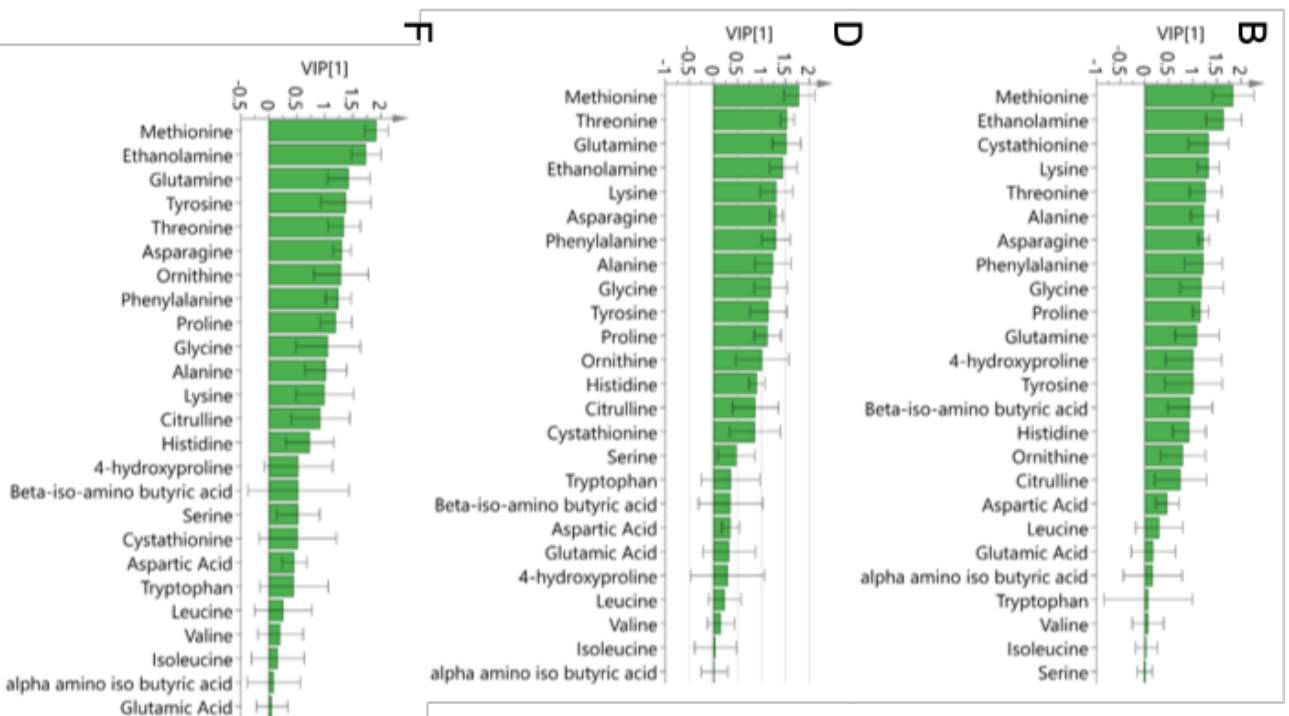
A supervised OPLS-DA analysis of the UPLC-MS data with 30- and 90-day survival data revealed invalid models (data not shown). Furthermore, any associations between the quantitative UPLC-MS data and clinical metadata were interrogated by PLS regression analysis. A summary of all valid PLS regression models computed is summarised in Table 11.

Regression with all liver disease groups produced valid models when the UPLC-MS data was regressed with the MELD score (Figure 30A & B; $R^2Y=0.42$; $Q^2Y=0.32$). The scores plot, coloured by class, shows a gradient according to MELD score along the predictive component (Tcv). The VIP plot identifies ethanolamine and methionine as most important to the model with a VIP score >1.5 .

A further PLS regression model was computed from SC, AD, ACLF, ALI and ALF patients against INR, a marker of clotting, (Figure 30C & D; $R^2Y=0.45$; $Q^2Y=0.33$) and for AD, ACLF, ALI and ALF patients against fibrinogen (Figure 30E & F; $R^2Y=0.45$; $Q^2Y=0.27$), which is also associated with clotting and wound healing. The VIP plot for the model computed against INR showed methionine, threonine and glutamine had the highest VIP scores >1.5 and the VIP plot for PLS regression against fibrinogen concentration showed methionine, ethanolamine and glutamine had the highest VIP scores >1.5 .

PLS regression models were also computed against: urea (Figure 30G & H; $R^2Y=0.36$; $Q^2Y=0.24$), GGT (Figure 30K & L; $R^2Y=0.39$; $Q^2Y=0.27$), albumin (Figure 30M & N; $R^2Y=0.34$; $Q^2Y=0.27$), CRP (Figure 30O & P; $R^2Y=0.33$; $Q^2Y=0.23$), lactate (Figure 30I & J; $R^2Y=0.42$; $Q^2Y=0.26$) and cholesterol (Figure 30Q & R; $R^2Y=0.34$; $Q^2Y=0.22$). The VIP plot for urea identified methionine, cystathionine and ethanolamine and AABA with a VIP score >1.5 . PLS regression against GGT identified VIP scores >1.5 for glutamic acid, methionine and glutamine. For albumin metabolites with $VIP>1.5$ included ethanolamine, methionine and lysine. For CRP metabolites with $VIP>1.5$ included citrulline and AABA. For total cholesterol metabolites with $VIP>1.5$ included cystathionine, ethanolamine and BAIBA. Finally, for lactate metabolites with $VIP>1.5$ included alanine, glutamine and proline.

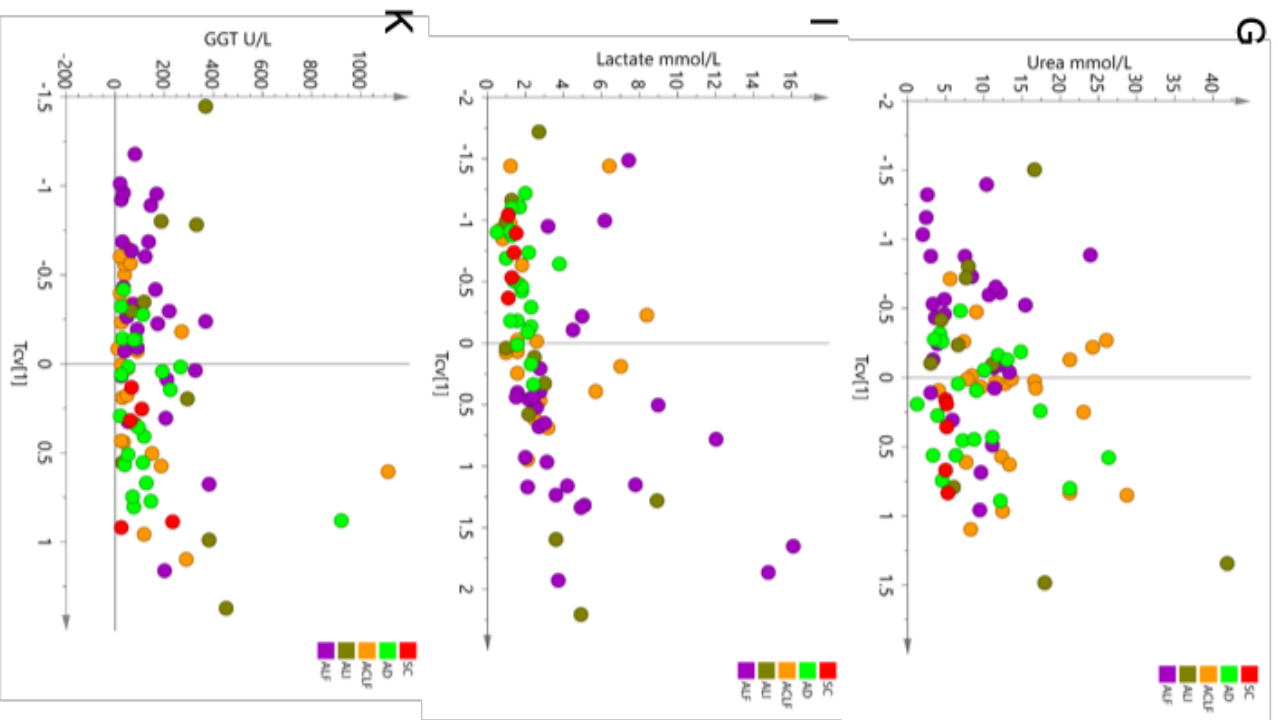
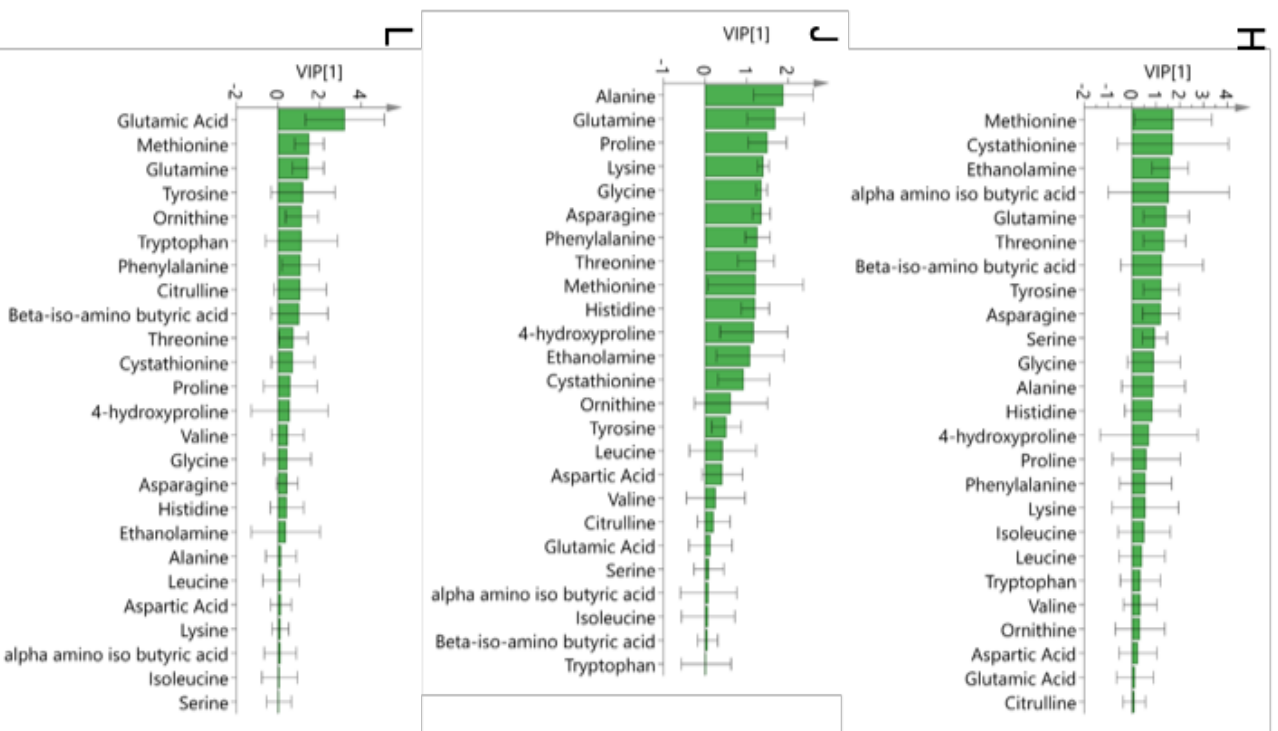
PLS regression models were also computed separately for the chronic liver disease groups (SC, AD and ACLF) which produced valid models for regression against the UKELD (Figure 30S & T; $R^2Y=0.51$; $Q^2Y=0.33$) and the Child Pugh scores (Figure 30U & V; $R^2Y=0.51$; $Q^2Y=0.27$). The VIP plots for these models identified ethanolamine, 4-hydroxyproline and methionine as the metabolites with the highest VIP scores (>1.5), whilst, tryptophan also had a VIP score >1.5 in the Child Pugh score PLS regression model (Figure 30U & V).



$R^2Y=0.45$
 $Q^2Y=0.37$

$R^2Y=0.45$
 $Q^2Y=0.32$

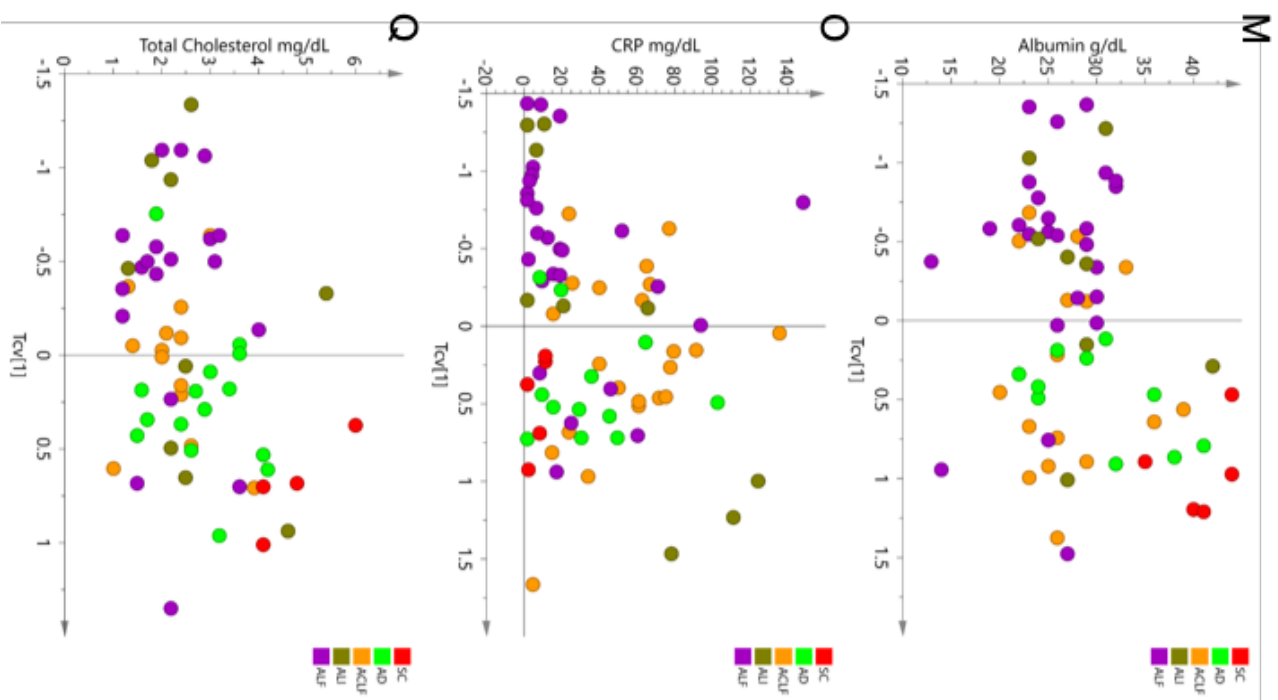
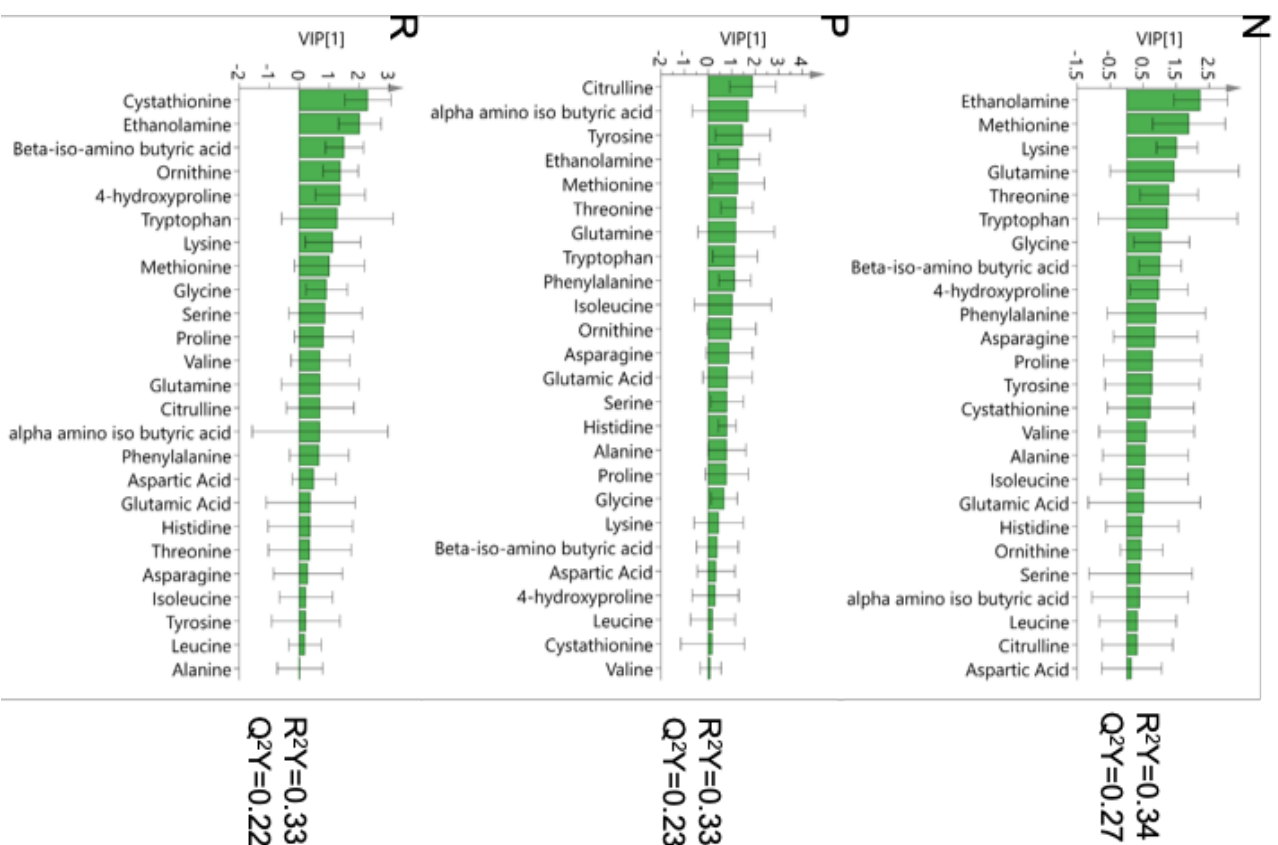
$R^2Y=0.40$
 $Q^2Y=0.27$



$R^2Y=0.36$
 $Q^2Y=0.24$

$R^2Y=0.41$
 $Q^2Y=0.26$

$R^2Y=0.39$
 $Q^2Y=0.27$



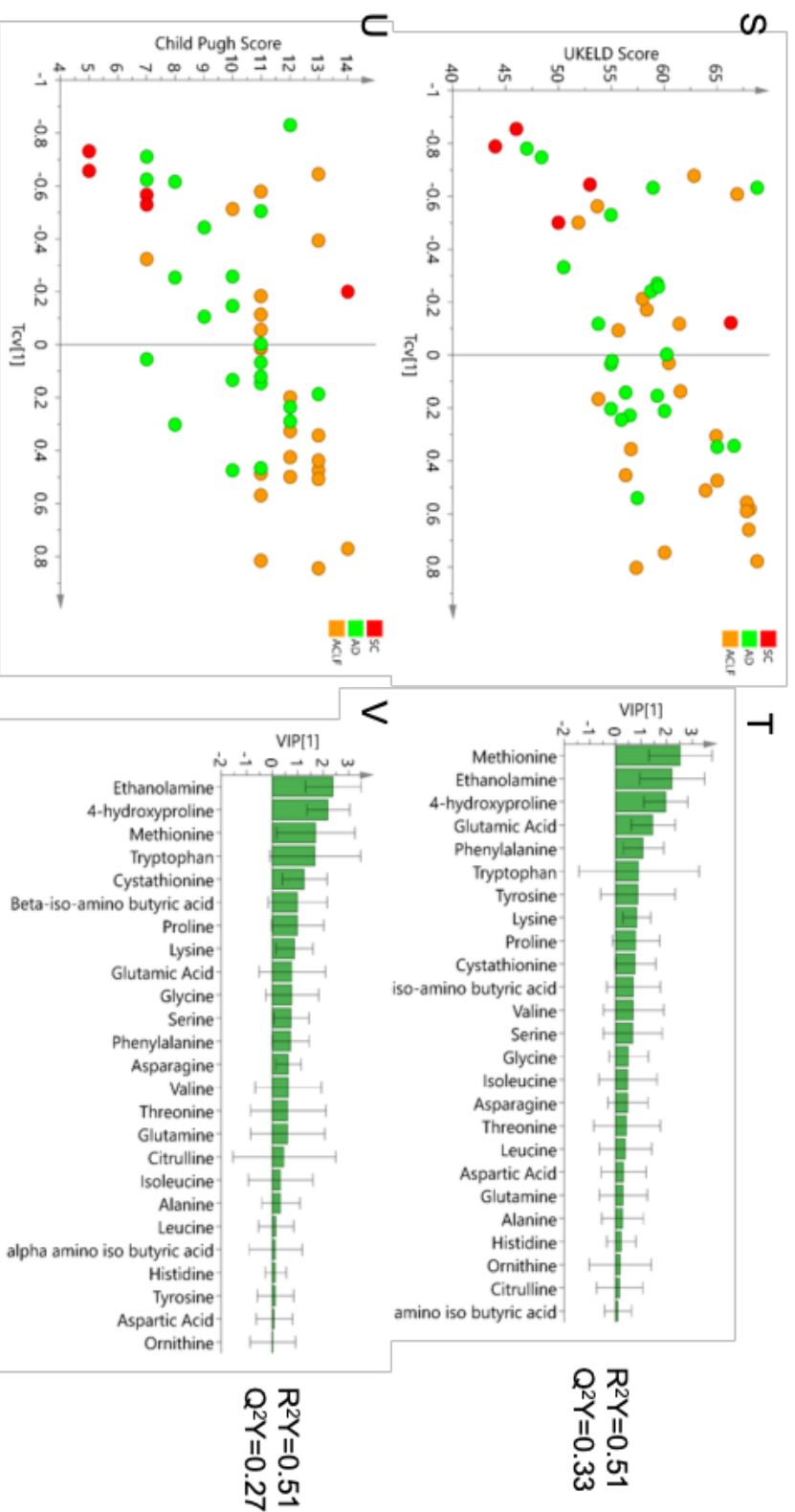


Figure 30. PLS regression of acute and chronic liver disease against metadata

All quantitative amino compound data was compared to clinical metadata (Y-variable) by PLS-regression modelling. All models displayed were permuted (n=999; CV-ANOVA p-value <0.05.) For each model the cross validated scores are plotted against the Y-variable and a corresponding VIP (variable of importance) plot for the model is also displayed. Cross-validated scores plots: A, C, E, G, I, K, M, O, Q, S & U; VIP plots: B, D, F, H, J, L, N, P, R, T, V; Y=MELD Score – (A & B); Y=INR – (C & D); Y=Fibrinogen plots (E & F); Y=Urea plots (G & H); Y=Lactate (I & J); Y=GGT (K & L); Y=Albumin (M & N); Y=CRP (O & P); Y=Total Cholesterol (Q & R); Y=UKELD plots (S & T); Y=Child Pugh Score (U & V);

Class	N=	Y- variable	No components	R ² Y	Q ² Y	Permuted R ² Y	Permuted Q ² Y	No. permutations	CV-ANOVA p-value
SC + AD + ACLF +ALI + ALF	86	MELD	2	0.42	0.32	0.11	-0.13	999	2.74E-06
SC + AD + ACLF +ALI + ALF	86	INR	2	0.45	0.33	0.11	-0.14	999	2.22E-06
SC + AD + ACLF +ALI + ALF	63	Fibrinogen	2	0.40	0.27	0.16	-0.13	999	7.30E-04
SC + AD + ACLF +ALI + ALF	87	Urea	2	0.36	0.24	0.12	-0.12	999	3.94E-04
SC + AD + ACLF +ALI + ALF	87	GGT	2	0.39	0.27	0.12	-0.13	999	5.88E-05
SC + AD + ACLF +ALI + ALF	64	Albumin	2	0.34	0.27	0.16	-0.12	999	1.26E-03
SC + AD + ACLF +ALI + ALF	74	CRP	2	0.33	0.23	0.14	-0.12	999	1.06E-03
SC + AD + ACLF +ALI + ALF	61	Total Cholesterol	2	0.34	0.22	0.17	-0.12	999	1.45E-02
SC + AD + ACLF +ALI + ALF	83	Lactate (Blood gas)	2	0.42	0.26	0.12	-0.13	999	8.15E-05
SC + AD + ACLF	50	UKELD	2	0.51	0.33	0.19	-0.22	999	7.38E-04
SC + AD + ACLF	50	Child Pugh Score	2	0.51	0.27	0.20	-0.20	999	2.82E-03

Table 11. Summary of UPLC-MS amine PLS regression models

UPLC-MS amino compound data was regressed with all metadata. Valid models are shown with full model statistics. MELD – model for end stage liver disease; UKELD – UK end stage liver disease; INR – international normalised ratio; GGT – gamma glutamyl transferase; CRP – C-reactive protein; ALT – alanine aminotransferase; NH4⁺ - ammonia;

4.5 Discussion

In this study multivariate and univariate analysis for a range of amino compounds present in plasma from patients with acute and chronic liver disease revealed a common metabolic phenotype for ALF, AD and ACLF (See Figure 31). Furthermore, a robust OPLS-DA model that discriminated ALF from ACLF was also computed. Minor changes in amino compounds that were discriminant within acute and chronic liver disease groups were also identified. These included a progressive rise in alanine from ALI to ALF, and also a progressive depletion of serine from AD to ACLF. Pathway analysis comparing ALF and ACLF, identified pathways discriminately perturbed that were linked to known pathophysiological mechanisms present in both groups (which are discussed below). PLS regression modelling of the data with the clinical metadata, produced valid models when data was regressed with end stage liver disease scoring systems and known markers of liver damage.

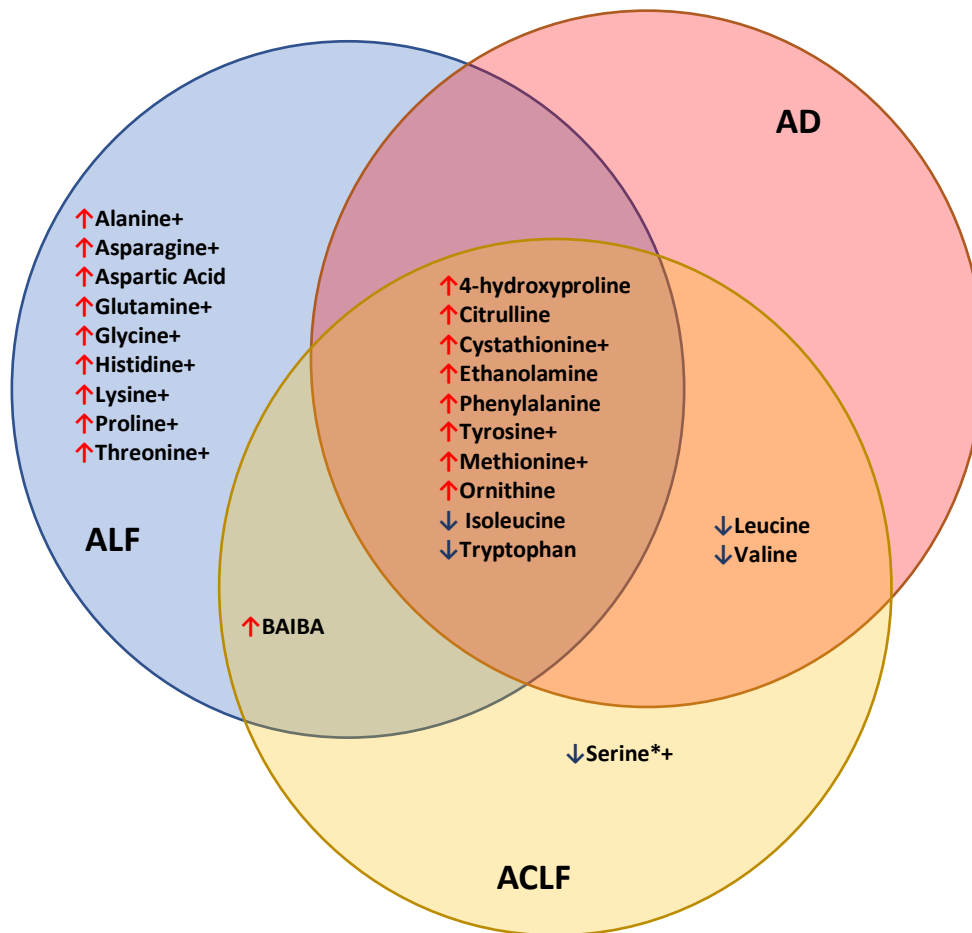


Figure 31. Venn diagram summarising metabolic differences observed from quantified plasma amino compound concentrations from AD, ACLF and ALF patients

These diagrams summarise the metabolic changes observed in AD, ACLF and ALF relative to HC as well as changes between groups. Statistical significance was derived from earlier univariate analyses see Figure 25. Significant changes in metabolites between AD vs ACLF are marked with an asterisk (*) and significant changes in metabolites between ALF and ACLF are marked with a cross (+).

4.5.1 Perturbation of the Cysteine and Methionine pathway is common to ALF and ACLF

A significant rise in sulphur containing metabolites linked to the cysteine and methionine pathway was detected in the plasma of ALF and ACLF patients (see Figure 25). This pathway is comprised of the folate cycle, methionine cycle, transsulfuration pathway and the gamma-glutamyl cycle and regulates production of glutathione and is involved in mechanisms of detoxification and cytoprotection. The metabolites which showed changes in both ALF and ACLF in this pathway are summarised in Figure 32.

Enzyme number	Enzyme Name	KEGG ID
1	Methionine adenosyltransferase 2B	2.5.1.6
2	DNA methyltransferase 1	2.1.1.37
3	Adenosylhomocysteinase	3.3.1.1
4	Choline dehydrogenase	1.1.99.1
5	Betaine homocysteine methyltransferase	2.1.1.5
6	Methionine synthase	2.1.1.14
7	Serine hydroxymethyltransferase	2.1.2.1
8	Methylenetetrahydrofolate reductase	1.5.1.20
9	Cystathionine beta-synthase	4.2.1.22
10	Cystathionine gamma-lyase	4.4.1.1
11	Branched chain amino acid transaminase/alanine-glyoxylate transaminase	2.6.1.42 / 2.6.1.44
12	Glutamate-cysteine ligase catalytic subunit	6.3.2.2
13	Glutathione synthase	6.3.2.3
14	L-cysteine hydrogen-sulfide-lyase	4.4.1.10
15	Aspartate aminotransferase	2.6.1.1
16	Malate dehydrogenase 1	1.1.1.37
17	Aspartate aminotransferase	2.6.1.1
18	3-Mercaptopyruvate sulfurtransferase	2.8.1.2
19	Cystathionine gamma-lyase	4.4.1.1
20	Aspartate aminotransferase	2.6.1.1
21	Lactate dehydrogenase	1.1.1.27
22	Cysteine dioxygenase	1.13.11.20
23	CoA-glutathione reductase	1.8.1.10
24	Glutathione-CoA transhydrogenase	1.8.4.3
25	Glutathione-CoA transhydrogenase	1.8.4.3
26	Cystathionine gamma-lyase	4.4.1.1

Table 12. Summary of enzymes from the Cysteine and Methionine pathway

Enzyme number corresponds to numbering of enzymes in Figure 32, KEGG ID per enzyme is also listed. Rows in bold indicate enzymes highlighted which might be perturbed in acute and chronic liver disease and warrant further investigation.

The cysteine and methionine pathway was one of the top pathways discriminating between ALF and ACLF (Figure 29B) and univariate analyses revealed an increase in plasma concentrations of methionine and cystathionine in ALF compared to ACLF (Figure 25). Perturbation of this pathway has been previously observed in experimental models of liver damage^{164,165} and patients with cirrhosis¹⁶⁶ and ALF¹⁶⁷ and has been shown to exacerbate liver injury and hepatic dysfunction. Studies on this pathway have mainly focused on methionine metabolism, although more studies on downstream sulphur metabolites are emerging, however, these are largely focused on chronic liver disease.

Methionine is metabolised to S-adenosylmethionine (SAME), which is an important metabolically pleiotropic molecule involved in transmethylation, transsulfuration and amine

propylation via the enzyme methionine adenosyl transferase (MAT). Reduced MAT leads to methionine elevation in chronic liver disease, preventing protective mechanisms and exacerbating injury, hence, supplementation with SAdMe is used as a treatment in patients with chronic liver disease in several countries.¹⁶⁸ Similarly, in ALF methionine elevation is also a consequence of liver injury where destruction of liver tissue leads to depletion of hepatic SAdMe concentrations, resulting in high methionine concentrations which are a predictor of mortality in ALF.¹⁶⁷

In addition to methionine, changes in other metabolites in the pathway have also been observed in patients with cirrhosis¹⁶⁹ and alcoholic liver disease.¹⁷⁰ These include a rise in circulating cystathionine, dimethylglycine, homocysteine, S-adenosylmethionine, S-adenosylhomocysteine concentrations, which correlate with fibrosis and severity of disease.^{168,170} Furthermore, there are no changes in plasma cysteine concentrations reported which is also confirmed by our data (see Figure 27) and indicates a downregulation of cystathionine- γ -lyase activity in acute and chronic liver disease. Overall, the findings from the literature and our data suggest that there is an impairment of transsulfuration at the level of cystathionine degradation in AD and ACLF, this mechanism is also very likely in ALF. However, no studies focusing on this pathway in ALF patients appear to have been published and therefore this pathway requires further characterisation in ALF patients.

Furthermore, a fold change increase in plasma cystine levels are reported in both acute and chronic liver disease and occur likely due to an upregulation of glutathione-CoA transhydrogenase. However, the mechanistic implications of this finding are unclear, and enzymes and metabolites linked to cystine which include glutathione-CoA transhydrogenase and cystathionine- γ -lyase and thiocystine and S-sulfo-L-cysteine respectively require further investigation in these patients. In summary, abnormalities in the methionine and cysteine pathway most likely reflect the impairment of multiple hepatic enzyme sites because of hepatocyte injury and cell death. Further characterisation of metabolites, highlighted in yellow (Figure 32), in these patient groups is required to confirm this hypothesis.

The key differences in the methionine and cysteine pathway between ALF and ACLF is the selective depletion of serine and no change in glycine concentrations in ACLF compared to ALF. These differences appear to indicate that the perturbation of methionine and cysteine metabolism may have a different mechanism in ACLF compared to ALF and may be linked to cystathionine- γ -lyase or serine hydroxymethyltransferase activity (Figure 32). In addition, serine and glycine are also central to the glycine, serine and threonine pathway

which is also discriminately perturbed in ALF vs ACLF and discussed further in the next section.

4.5.2 Serine depletion is unique for ACLF in the glycine serine and threonine pathway

The glycine, serine and threonine pathway was the second most discriminant pathway identified for ALF vs ACLF (see Figure 29). The main differences were a depletion of serine in ACLF and a significant rise in glycine and threonine concentrations ALF (summarised in Figure 33). This pathway is important for glycine synthesis from L-serine and L-threonine. Glycine is an important precursor for several vital metabolites including heme, glutathione, creatine and purines, as well as for conjugation to bile acids.

Significant elevations in circulating glycine and threonine concentrations have been observed previously in patients with ALF¹⁷¹ and cirrhosis¹⁷² and correlates with the degree of liver injury. In our data, these metabolites only increase in ALF plasma, which could be explained by the higher rate of liver injury in ALF compared to AD and ACLF which have a slower rate of progression.

The significant rise in circulating concentrations of glycine could also be explained by a perturbation of neighbouring metabolites in the pathway such as glyoxylate. The glyoxylate pathway generates TCA cycle metabolites and was previously identified as discriminant for both AD vs ACLF and ALF vs ACLF from the pathway analysis of the ¹H NMR spectroscopic data (See Chapter 3). Furthermore, the rise in glycine may suggest a downregulation of the hepatic enzyme 5-aminolevulinate synthase which converts glycine to 5-aminolevulinate which is linked to the porphyrin pathway (Figure 33). This pathway is vital for heme production a component of haemoglobin which is markedly reduced in ALF.¹⁷³ Finally, a further downstream metabolite affected by glycine elevation in ALF is sarcosine which was present at very high concentrations in six ALF patients (Figure 25). In health sarcosine (N-methylglycine) is an intermediate byproduct in glycine synthesis and degradation¹⁷⁴ and this is the first association of this compound with ALF in some of the patients in this unique clinical cohort. A selective down-regulation of the enzyme sarcosine dehydrogenase (Figure 33) which converts sarcosine to glycine is a likely mechanism for sarcosine elevation in the subpopulation of ALF patients with elevated sarcosine concentrations, however, this warrants further investigation.

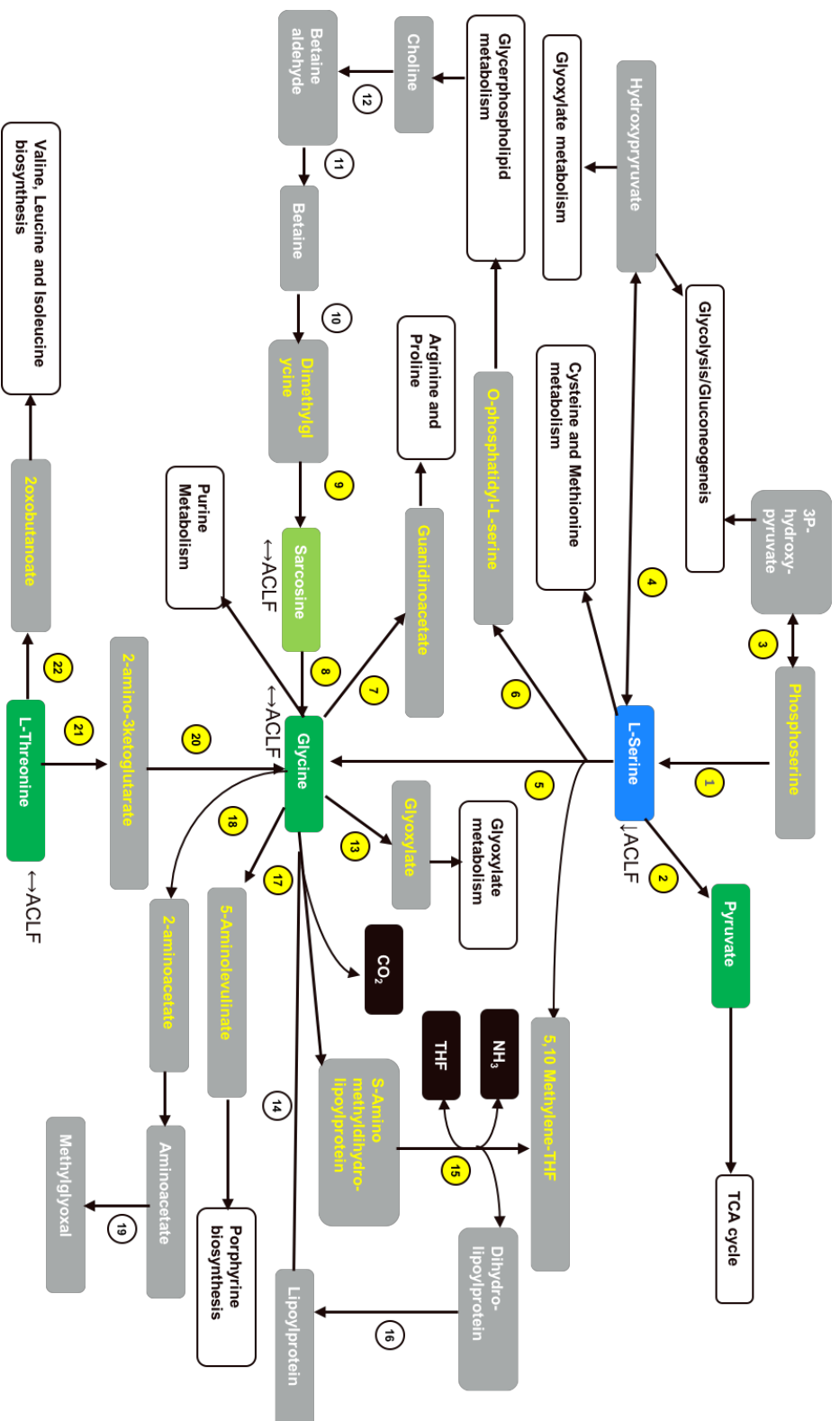


Figure 33. Glycine, Serine and Threonine pathway in ALF and ACLF
 Pathway adapted from KEGG database and metabolites coloured according to known changes in ALF relative to HC and differences in ACLF compared to ALF are indicated in text in the diagram: grey – compound not quantified; blue – no changes in concentration; green – increase in concentration; light green – concentration increase not statistically significant; Enzymes are numbered 1-22 and summarised in the table below. Enzymes and metabolites which require further investigation based on existing changes in amino compound concentrations are indicated in yellow. THF - tetrahydrofolate; NH₃ - ammonia

Enzyme number	Enzyme Name	KEGG ID
1	Phosphoserine Phosphatase	3.1.3.3
2	L-serine lyase	4.3.1.17
3	Phosphoserine aminotransferase	2.6.1.52
4	Serine-pyruvate transaminase	2.6.1.51
5	Glycine hydroxymethyltransferase	2.1.2.1
6	Phosphatidylserine synthase	2.7.8.8
7	Glycine amidinotransferase	2.1.4.1
8	Sarcosine oxidase / Sarcosine dehydrogenase	1.5.3.1/1.5.8.3
9	Dimethylglycine dehydrogenase	1.5.8.4
10	Betaine-homocysteine S methyltransferase	2.1.1.5
11	Aldehyde dehydrogenase	1.2.1.8
12	Choline dehydrogenase	1.1.99.1
13	D-amino acid oxidase	1.4.3.3
14	Glycine decarboxylase	1.4.42
15	Aminomethyltransferase	2.1.2.10
16	Dihydrolipoamide dehydrogenase	1.8.1.4
17	5-aminolevulinate synthase	2.3.1.37
18	Glycine C-acetyltransferase	2.3.1.29
19	Monoamine oxidase / primary-amine oxidase	1.4.3.4/1.4.3.21
20	2-amino-3-ketobutyrate coenzyme A ligase	2.3.1.29
21	L-threonine dehydrogenase	1.1.1.103
22	L-threonine ammonia lyase	4.3.1.19

Table 13. Summary of enzymes from the Glycine, Serine and Threonine pathway

Enzyme number corresponds to numbering of enzymes in Figure 33, KEGG ID per enzyme is also listed. Rows in bold indicate enzymes highlighted for further analysis in future work.

Interestingly, the only amino acid change unique to ACLF was serine depletion. Studies have shown that glutamic acid (glutamate) is an amino donor for the synthesis of serine, furthermore this is also regulated by plasma ammonia concentrations.⁶³ However, in our study population none of the groups showed any changes in glutamic acid concentrations (see Figure 25). A recent cell-based metabolomics study found that depletion of serine causes mitochondrial fragmentation and perturbs glycerophospholipid metabolism causing phosphatidylethanolamine elevation¹⁷⁵ suggesting an upregulation of L-serinephosphatidyltransferase (pathway summarised in Figure 34).

Furthermore, previous studies which have measured circulating serine concentrations in patients with cirrhosis and ACLF, either report a rise in serine concentrations,¹⁷² or no significant change.¹⁷⁰ Whilst studies in NASH also see a depletion in serine^{176,177} associating it with the progression of fibrosis¹⁷⁷ and a downregulation of the glycine, serine pathway as a result of glutathione depletion.¹⁷⁸ Furthermore, the enzymes

phosphoserine phosphatase (PSPH) and serine hydroxymethyltransferase (SHMT1) were identified as targets to restore concentrations and prevent progression to fibrosis.¹⁷⁶

Further investigation into enzymes and metabolites upstream and downstream of L-serine in the glycine, serine and threonine pathway, including the pathways linked to glutathione generation (see Figure 34), are indicated by these results.

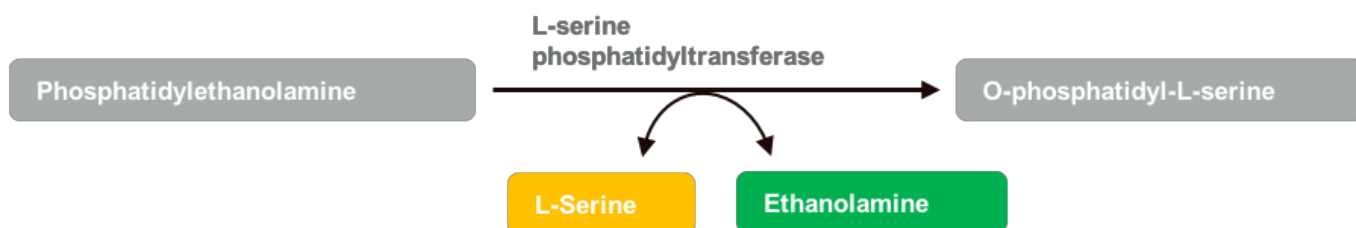


Figure 34. Serine depletion and ethanolamine elevation in ACLF

Metabolites coloured according to changes observed in ACLF relative to HC. Grey – metabolite not measured; Yellow – metabolite decreases; Green - metabolite increases.

4.5.3 An evaluation of amino compound changes linked to hepatic encephalopathy in ALF and ACLF

The development of hepatic encephalopathy (HE) is common to acute and chronic liver disease. HE leads to the accumulation of neurotoxic metabolites and molecules which perturb metabolism and neurotransmission in the brain of which ammonia is the primary neurotoxin. The Fischer ratio (low BCAA : high AAA) and high plasma glutamine concentrations are well characterised, and known to be strongly linked to the development of HE, these were confirmed by our data (Figure 25). Other metabolites also measured by UPLC-MS and linked to the development of HE includes tryptophan and 5-aminovaleric acid which are discussed below.

The phenylalanine pathway was one of the top ten pathways discriminant for ALF vs ACLF, where tyrosine which is produced from phenylalanine is present at a significantly higher concentration in ALF compared to ACLF (Figure 28) suggesting a greater upregulation of phenylalanine hydroxylase in ALF (Figure 35).

Raised plasma tyrosine and phenylalanine concentrations and the development of HE is linked to their increased uptake in the brain,¹⁷⁹ causing metabolism via alternative pathways. These give rise to metabolites, such as octopamine and phenylethanolamine, which act like false transmitters which interfere with neurotransmission, as well as causing an upregulation of the synthesis of cerebral catecholamines such as adrenaline, noradrenaline and dopamine⁶⁵. Therefore, it would be expected that there would be raised

amounts of octopamine, phenylethanolamine, noradrenaline and adrenaline in the plasma or brains of ALF and ACLF patients. Octopamine, was not detected in any of the plasma samples analysed by UPLC-MS (see Table 40 in the appendix). This suggests that either it was present at concentrations below the limits of detection, or it is rapidly taken up through the blood brain barrier and therefore is present at higher concentration in the brains of these patients.

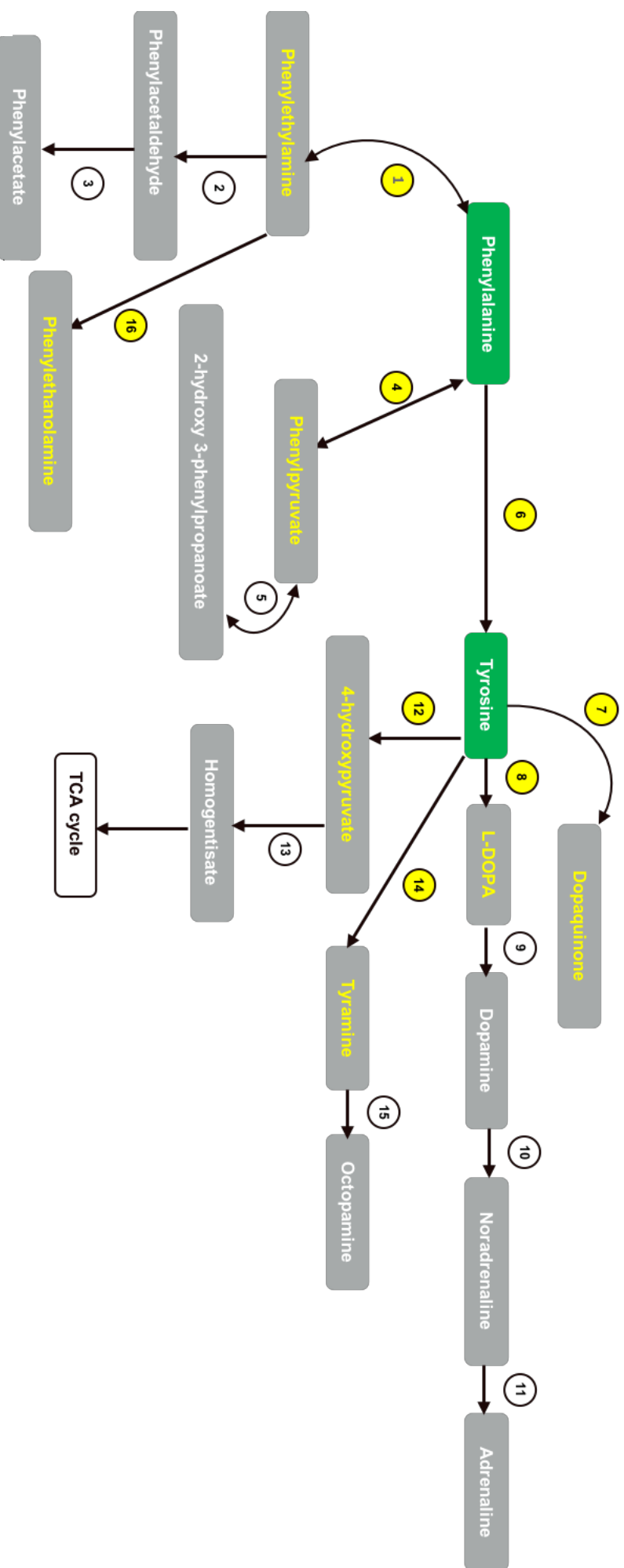


Figure 35. Phenylalanine and Tyrosine pathway in acute and chronic liver disease
 Metabolites coloured according to changes observed in AD, ACLF, ALI and ALF relative to HC. Grey – metabolite not measured; Green - metabolite increased in concentration; L-DOPA – L-3,4-dihydroxyphenylalanine;

Enzyme number	Enzyme Name	KEGG ID
1	Aromatic-L-amino-acid decarboxylase	41.1.28
2	Monoamine oxidase / Primary –amine oxidase	1.4.3.4/1.4.3.21
3	Aldehyde dehydrogenase	1.2.1.5
4	Aspartateaminotransferase/tyrosine aminotransferase	2.6.1.1/2.6.1.5
5	Phenylpyruvate tautomerase	5.3.2.1
6	Phenylalanine-4-hydroxylase	1.14.16.1
7	Tyrosinase	1.14.18.1
8	Tyrosinase /tyrosine 3-monooxygenase	1.14.18.1/1.14.16.2
9	Aromatic-L-amino acid decarboxylase	4.1.1.28
10	Dopamine beta-monooxygenase	1.14.17.1
11	Phenylethanolamine N-methyltransferase	2.1.1.28
12	Aspartate aminotransferase/tyrosine aminotransferase	2.6.1.1/2.6.1.5
13	4-hydroxyphenylpyruvate dioxygenase	1.13.11.27
14	Aromatic-L-amino-acid decarboxylase	4.1.1.28
15	Dopamine Beta hydroxylase	1.14.17.1
16	Dopamine Beta hydroxylase	1.14.17.1

Table 14. Summary of enzymes from the Phenylalanine and Tyrosine pathway

Enzyme number corresponds to numbering of enzymes in Figure 35, KEGG ID per enzyme is also listed. Rows in bold indicate enzymes highlighted for further analysis in future work.

A further amino acid linked to HE is tryptophan, which is an essential amino acid, that is depleted in concentration in the plasma of AD, ACLF, ALI and ALF patients relative to HC (see Figure 26). This is in contrast to some studies, which show tryptophan is elevated in the plasma of individuals with liver failure.^{64,180} However, a recent study found tryptophan was present at high concentrations in the CSF, but not plasma of cirrhotics with HE.¹⁸¹ The depletion of plasma concentrations of tryptophan implies a potential upregulation of two pathways linked to tryptophan metabolism which include the kynurenine pathway and the serotonin pathway (see Figure 36).

An upregulation of the serotonin pathway, leading to the disruption of neurotransmission in liver disease, has been suggested previously as a mechanism for the development of HE.¹⁸² Research into the physiological role of the kynurenine pathway in the brain, which results in the production of NAD⁺ is currently ongoing.¹⁸³ This pathway is not well understood but is known to be disrupted in several diseases including HIV¹⁸⁴, rheumatoid arthritis¹⁸⁵ and neurodegenerative diseases.¹⁸⁶ An upregulation of the pathway

has been linked to neurosignalling via glutamate receptors via kynurenic acid and quinolinic acid¹⁸⁷ however, these are yet to be investigated in ALF and ACLF.

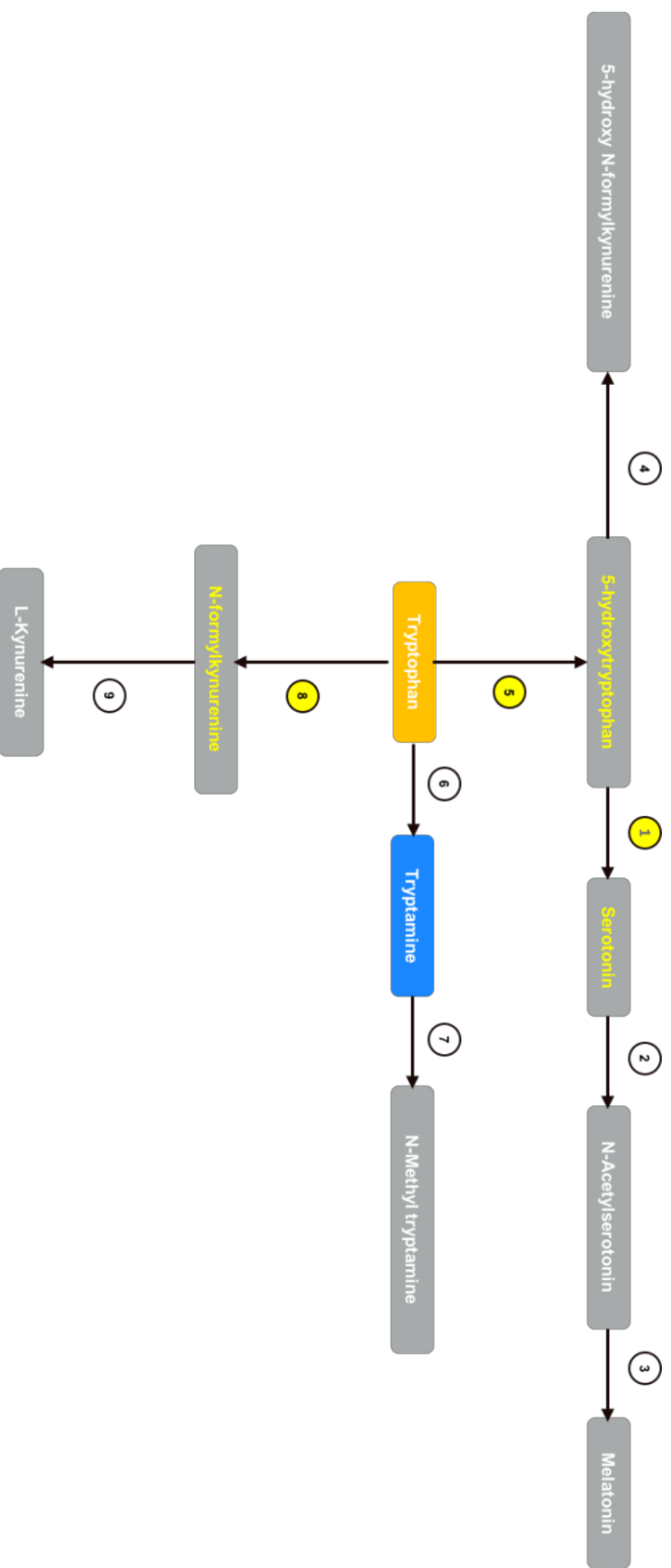


Figure 36. Tryptophan pathway in acute and chronic liver disease

Metabolites coloured according to changes observed in AD, ACLF, ALI and ALF relative to HC. Grey – metabolite not measured; Yellow – decrease in concentration; Blue – no change in concentration

Enzyme number	Enzyme Name	KEGG ID
1	Aromatic-L-amino-acid decarboxylase	4.1.28
2	Arylalkylamine N-acetyltransferase	2.3.1.87
3	Acetylserotonin O-methyltransferase	2.1.1.4
4	Indoleamine 2,3-dioxygenase	1.13.11.52
5	Tryptophan 5-monooxygenase	1.14.16.4
6	Aromatic-L-amino-acid decarboxylase/L-tryptophan decarboxylase	4.1.1.28/4.1.1.105
7	Methyltransferase	2.1.1.49
8	Tryptophan 2,3-dioxygenase/Indoleamine 2,3-dioxygenase	1.13.11.11/1.13.11.52
9	Arylformamidase	3.5.1.9

Table 15. Summary of enzymes from the Tryptophan pathway

Enzyme number corresponds to numbering of enzymes in Figure 36, KEGG ID per enzyme is also listed. Rows in bold indicate enzymes highlighted for further analysis in future work.

A marked increase in 5-aminovaleric acid (also known as 5-aminopentanoic acid) concentration relative to HC was also observed. The main role of 5-aminovaleric acid is not fully clear, although it has been identified in two animal studies as an analogue of GABA (which showed no changes in plasma from our data – see Figure 26) involved in neuro-signalling and therefore cognitive function.^{188,189} Hence, a rise in 5-aminovaleric acid concentrations could be linked to the disruption of neuro-signalling and the subsequent development of HE observed in AD, ACLF and ALF, although this hypothesis clearly requires further testing.

All of the amino compounds (5-aminovaleric acid, glutamine, phenylalanine, tyrosine and tryptophan) associated with HE, were assessed for correlation to HE grade and the Glasgow comma score (GCS) however, none showed significant correlations (data not shown). The reason for this lack of correlation is unlikely to be that they are unrelated to HE but because determination of the HE grade is based on visual and behavioural changes in patients.

4.5.4 Perturbation of histidine metabolism and novel pathways linked to mechanisms of muscle wasting in ALF and ACLF

Pathways potentially linked to muscle wasting were identified from analysis of the UPLC-MS data and included BCAA depletion and increased concentrations of 3-

methylhistidine (3-MH) and BAIBA (Figure 25 & 26). Muscle wasting, which is a consequence of malnutrition in liver disease occurs in chronic liver disease.¹⁹⁰

Depletion of the BCAA's isoleucine and valine was observed in AD and ACLF, whilst leucine was depleted in AD and isoleucine in ALF. Decreased BCAA concentrations have been previously observed in liver failure and are thought to be markers of muscle wasting.¹⁹¹

Histidine metabolism was also discriminant between ALF and ACLF where histidine was selectively elevated in ALF. Histidine plasma concentrations have previously been observed to increase systemically in patients with liver failure.^{171,181} The precise mechanisms which lead to elevated histidine concentrations are unclear, but to are likely occur as a consequence of liver injury and hepatocyte death leading to a decrease in hepatic capacity for histidine breakdown catalysed by the hepatic enzyme histidine ammonia lyase (Figure 37).

Furthermore, 3-MH, which is produced by the methylation of histidine residues in actin and myosin, is linked to muscle wasting,¹⁹² was selectively decreased in ALF plasma (Figure 26). An elevation of 3-MH in urine and plasma has been linked to muscle wasting,^{193,194} however, we find 3-MH is selectively decreased in the plasma of patients with acute liver disease and the mechanism explaining this observation is currently unknown. Furthermore, changes were also observed in 1-methylhistidine (1-MH) which is a muscle protein breakdown product in rats and other mammals. However, humans lack this enzyme and the 1-MH present in plasma is likely to be a breakdown product of the dipeptide anserine (comprised of 1-MH and β -alanine) which is obtained from dietary sources.¹⁹⁵ 1-MH showed a fold increase in AD, ACLF and ALI (see Figure 26) which is likely due to an upregulation of anserine catabolism in these patients as protein catabolism is upregulated. However, β -alanine showed no differences across all groups (see Figure 26) hence, the mechanism causing 1-MH upregulation in these patients is unclear.

Finally, an additional metabolic change linked to muscle wasting was an increase in plasma BAIBA concentrations (see Figure 25). Previous studies show that BAIBA is a myokine released from skeletal muscles from the enhanced catabolism of valine following prolonged exercise.¹⁹⁶

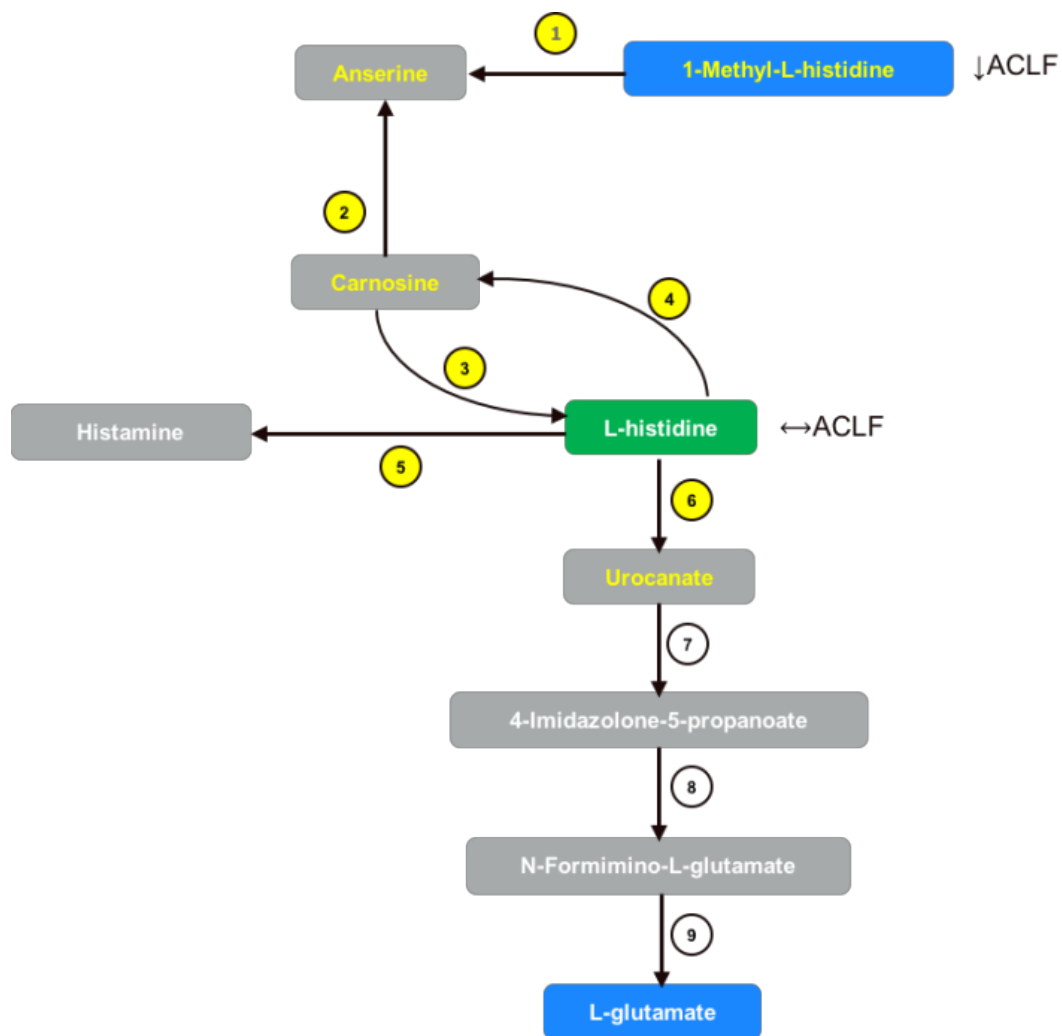


Figure 37. Histidine metabolism in acute liver disease

Metabolites coloured according to changes observed in AD, ACLF, ALI and ALF relative to HC. Changes that are different for ACLF are indicated in the diagram; Blue – no change in metabolite intensity; Grey – metabolite not measured; Green - metabolite increases;

Enzyme number	Enzyme Name	KEGG ID
1	Carnosine synthase	6.3.2.11
2	Carnosine N-methyltransferase	2.1.1.22
3	Cytosolic nonspecific dipeptidase/beta-ala-his dipeptidase	3.4.13.18/3.4.13.20
4	Carnosine synthase	6.3.2.11
5	Histidine decarboxylase	4.1.1.22
6	Histidine ammonia-lyase	4.3.1.3
7	Urocanate hydratase	4.2.1.49
8	Imidazolonepropionase	3.5.2.7
9	Glutamate formiminotransferase	2.1.2.5

Table 16. Summary of enzymes from the Histidine pathway

Enzyme number corresponds to numbering of enzymes in Figure 37, KEGG ID per enzyme is also listed. Rows in bold indicate enzymes highlighted for further analysis in future work.

4.5.5. Raised 4-hydroxyproline and proline plasma concentrations are not linked to fibrosis in chronic liver disease

We observed a significant elevation of proline concentrations in ALF plasma and increased 4-hydroxyproline in AD, ACLF, ALI and ALF plasma (see Figure 25). Previous studies have shown that hyperprolinemia occurs in cirrhosis, although less frequently in alcoholics.¹⁹⁷ Hyperprolinemia has also been associated with the upregulation of collagen biosynthesis in cirrhosis¹⁹⁸ through the enzymatic activity of collagenase and proline oxidase,¹⁹⁹ linking it to liver fibrosis.¹⁹⁷

Fibrotic lesions, which form during fibrosis, are largely comprised of the extracellular matrix protein - collagen which is comprised largely of proline and 4-hydroxyproline.²⁰⁰ Furthermore, fibrosis occurs in the earlier stages of chronic liver disease and its progressive worsening leads to cirrhosis.²⁰¹ This mechanism is not present in acute liver disease (ALI or ALF), hence indicating raised plasma concentrations of proline and 4-hydroxyproline are not mechanistically linked to fibrosis of the liver in this cohort.

Instead, the significance of ALF hyperprolinemia could be its link to high lactate in ALF. Previous studies have found that lactate inhibits the breakdown of proline to glutamate and alpha-ketoglutarate which is catalysed by proline oxidase and 1-pyrroline-5-carboxylate dehydrogenase.^{197,202} Furthermore, the selective rise in proline in ALF suggests either a saturation of the enzyme prolyl-4-hydroxylase or a downregulation of the hepatic enzymes

proline dehydrogenase/ pyrroline-5-carboxylate reductase in ALF (see Figure 38). The rise in 4-hydroxyproline in acute liver disease is a novel finding and may be linked to a downregulation of hepatic hydroxyproline dehydrogenase in acute and chronic liver disease (see Figure 38). Further investigation of the downstream targets in the arginine and proline pathway is required.

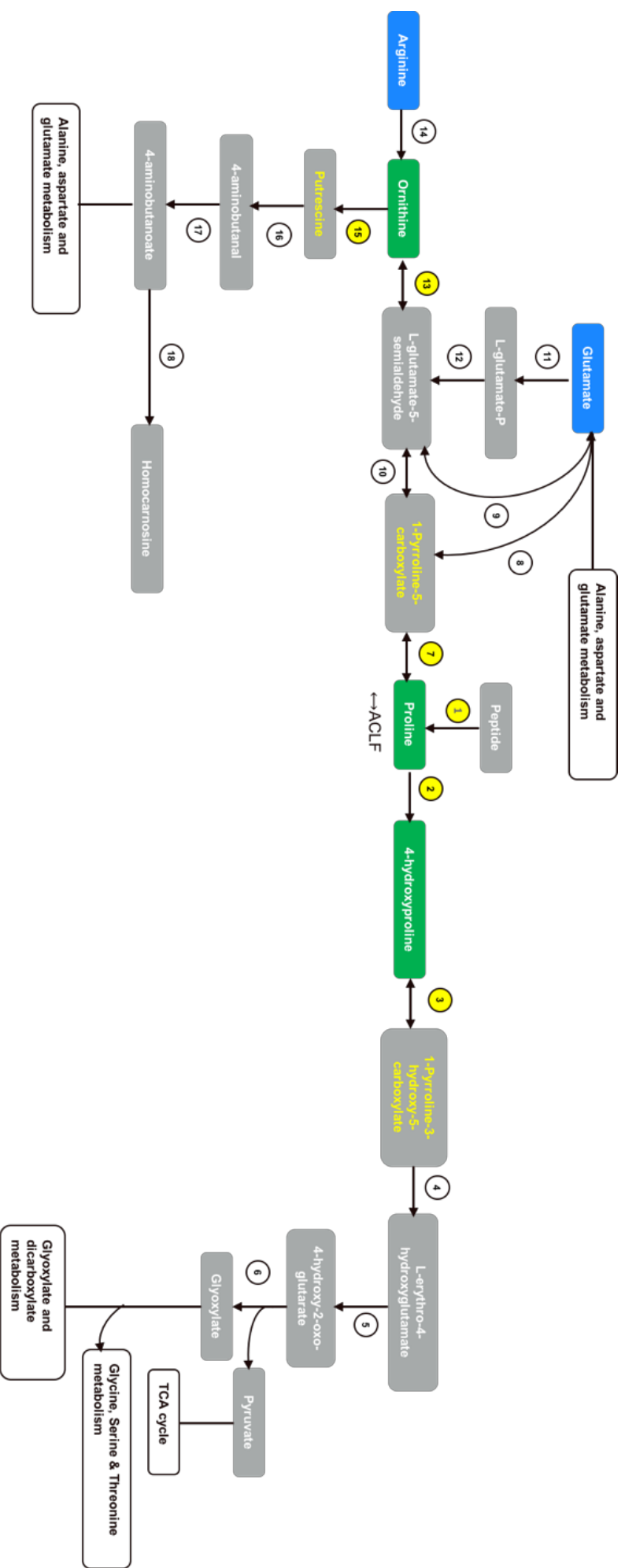


Figure 38. Arginine and Proline metabolism in acute and chronic liver disease
Metabolites coloured according to changes observed in ALF relative to HC. Changes that are different for ACLF are indicated. Grey – metabolite not measured; Green - metabolite increases.

Enzyme number	Enzyme Name	KEGG ID
1	Cytosol aminopeptidase	3.4.11.5
2	Prolyl 4-hydroxylase	1.14.11.2
3	Pyrroline-5-carboxylate reductase/hydroxyproline dehydrogenase	1.5.1.2/1.5.5.3
4	1-pyrroline-5-carboxylate dehydrogenase	1.2.1.88
5	Cytoplasmic Aspartate aminotransferase	2.6.1.1
6	4-hydroxy-2-oxoglutarate aldolase	4.1.3.16
7	Pyrroline-5-carboxylate reductase/Proline dehydrogenase	1.5.1.2/PRODH
8	1-pyrroline-5-carboxylate dehydrogenase	1.2.1.88
9	1-pyrroline-5-carboxylate dehydrogenase	1.2.1.88
10	unknown	-
11	Delta-1-pyrroline-5-carboxylate synthetase	2.7.2.11
12	Glutamate-5-semialdehyde dehydrogenase	1.2.1.41
13	Ornithine-oxo-acid transaminase	2.6.1.13
14	Arginase	3.5.3.1
15	Ornithine decarboxylase	4.1.1.17
16	Diamine oxidase	1.4.3.22
17	Aldehyde dehydrogenase	1.2.1.3
18	Carnosine synthase/Cytosolic nonspecific dipeptidase/beta-ala-his dipeptidase	6.3.2.11/3.4.13.18/3.4.13.20

Table 17. Summary of enzymes from the Arginine and Proline Pathway

Enzyme number corresponds to numbering of enzymes in Figure 38, KEGG ID per enzyme is also listed. Rows in bold indicate enzymes highlighted for further analysis in future work.

4.5.6. Perturbation of Aminoacyl-tRNA biosynthesis in ALF and ACLF

A further pathway discriminant for ALF vs ACLF was the aminoacyl-tRNA biosynthesis pathway which was identified by pathway analysis of both the ¹H NMR data (Chapter 3, Table 4) and the UPLC-MS data (Table 10). This pathway regulates tRNA charging where aminoacyl-tRNA synthetases (AARSs) catalyse the ligation of amino acids to their cognate tRNAs, an important mechanism for protein synthesis.²⁰³ In addition, AARSs have also been found to have a regulatory role in cell signalling pathways, cytokine signalling, immune cell activation and mitochondrial function, although the precise interactions between AARSs and these mechanisms are not yet fully characterised.²⁰⁴ Furthermore, specific mutations in genes encoding these groups of enzymes have been found in many diseases including diabetes, Charcot-Marie-tooth disease, Parkinson's disease and cancer.²⁰⁴ The distinction between ALF

vs ACLF therefore could be attributed to changes in AARS's activity or expression, where downregulation of AARS is discriminant for ALF vs ACLF, however this warrants further investigation.

Furthermore, according to a recent study, the aminoacyl-tRNA biosynthesis pathway is a common hit and is often highlighted as significant by metaboanalyst if several amino acids show significant changes.²⁰⁵ Hence, it is advisable, in the absence of direct measurements of aminoacyl-tRNA, to use this information with a degree of caution.²⁰⁵ Currently, disruption of AARS activity and its impact on pathophysiological mechanisms of disease is an emerging area and the therapeutic value in targeting AARSs is being investigated in a number of diseases.²⁰⁶ Hence, at present it is unclear if this pathway is perturbed, and if so what the mechanistic implications are in acute and chronic liver disease.

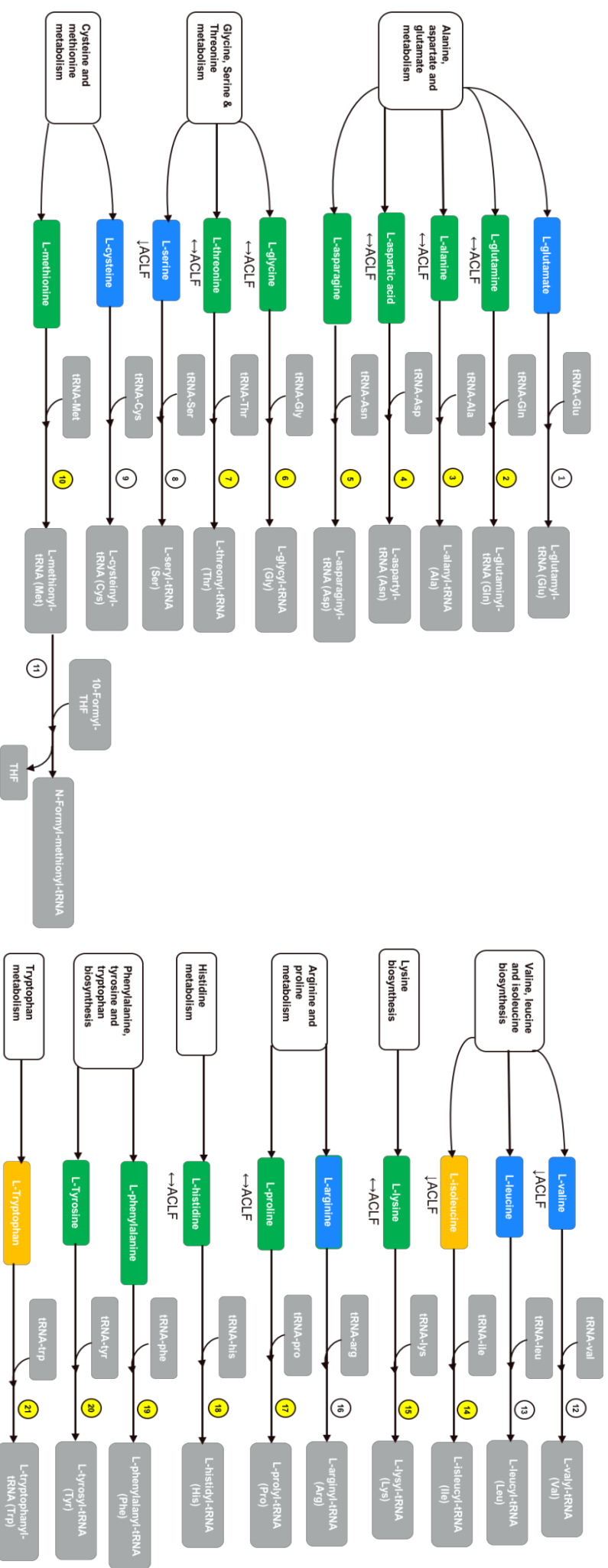


Figure 39. Aminoacyl-tRNA biosynthesis in acute and chronic liver disease

Metabolites coloured according to changes observed in ALF relative to HC. Changes in ACLF that are different to ALF are indicated in the diagram. Grey – metabolite not measured; Green - metabolite increases; Blue - no change in metabolite.

Enzyme number	Enzyme Name	KEGG ID
1	Glutamyl-tRNA synthetase	6.1.1.17
2	Glutaminyl-tRNA synthetase	6.1.1.24
3	Alanyl-tRNA synthetase	6.1.1.7
4	Aspartyl-tRNA synthetase	6.1.1.12
5	Asparaginyl-tRNA synthetase	6.1.1.22
6	Glycyl-tRNA synthetase	6.1.1.14
7	Threonyl-tRNA synthetase	6.1.1.3
8	Seryl-tRNA synthetase	6.1.1.11
9	Cysteinyl-tRNA synthetase	6.1.1.16
10	Methionyl-tRNA synthetase	6.1.1.10
11	Methionyl-tRNA formyltransferase	2.1.2.9
12	Valyl-tRNA synthetase	6.1.1.9
13	Leucyl-tRNA synthetase	6.1.1.4
14	Isoleucyl-tRNA synthetase	6.1.1.5
15	Lysyl-tRNA synthetase	6.1.16
16	Arginyl-tRNA synthetase	6.1.1.19
17	Prolyl-tRNA synthetase	6.1.1.15
18	Histidyl-tRNA synthetase	6.1.1.21
19	Phenylalanyl-tRNA synthetase	6.1.1.20
20	Tyrosyl-tRNA synthetase	6.1.1.1
21	Tryptophanyl-tRNA synthetase	6.1.1.2

Table 18. Summary of enzymes from the aminoacyl-tRNA biosynthesis pathway

Enzyme number corresponds to numbering of enzymes in Figure 39, KEGG ID per enzyme is also listed. Rows in bold indicate enzymes highlighted for further analysis in future work.

4.5.7 Prognostic markers of liver damage, coagulopathy and lipid metabolism correlate with metabolic changes seen in the UPLC-MS amine data

Regression of the quantitative plasma UPLC-MS amino compound profiles with the clinical metadata, identified amino compounds that showed a strong association with clinical markers (see Table 11).

PLS-regression of the UPLC-MS amine data with clinical scoring systems such as MELD (Figure 30A & B), Child Pugh (Figure 30S & T), and UKELD (Figure 30U & V), revealed amino compounds with strong associations to these scores that could improve or aid prognostic scoring. These models revealed that ethanolamine and methionine showed the strongest associations and might therefore be of prognostic value. Furthermore, 4-hydroxyproline also showed a strong association in the comparison with Child Pugh Scores for chronic liver disease (SC, AD & ACLF). Therefore, we conclude that methionine and ethanolamine are the best overall prognostic metabolites for both acute and chronic liver disease, whilst 4-hydroxyproline is most prognostic for chronic liver disease. Methionine, is previously reported to have prognostic qualities in end stage liver disease and cirrhosis, whilst phospholipid metabolism which is linked

to ethanolamine has also been found to predict survival in cirrhotics,¹⁵⁶ showing these amino compounds are good correlates of liver disease severity.

Interestingly, similarly to regression with ¹H NMR data, valid models were produced from regression with blood coagulation factors synthesised by the liver: INR (Figure 30C & D) and Fibrinogen (Figure 30E & F), which is important for clotting and wound healing mechanisms. Whilst, INR is widely to have prognostic value in acute and chronic liver disease²⁰⁷ fibrinogen is not a routine marker for prognostication but has been shown to have prognostic value in chronic liver disease.²⁰⁸ The models of regression with INR and fibrinogen showed a strong association with methionine, ethanolamine and glutamine. Although our data shows methionine and ethanolamine correlate with prognostic markers, based on the literature and the data presented in this thesis it is unclear if glutamine has prognostic value.²⁰⁹

4.5.8 Semi quantified and monitored amino compounds

Peak area or fold change differences were compared when analysing data generated from semi-quantified amino compounds and the monitored compounds. The results from both sets of compounds showed the compound was not detectable in a number of samples which limited analysis and power of statistical testing. Hence this data was not subject to multivariate analysis as it would require imputation which would have complicated the analysis. Any differences observed across groups from the semi-quantified compounds will require further validation by rerunning samples and optimising the method to ensure the compounds are within the detection range.

Furthermore, some of the monitored compounds such as creatine and creatinine showed no change between groups which suggests the results from the monitored compound should be interpreted with a degree of caution as this result contradicts the clinical creatinine results. Also, this suggests that the ion peak used to detect creatinine is unstable and sensitive to changes in sample volume which are not accounted without the use of an internal standard.

4.6 Study Limitations

The main limitation was the dynamic range of the UPLC-MS method to quantify concentrations. Whereby, compounds such as glutamine and glycine which are significantly elevated in liver disease were above the upper limit of quantification, this was overcome by

diluting the samples and re-acquiring data for these particular metabolites. Whilst, compounds such as cystathionine and BAIBA were below the limits of quantification, however, these were only below the limits in a small number of samples and therefore did not affect the main trends observed in the reported concentrations.

4.7 Conclusions

The main findings from analysis of the plasma amino compound metabolic profiles acquired from this cohort was that the data can discriminate between ALF and ACLF, producing a valid OPLS-DA model of ALF vs ACLF using metabonomics for the first time. Furthermore, metabolic pathways that are discriminant for ALF vs ACLF are linked to many relevant mechanisms of liver failure linked to glutathione generation and antioxidant action of the liver, protein synthesis, muscle wasting and liver fibrosis/injury which are more severely perturbed in ALF compared to ACLF. Furthermore, the depletion of serine is unique to ACLF and may be linked to perturbation of the transsulfuration pathway or glycerophospholipid metabolism. Further investigation of these pathways by measuring metabolites and enzyme expression/activity is required. Similarly, ethanolamine and methionine appear to be prognostic and correlate with MELD, UKELD and Child Pugh scores.



Chapter 5

UNTARGETED UPLC-MS LIPID ANALYSIS

5.1 Introduction

The liver plays a central role in lipid metabolism, where fatty acid (FA) uptake, oxidation, esterification and secretion all occur.²¹⁰ A global reduction in circulating amounts of lipid such as cholesterol and triglycerides have been observed in numerous studies on acute and chronic liver disease.^{69,211,212} These effects are traditionally interpreted as an impairment of the liver's metabolic capacity for processes such as lipoprotein synthesis and formation of lipid droplets.²¹⁰ In addition, to being vital sources of energy and structural components of cell membranes, lipids also play an important role in cell signalling processes such as inflammation and cell death and research in this area is emerging.^{35,36}

Both ALF and ACLF are associated with mechanisms of cell death including apoptosis and necrosis, as well as, systemic immune dysfunction, which are believed to be the main mechanistic drivers of mortality in patients.^{8,41,47} Previous analysis of the plasma ¹H NMR spectroscopic data (see Chapter 3), found an overall depletion of resonances arising from fatty acid groups of lipids, including phosphatidylcholine, in both ALF and ACLF. Analysis of the ¹H NMR spectroscopic data identified broad spectral resonances arising from the major lipid classes but is unable to identify individual lipid characteristics, for example, chain length or degree of unsaturation. A previous study using both ¹H NMR spectroscopy and UPLC-MS to profile these samples identified lysophosphatidylcholines (LysoPCs) as the main lipid class driving change seen in survivors vs non-survivors with AD.¹⁵⁶ Based on these findings, an untargeted UPLC-MS lipid profiling method¹⁰² was applied to sera from the same cohort of patients metabotyped in Chapters 3 and 4. Data was acquired in both positive and negative ionisation modes and both sets of data were subjected to multivariate analysis. Furthermore, tandem mass spectrometry was applied following multivariate analysis, following selection of discriminant and high intensity features, to identify individual lipids. Metabolite identification was then performed on the positive mode data by checking fragmentation patterns from the MS/MS fragmentation data and comparing these to online databases. Following metabolite identification, the identified features were then analysed using a univariate approach. Metabolite identification of discriminant features from the negative mode UPLC-MS data is currently outstanding and MS/MS fragmentation data to aid identification have been acquired.

5.2 Aims and Objectives

The main aims of this study were to characterise class specific changes in lipid profiles in acute and chronic liver disease and identify any associations/correlation to the clinical metadata. A further aim was to apply multivariate modelling to identify lipids which correlated with survival at 30 and 90 days and 1-year post admission in acute and chronic liver disease.

5.3 Materials and Methods

5.3.1 Cohort characteristics

Serum samples from the cohort initially profiled by ^1H NMR, were analysed by untargeted UPLCMS in this chapter. The cohort phenotyped in this chapter are summarised in Table 2 in Chapter 3.

5.3.2. Untargeted UPLC-MS Lipid analysis

Sample preparation and data acquisition for UPLC-MS analysis of lipids from serum was performed according to the recently published NPC (National Phenome Centre) blood lipids standard operating procedure.¹⁰² A full explanation of the methods are outlined below.

5.3.2.1 Chemicals

All mobile phases were prepared with LC-MS grade solvents: water (Fischer Scientific, New Jersey, USA), HPLC grade isopropanol (IPA), LC-MS grade acetonitrile (ACN), ammonium acetate, acetic acid and phosphoric acid (the latter all from Sigma-Aldrich (Dorset, U.K.)). The 12 lipid standard mix run was made up of LysoPC(9:0) (855276P), PC(11:0) (850399P), C(17:0) (H3500), PG(15:0) (840446P), PE(15:0) (840446P), PS(17:0) (840028P), PA(17:0) (830856P), Cer(17:0) (860517P), DG(19:0) (68633), PC(23:0) (850372P), TG(15:0) (T4257) and TG(17:0) (T2151) (Avanti Polar Lipids, Alabaster, Alabama) which are selected to be representative of each lipid class found in human blood, these have odd number FA chains as they are less likely to overlap with endogenous lipids in the samples being analysed. Furthermore, lipid class mixtures which were analysed by subsequent MS/MS experiments were obtained from Avanti Polar Phospholipid mixes, which included soy PS mixture (870336), egg PG mixture (841138), egg PE mixture (841118), porcine brain ceramide mixture (860052P) and bovine milk SM

mixture (860063). Fragmentation data from these lipid mixtures were used to aid metabolite identification of features from the sample by comparing to fragments obtained from known lipid classes.

5.3.2.2 Sample preparation

Serum samples were thawed at 4°C and vortexed in batches according to plate number. An aliquot of 100µl per sample was added to each well of the lipid preparation plate (see Table 19 for the full plate plan) and an additional 10µl per sample was aliquoted into a falcon tube to make up pooled QC. The pooled QC was added to the lipid preparation plate in column 12 at 100µl per well (see Table 19).

In addition, 400µl of the 12 Lipid standard mix, dissolved in isopropanol (IPA), was added to each study and QC sample in the sample preparation plate. The lipid samples were prepared by protein precipitation using the IPA present in the lipid standard mix. Precipitation was carried out as follows: the samples were mixed with IPA (12 Lipid standard mix) using an Eppendorf MixMate for 2 hours at 1400rpm at 4°C, followed by centrifugation using RCF 3486 x g for 10 minutes at 4°C. After centrifugation 125µl of the supernatant (organic phase) was taken (carefully avoiding the protein pellet at the bottom) from each well of the preparation plate and added to the corresponding well of the analytical plate. For each lipid preparation plate three lipid analytical plates were prepared: two for analysis per ionisation mode (positive and negative) and one as a backup. The analytical plates were then heat-sealed using a heat seal foil, centrifuged at RCF 3486 x g for 5 minutes at 4°C and stored at -80°C until analysis.

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	S	S	S	S	S	S	S	S	S	S	Lipid mix	QC
B	S	S	S	S	S	S	S	S	S	S	Lipid mix	QC
C	S	S	S	S	S	S	S	S	S	S	Lipid mix	QC
D	S	S	S	S	S	S	S	S	S	S	Lipid mix	QC
E	S	S	S	S	S	S	S	S	S	S	Lipid mix	QC
F	S	S	S	S	S	S	S	S	S	S	Lipid mix	QC
G	S	S	S	S	S	S	S	S	S	S	Lipid mix	QC
H	S	S	S	S	S	S	S	S	S	S	Lipid mix	QC

Table 19. Lipid plate plan

Each plate consisted of 80 study samples and 8 pooled QCs and the 12-lipid standard mix which were injected every 10 samples throughout the run. S – study sample; QC – quality control;

Furthermore, the pooled QC was aliquoted into seven tubes and diluted to make up a dilution series spanning seven points (see Table 20 for details). The remaining QC serum was separated into 175µl aliquots and placed into eppendorfs, to be used for conditioning of the UPLC-MS instrument. The lipid standard mix was added to both the dilution series and QC for conditioning prior to instrument set up, 700µl was added per 175µl of QC, whilst the amounts for the dilution series are specified in Table 20. All eppendorfs were then centrifuged at 3486g for 10 minutes at 2-8°C. The supernatant (organic phase) was taken and transferred into 300µl glass recovery vials and sealed with foil lined caps.

Dilution Point	Percentage of SR (%)	Vol. of SR (µL)	Vol. of water (µL)	Total vol. (µL)	vol. in aliquot (µL)	Vol. of Lipid standard mixture (µL)
1	100	450	0	450	70	280
2	80	240	60	300	45	180
3	60	150	100	250	35	140
4	40	100	150	250	35	140
5	20	100	400	500	45	180
6	10	50	450	500	70	280
7	1	10	990	1000	70	280
Blank	0	0	990	990	70	280

Table 20. UPLC-MS QC dilution series

An eight-point dilution series was prepared from pooled QC serum ranging from 0 – 100% concentrations

5.3.2.3 Chromatography

Chromatographic analysis was performed using an Acquity UPLC system (Waters Ltd., Elstree, U.K.) via a C8 column (100mm × 2.1mm, 1.7µm; Waters Acquity, p/n:186002878) maintained at 55°C. Mobile phase A was comprised of water:IPA:ACN (2:1:1 v/v) containing 5mM ammonium acetate, 0.05% acetic acid and 20µM phosphoric acid. Mobile phase B was made up of IPA:ACN, containing 5mM ammonium acetate and 0.05% acetic acid. The flow rate used was 600µL/min. The injection volume per sample was 15µl and a 1µl sample loop was used for positive ionisation mode, whilst a 2µl sample loop was used for negative ionisation mode. The chromatographic gradient is shown in Table 21 and chromatograms acquired from QC injections are shown for both positive and negative ionisation modes in Figure 40.

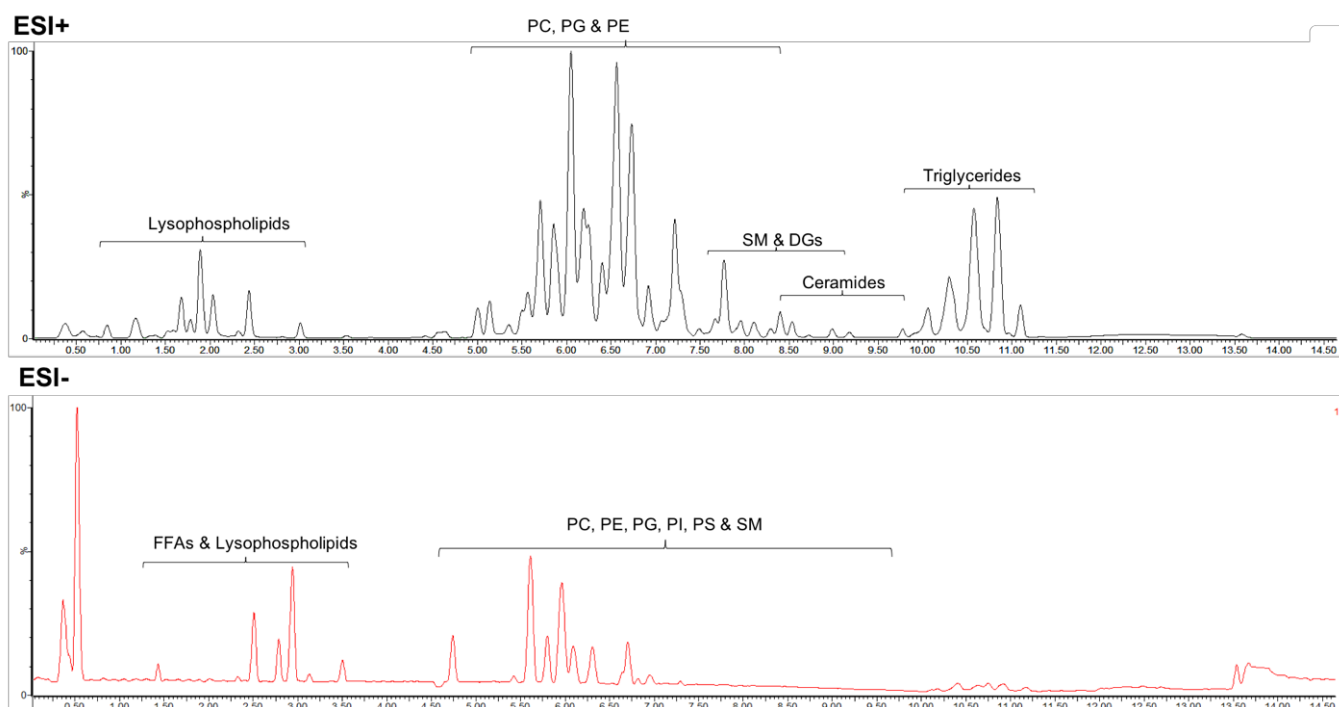


Figure 40. Base Peak Ion (BPI) chromatograms of QC serum in ESI+ and ESI- ionisation modes
 Abbreviations: FFA - free fatty acids; PC - phosphatidylcholine; PG - phosphatidylglycerol; PE - phosphatidylethanolamine; PI - phosphatidylinositol; PS - phosphatidylserine; SM - sphingomyelin; DG - diacylglycerol;

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.6	99.0	1.0	Initial
0.10	0.6	99.0	1.0	6
2.00	0.6	70.0	30.0	6
11.50	0.6	10.0	90.0	6
12.00	1.0	0.1	99.9	6
12.50	1.0	0.1	99.9	6
12.55	0.9	35.0	65.0	6
12.65	0.8	70.0	30.0	6
12.75	0.7	99.0	1.0	6
12.95	0.6	99.0	1.0	6
13.25	0.6	99.0	1.0	6

Table 21. Chromatographic gradient used for serum lipid analysis

5.3.2.4 Mass Spectrometry

After separation by UPLC, mass spectrometry was performed on a Xevo G2S Q-TOF (Waters, Manchester, U.K.), operated in positive and negative electrospray ionisation (ESI) modes. For subsequent MS/MS and MS^E experiments, mass spectrometry was performed on a Synapt Q-ToF, also operated in positive and negative ESI modes. The Synapt Q-ToF was used for subsequent MS/MS analyses, due to its greater sensitivity, allowing better detection of low

intensity lipids, as the lipids of interest were present at low intensities in the serum samples being analysed.

Positive and negative ESI parameters were as follows: capillary voltage was set at 2.0kV for positive mode and 1.5kV for negative mode, cone voltage at 40V, source temperature at 120°C, desolvation temperature at 600°C, desolvation gas flow at 1000L/h and the cone gas flow at 150L/h. Data for both positive and negative ESI were acquired in sensitivity mode, using a scan time of 0.1 seconds and an interscan time 0.014 seconds. Leucine enkephalin (m/z 556.2771 in positive mode, m/z 554.2615 in negative mode, 600pg/ μ l) was continuously infused at 15 μ L/min and used as a lock mass correction. Acquisition was performed from 50 – 2000 m/z in both positive and negative ESI, and the total acquisition time per injection was 14.65 minutes. MassLynx software version 4.1 was used for data acquisition. All data were acquired in centroid mode to accommodate data storage issues.

Prior to analysis of the study samples a number of blanks, conditioning injections, 5 start QC injections, the dilution series and 5 end QC injections were acquired (see Figure 41). The QC injections were also acquired in data dependent acquisition mode (DDA). This order was then repeated (excluding the DDA experiments) after all the analytical plates for each ionisation mode were analysed. See Chapter 2 for details on the purpose of instrument conditioning and sample blanks during MS set up.

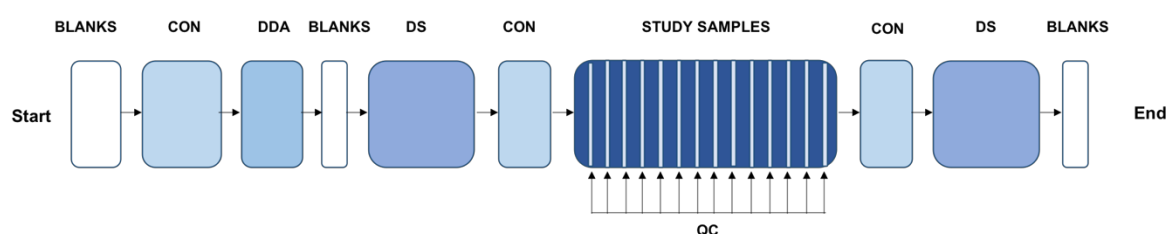


Figure 41. UPLC-MS lipid analytical run order

Schematic of the UPLC-MS lipid analysis run order. QCs were injected at regular intervals during the study sample analysis. Sample blanks injected were 100% water. Abbreviations: CON (QC conditioning); DDA (data dependent analysis); DS (dilution series);

5.3.2.5 MS Data processing

The Waters raw data files were converted to the netCDF format using the Databridge tool provided in the MassLynx 4.1 software (Waters, Milford, USA). Peak picking was then performed using the XCMS package¹⁰⁹ implemented in R²¹³ (v.3.2.5 using RStudio v.0.99.482). The centWave algorithm²¹⁴ was used for peak picking, whilst the peak grouping step was

performed using the density method. After obtaining a final feature list with XCMS, the data sets were then ran through the nPYc-Toolbox pipeline²¹⁵ for MS data quality control, feature filtering and batch correction.²¹⁶ Batch effect correction was performed by applying the LOESS based correction available in the nPYc-Toolbox. Briefly, for each feature a LOESS smoothing curve is fitted to the intensity values detected in the QC samples, and the LOESS fit is then interpolated to cover the whole run order. Each sample is then divided by the value estimated in the LOESS curve. Features where negative or zero values were obtained (typically caused by very low intensity features) were removed, as no batch correction is possible in that case. Whenever there are multiple separate batches (caused by stoppages in the data acquisition, for example), a separate LOESS curve is estimated for each batch. These are then corrected individually using their respective LOESS fits, and then each batch is aligned to the median of the batch medians. The LOESS window parameter was set to 11, the default value in the nPYc-Toolbox (meaning that the LOESS fit at each point is estimated from the adjacent 11 samples).

As part of the feature filtering process, all features whose relative standard deviation (RSD) calculated on the study samples was smaller than the one measured on the QC samples or whose RSD estimated from the latter was smaller than 30% were removed. Features whose Pearson correlation with dilution estimated in the dilution series was lower than 0.7 were also removed.²¹⁷ XCMS parameters were selected by manual analysis of the data. The parameters are shown in Table 22.

Parameter	Positive ESI mode	Negative ESI mode
ppm tolerance	20	15
Peak widths (s)	2-20	2-15
m/z tolerance	0.01	0.01
Intensity prefilter	8 scans, 6000 intensity	10 scans, 10000 intensity
S/N threshold (intensity)	600	600

Table 22. XCMS parameters for peak picking of ESI+ and ESI- data

For both positive and negative mode datasets, the LOESS batch effect correction method provided in the nPYc-Toolbox was applied to remove within-run intensity drift effects. In the case of the positive ionisation mode dataset, the correction was applied to two

independent batches (caused by an interruption of analytical data acquisition due to an instrument failure).

33,206 features were extracted with XCMS in the positive ion mode data. Furthermore, 6144 features were excluded following data filtering, leaving 27,062 features in the final dataset. A total of 40,565 peaks were obtained with XCMS for the negative mode data. After quality control and feature filtering through the nPYc-Toolbox, 17,971 features were excluded, leaving a remaining set of 27,062 features in the final dataset for subsequent modelling.

5.3.2.6 Tandem mass spectrometry

Tandem mass spectrometry and MS^E fragmentation data from QC and lipid mixes were acquired from a Synapt G2-S following initial multivariate modelling of UPLC-MS data. Data was acquired in resolution mode for both positive and negative ionisation modes using the same parameters outlined above for initial MS profiling. Tandem MS targeted both discriminant features identified by OPLS-DA modelling and also features with the highest intensity across the run.

5.3.3. Statistical Analyses

5.3.3.1 Multivariate modelling

Prior to multivariate analysis, the UPLC-MS positive and negative mode lipid data were log₁₀ transformed and mean-centred. Multivariate analysis of both positive and negative ESI data was performed using SIMCA P+ v14.2 (Umetrics, Umea, Sweden), as described previously for UPLC-MS amine data see Chapter 4.

5.3.3.2 Univariate statistical analysis

Univariate statistical analysis was performed on Log₁₀ transformed positive mode UPLC-MS intensity data, where a 1-way ANOVA was performed for class comparisons as previously described for ¹H NMR spectral data (see Chapter 3). Pairwise comparisons of survivors vs non-survivors were performed using student's t-test in Prism 7.0 (Graphpad, California, USA), and the p-values reported were subject to FDR correction as previously described (see Chapter 3).

5.3.3.3 Hierarchical cluster analysis

Hierarchical cluster analysis (HCA) was performed on the identified positive ESI lipids as previously described (see Chapter 3).

5.3.3.4 ROC analysis

ROC curves were computed from the predictive Y-variable generated by OPLS-DA analysis of survival from UPLC-MS data and compared to clinical scores using the Hanley McNeil method within MedCalc (v18.9) software.

5.3.4. Metabolite Identification

Discriminant features from OPLS-DA models, computed from positive mode UPLC-MS data, were chosen for metabolite identification, and were selected according to a VIP score > 2.0 and S-plot cutoffs including $p(1)$ and $p(\text{corr})$. Where $p(1)$ represents the covariance of variables is shown along the x-axis, where variables furthest horizontally from the zero value display the highest contribution towards the model and $p(\text{corr})$ the correlation coefficient of each feature with their predicted class is plotted along the y-axis ($p(\text{corr})[1]$), represents the reliability of that feature the most reliable being furthest vertically away from zero in the y-axis.

Features were further filtered according to retention time and feature intensity, where all features eluting before 0.45 seconds were excluded, as these overlap with the solvent front, and features with an intensity of <5000 were also excluded as these would be too low intensity for tandem mass spectrometry. Features were then re-ordered in order of importance according to VIP, $p(1)$ and $p(\text{corr})$ for metabolite identification. Each feature was then subject to a database search, according to m/z , using an “in-house tool” called CompoundDB which automates database searching of HMDB and Lipid Maps by ppm matching of MS adducts. These search results were then used to exclude any drugs (Table 23) and tentatively assign a lipid identity.

Drug	Drug class
Tazocin	Antibiotic
Meropenem	Antibiotic
Vancomycin	Antibiotic
Amikacin	Antibiotic
Linezolid	Antibiotic
Teicoplanin	Antibiotic
Clindamycin	Antibiotic
Cefalexin	Antibiotic
Co-amoxiclav	Antibiotic
Metronidazole	Antibiotic
Clarithromycin	Antibiotic
Ciprofloxacin	Antibiotic
Flucoxacin	Antibiotic
Norfloxacin	Antibiotic
Rifaximin	Antibiotic
Co-trimoxazole	Antibiotic
Fluconazole	Antibiotic
Anidulafungin	Antibiotic
Ambisome	Antibiotic
Amphotericin	Antibiotic
Ceftriaxone	Antibiotic
Noradrenaline	Sympathomimetic
Adrenaline	Sympathomimetic
Dobutamine	Sympathomimetic
Vasopressin	Vasoactive
Hydrocortisone	Steroid
Prednisolone	Steroid
Furosemide	Diuretic
Terlipressin	Vasopressor
Propofol	Anaesthetic
Fentanyl	Opioid
Midazolam	Sedative

Table 23. Documented medications administered to patients

A summary of drugs administered to patients in study cohort recorded from clinical metadata collected during patient recruitment.

Following tentative assignment of metabolite identifications, MS/MS fragmentation data, MS^E and DDA data were used to check tentative metabolite assignments. Fragmentation data was also compared to *in silico* MS/MS data in Metlin.²¹⁸

5.4 Results

5.4.1 Class comparisons by multivariate modelling

Initial unsupervised PCA modelling of the positive and negative ESI data included pooled QCs, HCs and patients sampled at day 1 of admission (See Figure 42). A PCA model computed from the positive mode data (4 component model, $R^2=0.57$; $Q^2=0.55$) shows tight clustering of the QC group and separation between HC and AD, ACLF and ALF groups in PC1 which accounts for 24% of the total variance (Figure 42A). For the negative ESI data, an initial PCA model revealed four outliers, the chromatography for these samples showed a sparse chromatogram indicating the injections had failed (data not shown). These four samples were then excluded, and a new PCA model was computed (4 component model, $R^2=0.62$; $Q^2=0.60$), which also showed tight clustering of the QC group, and separation between HC and AD, ACLF, ALI and ALF in PC1 which accounted for 38% of the total variance in the dataset (Figure 42B).

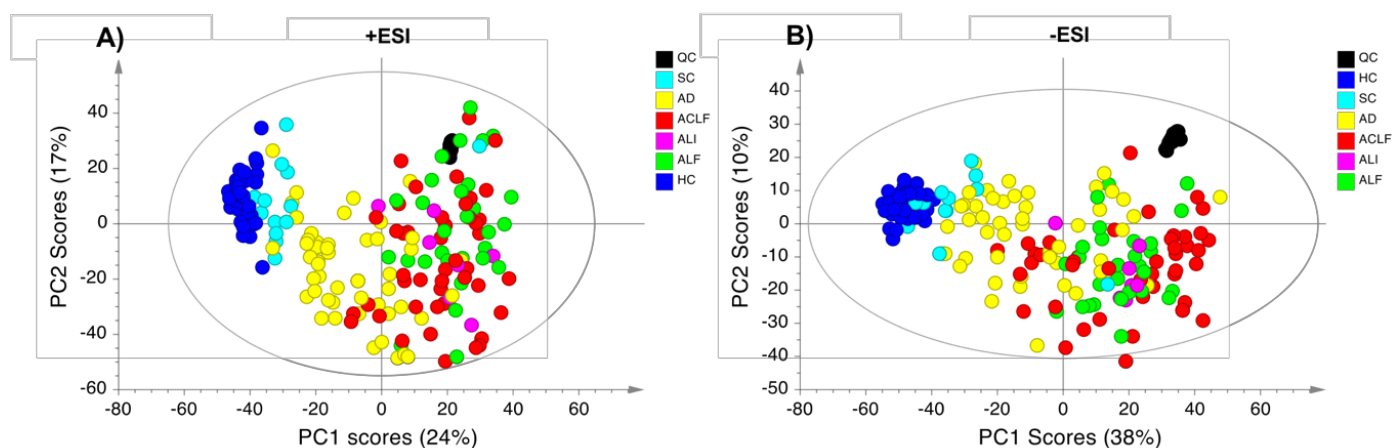


Figure 42. PCA modelling of QC and study samples

Unsupervised multivariate modelling of the UPLC-MS lipid data acquired in positive and negative ESI for all groups and QCs. PCA scores plots are shown. (A) 4 component PCA model computed from positive ESI data ($R^2=0.57$; $Q^2=0.55$). (B) 4 component PCA model from negative ESI data ($R^2=0.62$; $Q^2=0.60$) with 4 outliers removed due to poor chromatographic data quality.

PCA modelling was also performed separately on the ALF and cirrhosis group data (Figure 43). PCA modelling of the chronic groups revealed a clear separation between them in PC1 for the positive ESI data (Figure 43A, 4 component model, cumulative $R^2=0.56$; $Q^2=0.52$) and the negative ESI data (Figure 43B, 4 component model, cumulative $R^2=0.60$; $Q^2=0.56$). The

separation between the groups in PC1 is in order of severity of disease beginning with HC, followed by SC, AD and ACLF.

PCA modelling of HC and ALF for both positive and negative ESI data, revealed a distinct separation between HC and ALF for both positive (Figure 43C, 2 component model, cumulative $R^2=0.59$; $Q^2=0.52$) and negative mode data (Figure 43D, 2 component model, cumulative $R^2=0.59$; $Q^2=0.52$).

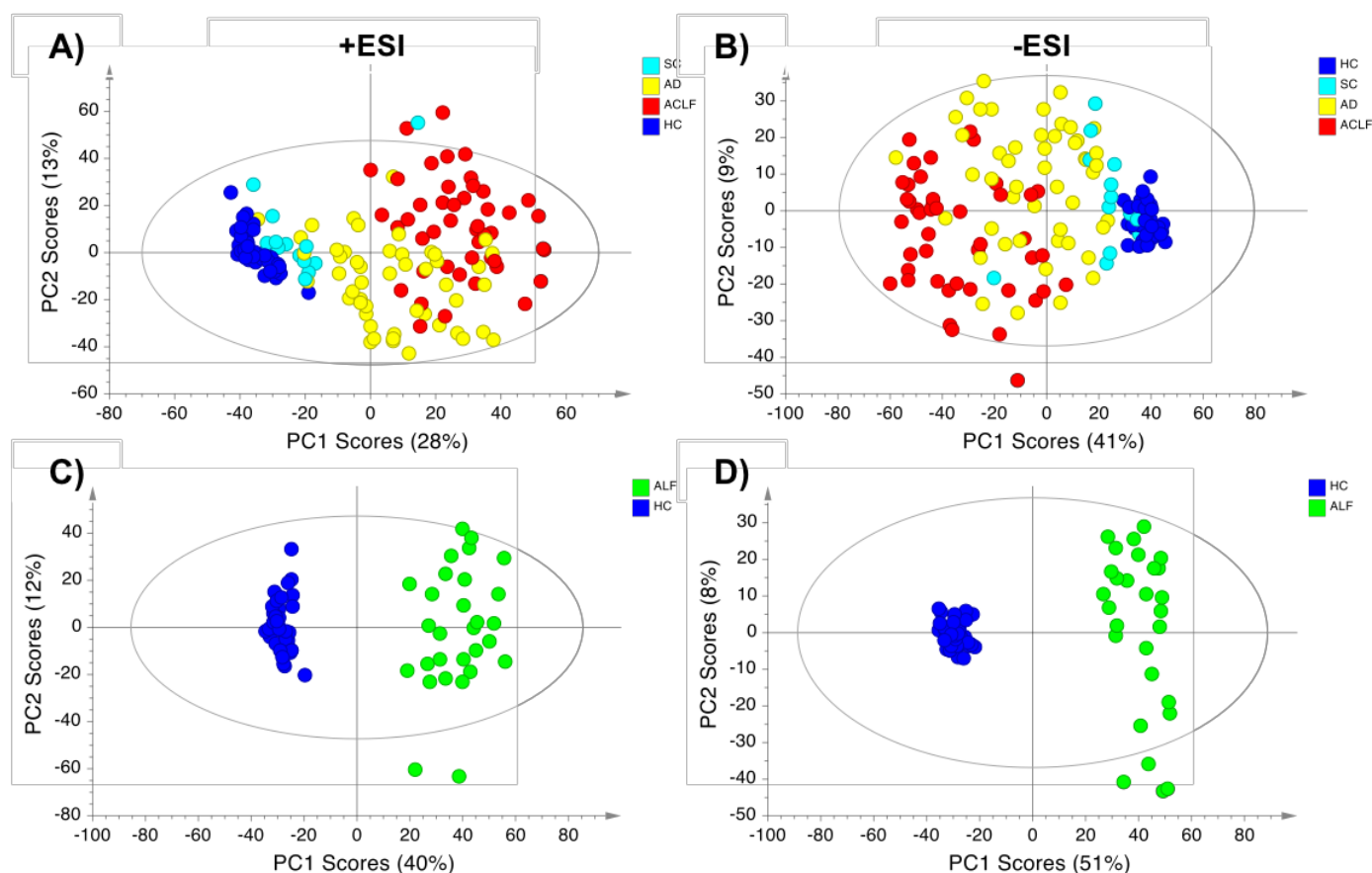


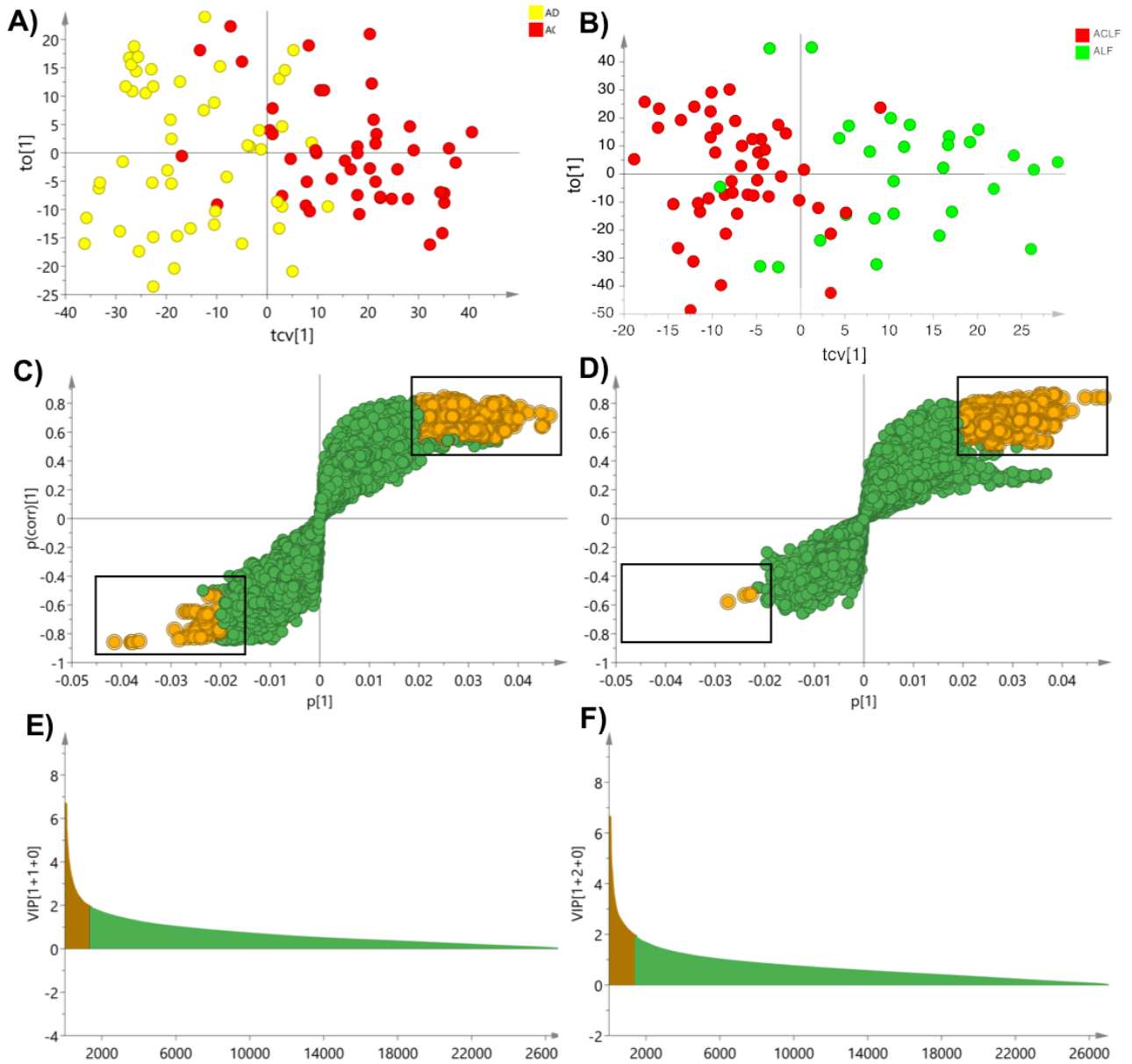
Figure 43. PCA modelling of ALF and Cirrhosis

Unsupervised multivariate modelling of the UPLC-MS lipid data acquired in positive and negative ESI for patients with ALF and cirrhosis (A-D). PCA scores plots (A-D) show clustering of the data and reveal separation between groups in PC1. PCA modelling of positive ionisation ESI data shown in A & C and PCA modelling of negative ESI data shown in B & D. 4 component models of HC, SC, AD & ACLF for positive ESI data (A), cumulative $R^2=0.56$; $Q^2=0.52$ and negative ESI data (B), cumulative $R^2=0.60$; $Q^2=0.56$. 2 component models of HC and ALF for positive ESI data (C), cumulative $R^2=0.59$; $Q^2=0.52$ and negative ESI data (D), cumulative $R^2=0.59$; $Q^2=0.52$.

To better understand discrimination between AD and ACLF and ALF and ACLF, OPLS-DA models (Figure 44) were computed from positive ESI data for AD vs ACLF ($R^2Y=0.71$, $Q^2Y=0.56$, CV-ANOVA p-value <0.0001), and ACLF vs ALF ($R^2Y=0.79$, $Q^2Y=0.58$, CV-ANOVA p-value <0.0001), where the cross-validated scores plots showed separation between the two classes (see Figure 44A and B respectively). Furthermore, the equivalent models were also computed

from the negative ESI data for AD vs ACLF ($R^2Y=0.63$, $Q^2Y=0.54$, CV-ANOVA p-value <0.0001) and ALF vs ACLF ($R^2Y=0.74$, $Q^2Y=0.60$, CV-ANOVA p-value <0.0001) and the cross-validated scores plots for these models also showed separation between the two classes (see Figure 44G and H respectively). The corresponding S-plot loadings for these models were used to identify the most discriminant metabolites (see Figure 44C, D, I and J, discriminant features are highlighted in orange) from these models using the cutoff: $p(1) < -0.02$ & > 0.02 ; $p(\text{corr}) < -0.7$ & > 0.7 . Furthermore, VIP plots (see Figure 44E, F, K and L, discriminant features are highlighted in orange) were also used to identify the metabolites most important to the ionisation mode by using a VIP score cutoff of >2.00 . Metabolites were then prioritised for identification based on both of these cutoffs.

+ESI



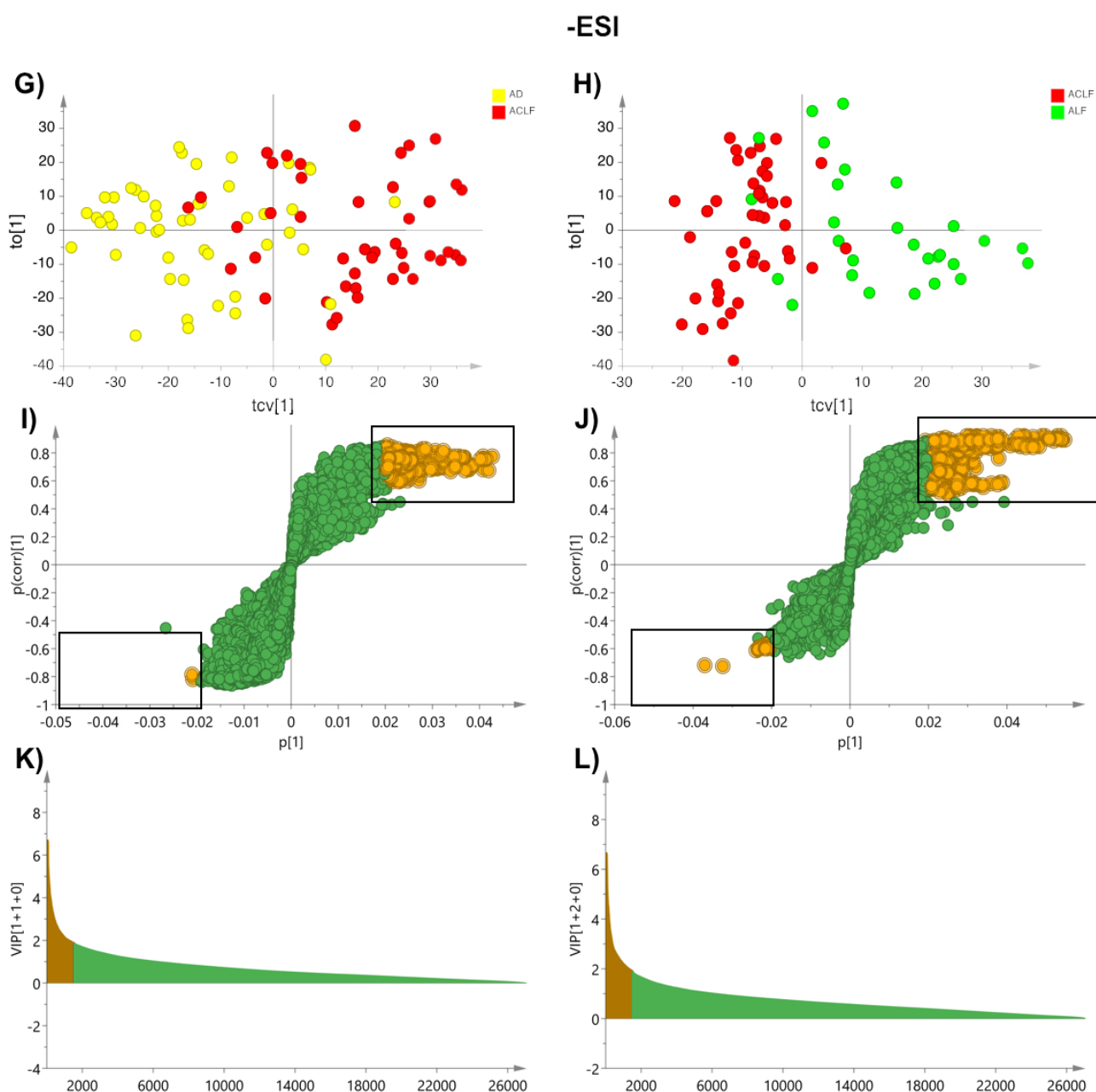


Figure 44. OPLS-DA comparing AD vs ACLF and ALF vs ACLF

Discriminant analysis of the UPLC-MS lipid data performed by OPLS-DA demonstrated discrimination between AD vs ACLF and ALF vs ACLF. OPLS-DA scores plots (A, B, G & H), S-plot loadings (C, D, I & J) showing p_1 vs $p[corr]$ and VIP plots (E, F, K and L) are shown, all discriminant features from the S-plots and VIP plots are highlighted in orange. All OPLS-DA models have 1-Y orthogonal component and 1-Y predictive component. See Table 24 for full model statistics.

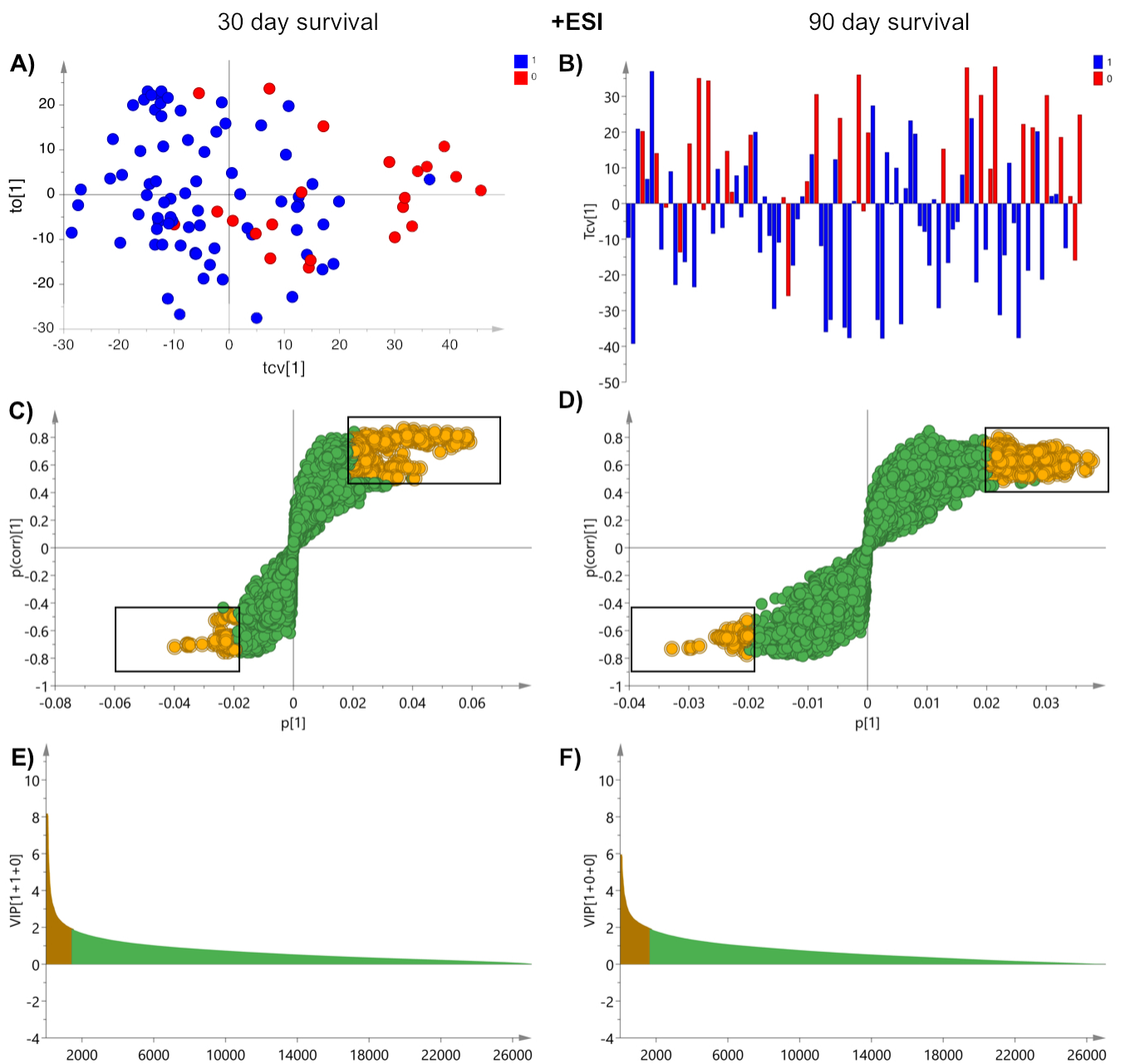
5.4.2 Survival modelling

Multivariate modelling using OPLS-DA was performed comparing survival at 30 and 90 days and 1-year post admission in acute and chronic liver disease. All analysis performed on ALF and ALI groups produced invalid models with low or negative Q^2Y values for 30, 90 and 1-year post admission (data not shown). In contrast, for the cirrhosis groups (AD and ACLF) valid

models were computed for survival at 30- and 90-days post admission from both positive and negative ESI lipid data see Figure 45.

Models computed for survival at 30 days: from positive ($R^2Y=0.56$; $Q^2Y=0.35$) and negative ESI data ($R^2Y=0.50$; $Q^2Y=0.35$) showed separation between survivors and non-survivors in the cross-validated scores (see Figure 45A & G). Whilst, models computed for survival at 90 days: from positive ($R^2Y=0.33$; $Q^2Y=0.23$) and negative ESI data ($R^2Y=0.45$; $Q^2Y=0.26$) showed some separation between survivors and non-survivors in the cross-validated scores (see Figure 45B & H), overall, there was less separation between survivors and non-survivors compared to models computed for 30 days.

Discriminant features were identified from the OPLS-DA S-plots (see Figure 45C, D, I and J) and VIP plots (see Figure 45E, F, K and L) using the same cutoffs as used for models in section 5.4.1: ($p(1) < -0.02$ & > 0.02 ; $p(\text{corr}) < -0.7$ & > 0.7) and VIP score > 2.00 .



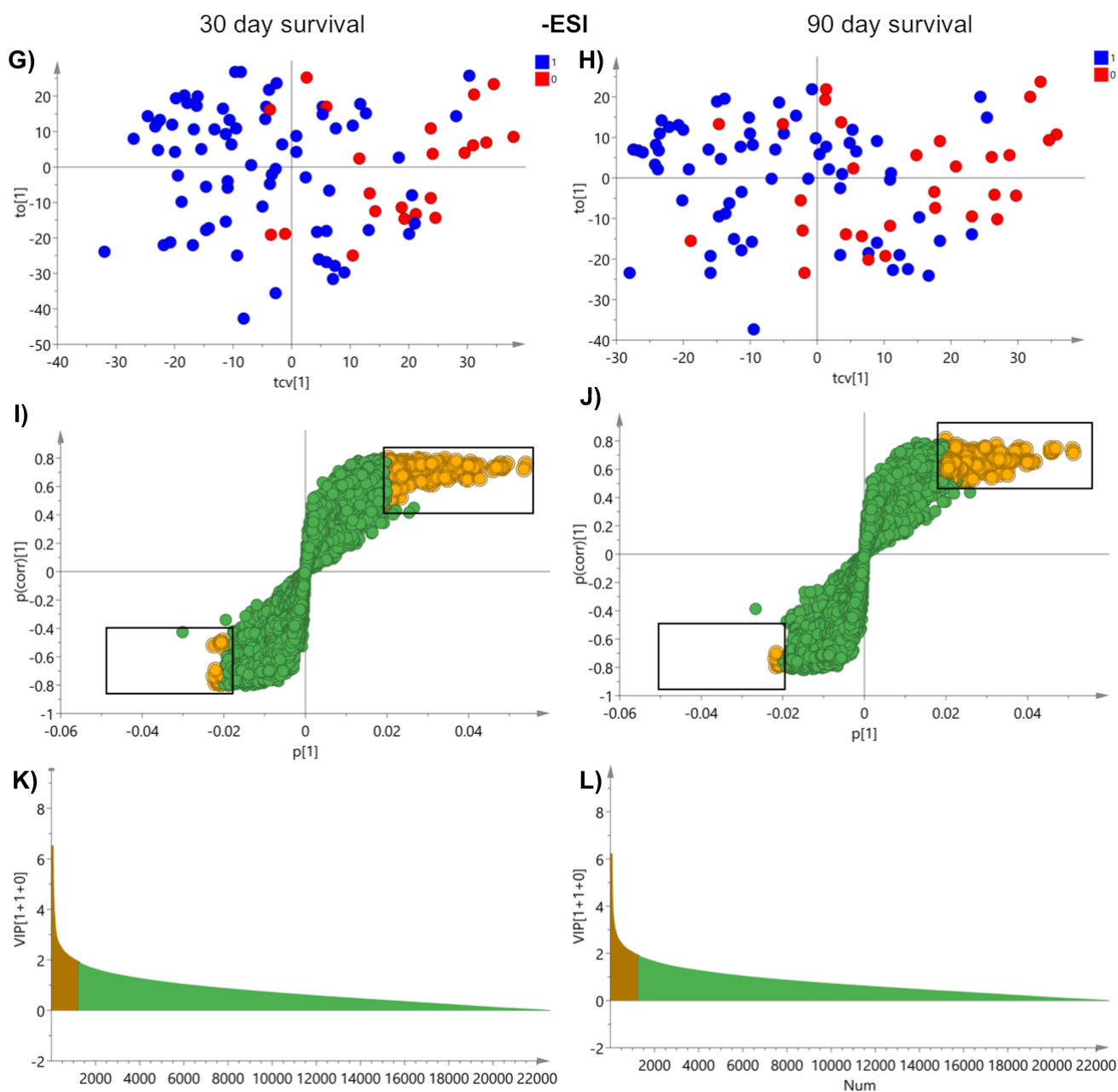


Figure 45. OPLS-DA analysis of survival at 30 and 90 days for AD and ACLF

Discriminant analysis of the UPLC-MS lipid data performed by OPLS-DA & PLS-DA demonstrated discrimination between survivors (blue scores) and non-survivors (red scores). Cross validated-scores plots (A, B, G & H), S-plot loadings (C, D, I & J) showing p_1 vs $p(\text{corr})$ and VIP plots (E, F, K and L) are shown, all discriminant features from the S-plots and VIP plots are highlighted in orange. All OPLS-DA models have 1-Y orthogonal component and 1-Y predictive component, whilst the PLS-DA model (B, D & F) has 1-Y predictive component. See Table 24 for full model statistics.

Ionisation mode	Figure ID	No. Components	Comparison	R ² Y	Q ² Y	Permutation p-value
ESI+	4 (A, C, E)	1 Y-orth & 1 Y-pred	AD vs ACLF	0.71	0.56	<0.0001
ESI+	4 (B, D, F)	1 Y-orth & 1 Y-pred	ALF vs ACLF	0.79	0.58	<0.0001
ESI-	4 (G, I, K)	1 Y-orth & 1 Y-pred	AD vs ACLF	0.63	0.54	<0.0001
ESI-	4 (H, J, L)	1 Y-orth & 1 Y-pred	ALF vs ACLF	0.74	0.60	<0.0001
ESI+	5 (A, C, E)	1 Y-orth & 1 Y-pred	30-day survival	0.56	0.35	<0.0001
ESI+	5 (B, D, F)	1 Y-pred	90-day survival	0.33	0.23	<0.0001
ESI-	5 (G, I, K)	1 Y-orth & 1 Y-pred	30-day survival	0.50	0.35	<0.0001
ESI-	5 (H, J, L)	1 Y-orth & 1 Y-pred	90-day survival	0.45	0.26	<0.0001

Table 24. Summary of PLS-DA models computed

The model statistics for all PLS-DA models of class comparisons (Figure 44) and 30 and 90 day survival analysis (Figure 45) are summarised. Y-orth: (orthogonal component); Y-pred: (predictive component);

5.4.3 Comparison of survival predictability of UPLC-MS lipid data against standard clinical scores

AUROC analysis was performed on the Y-predicted components from the OPLS-DA models of survival at 30 and 90 days shown in Figure 46, and compared to clinical scores Child-Pugh, CLIF-SOFA, MELD and UKELD and summarised in Table 25.

For survival up to 30 days (Figure 46A) the UPLC-MS positive and negative ESI lipid data demonstrated a higher AUC (0.96 and 0.94 respectively) (Table 25) compared to Child Pugh ($p=0.001$ and $p=0.004$), CLIF-SOFA ($p=0.012$ and $p=0.044$), MELD ($p=0.011$ and $p=0.032$) and UKELD ($p<0.0001$ and $p<0.0001$) scores, see Table 26. All pairwise comparisons of all AUROC analyses at 30 and 90 days are summarised in Table 26. Comparisons of the AUROC analyses between clinical scores revealed that UKELD had a significantly lower AUC compared to Child-Pugh ($p=0.027$), CLIF-SOFA ($p=0.001$) and MELD ($p=0.001$).

For survival at 90 days (Figure 46B) the AUC for UPLC-MS lipid positive and negative ESI data (AUC=0.85 and AUC=0.90 respectively) was higher than the AUC for UKELD score (AUC=0.68; $p=0.035$ and $p=0.003$) (Table 26). Furthermore, the AUC for the UPLC-MS negative ESI lipid data was significantly higher than the AUC for the CLIF-SOFA score at 90 days (AUC=0.82; $p=0.028$) (See Table 25 & 26). Comparisons of the UPLC-MS lipid AUROC with MELD, CLIF-SOFA and Child-Pugh scores did not show any significant differences. Whilst comparison between clinical scores, revealed that the AUC for MELD was significantly higher compared to UKELD ($p=0.012$) at 90 days post-admission (Table 26).

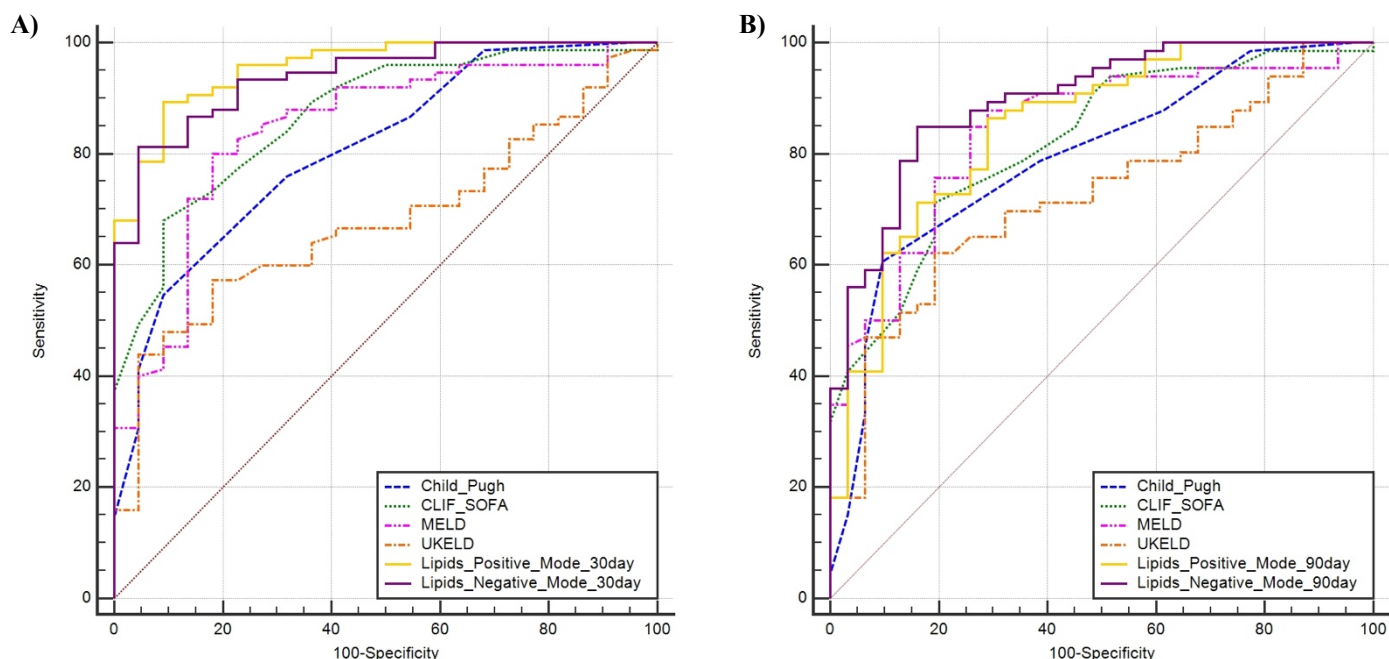


Figure 46. Comparisons of survival prediction at 30 days and 90 days post admission by Area Under Receiver Operating Curve (AUROC) analysis

AUROC comparisons of Y-predicted (survival) from UPLC-MS positive ESI and negative ESI lipid data, Child-Pugh Score, CLIF-SOFA Score, MELD Score, UKELD Score. (A) survival at 30 days (B) survival at 90 days

Survival	Variable	AUROC	95% CI	Sensitivity	Specificity
30-day survival	Child_Pugh	0.808	0.716 to 0.881	55%	91%
	CLIF_SOFA	0.870	0.786 to 0.930	68%	96%
	MELD	0.843	0.755 to 0.909	80%	91%
	UKELD	0.679	0.576 to 0.770	44%	91%
	Lipids_Positive_Mode_30day	0.956	0.894 to 0.987	89%	82%
	Lipids_Negative_Mode_30day	0.939	0.872 to 0.978	81%	96%
90-day survival	Child_Pugh	0.793	0.699 to 0.869	61%	90%
	CLIF_SOFA	0.820	0.729 to 0.891	71%	81%
	MELD	0.841	0.752 to 0.907	85%	74%
	UKELD	0.725	0.625 to 0.811	62%	81%
	Lipids_Positive_Mode_30day	0.848	0.760 to 0.912	90%	68%
	Lipids_Negative_Mode_30day	0.896	0.817 to 0.949	85%	84%

Table 25. Summary of AUROC survival analyses at 30- and 90-days post admission

The statistics for all AUROC curves plotted in Figure 46 are summarised here.

Survival	Comparison	Difference between areas	95% CI	P-value
30-day survival	Child-Pugh vs CLIF-SOFA	0.06	-0.03-0.15	0.1636
	Child-Pugh vs MELD	0.04	-0.06-0.12	0.4507

	Child-Pugh vs UKELD	0.13	0.02-0.24	0.0272
	Child-Pugh vs Lipids Positive mode	0.15	0.06-0.24	0.0010
	Child-Pugh vs Lipids Negative mode	0.13	0.04-0.22	0.0036
	CLIF-SOFA vs MELD	0.03	-0.05-0.10	0.4800
	CLIF-SOFA vs UKELD	0.19	0.08-0.30	0.0009
	CLIF-SOFA vs Lipids Positive mode	0.09	0.02-0.15	0.0117
	CLIF-SOFA vs Lipids Negative mode	0.07	0.02-0.14	0.0439
	MELD vs UKELD	0.16	0.71-0.26	0.0006
	MELD vs Lipids Positive mode	0.11	0.03-0.20	0.0112
	MELD vs Lipids Negative mode	0.10	0.09-0.19	0.0318
	UKELD vs Lipids Positive mode	0.28	0.17-0.39	<0.0001
	UKELD vs Lipids Negative mode	0.26	0.15-0.37	<0.0001
	Lipids Positive mode vs Lipids Negative mode	0.02	-0.02-0.05	0.3605
90-day survival	Child-Pugh vs CLIF-SOFA	0.03	-0.06-0.11	0.5267
	Child-Pugh vs MELD	0.05	-0.03-0.13	0.2538
	Child-Pugh vs UKELD	0.07	-0.04-0.18	0.2062
	Child-Pugh vs Lipids Positive mode	0.05	-0.03-0.14	0.2095
	Child-Pugh vs Lipids Negative mode	0.10	0.02-0.19	0.0183
	CLIF-SOFA vs MELD	0.02	-0.05-0.09	0.5625
	CLIF-SOFA vs UKELD	0.10	-0.02-0.21	0.1036
	CLIF-SOFA vs Lipids Positive mode	0.03	-0.04-0.10	0.4487
	CLIF-SOFA vs Lipids Negative mode	0.08	0.01-0.14	0.0276
	MELD vs UKELD	0.12	0.03-0.21	0.0120
	MELD vs Lipids Positive mode	0.01	-0.07-0.10	0.8603
	MELD vs Lipids Negative mode	0.06	-0.02-0.13	0.1408
	UKELD vs Lipids Positive mode	0.12	0.01-0.24	0.0345
	UKELD vs Lipids Negative mode	0.17	0.06-0.28	0.0028
	Lipids Positive mode vs Lipids Negative mode	0.05	-0.01-0.10	0.0860

Table 26. Pairwise comparisons of AUROC analyses

Pairwise comparisons were performed using the Hanley and McNeil method and are summarised. A significance threshold of $p < 0.05$ was used to determine significant differences between AUROCs and are highlighted in green.

5.4.4 PLS regression with metadata

The ESI+ and ESI- UPLC-MS lipid data was regressed with the clinical metadata using a multivariate approach by PLS. Metadata was regressed with ESI+/- data from SC, AD, ACLF, ALI and ALF groups to identify metabolites associations. The ESI+/- data was initially regressed against demographic data including age, gender, BMI to identify confounders, which produced invalid models based on R^2Y and Q^2Y values (data not shown). Furthermore, PLS-regression of positive and negative ESI data with clinical scores, used for prognostication of cirrhosis such as Child-Pugh, CLIF-SOFA and UKELD score, was performed on SC, AD and ACLF groups only.

A summary of all valid PLS regression models computed from the UPLC-MS positive ESI data are summarised in Table 27 and models computed from negative ESI data are summarised in Table 28. PLS-regression of the positive ESI data with cytokines produced valid models with IL-12p70 ($R^2Y=0.36$; $Q^2Y=0.28$), IL-13 ($R^2Y=0.32$; $Q^2Y=0.21$), IL-1 β ($R^2Y=0.37$; $Q^2Y=0.26$), IL-2 ($R^2Y=0.37$; $Q^2Y=0.26$), in contrast to the ^1H NMR spectroscopic and UPLC-MS amine data.

Classes	N=	Y- variable	Components	R ² Y	Q ² Y	CV-ANOVA p-value
HC+SC+AD+ACLF+ALI+ALF	173	IL-12p70	2	0.36	0.28	3.26E-010
HC+SC+AD+ACLF+ALI+ALF	172	IL-13	2	0.32	0.21	4.53E-008
HC+SC+AD+ACLF+ALI+ALF	172	IL-1 β	2	0.37	0.26	5.75E-010
HC+SC+AD+ACLF+ALI+ALF	173	IL-2	2	0.37	0.26	2.82E-010
SC+AD+ACLF+ALI+ALF	146	FiO ₂	2	0.52	0.28	7.57E-010
SC+AD+ACLF+ALI+ALF	146	GCS	2	0.74	0.65	2.85E-031
SC+AD+ACLF+ALI+ALF	145	HE grade	2	0.56	0.38	7.25E-014
SC+AD+ACLF+ALI+ALF	144	INR	2	0.62	0.49	2.05E-019
SC+AD+ACLF+ALI+ALF	86	Fibrinogen	2	0.58	0.39	4.15E-008
SC+AD+ACLF+ALI+ALF	146	Bilirubin	2	0.64	0.51	1.67E-020
SC+AD+ACLF+ALI+ALF	124	Conjugated Bilirubin	2	0.62	0.43	2.50E-013
SC+AD+ACLF+ALI+ALF	146	AST	2	0.64	0.48	3.31E-018
SC+AD+ACLF+ALI+ALF	106	ALT	2	0.77	0.64	9.85E-021
SC+AD+ACLF+ALI+ALF	113	Cholesterol	2	0.68	0.54	4.97E-018
SC+AD+ACLF+ALI+ALF	113	Triglycerides	2	0.54	0.29	3.66E-006
SC+AD+ACLF+ALI+ALF	129	Lactate	2	0.55	0.31	1.98E-009
SC+AD+ACLF+ALI+ALF	145	MELD	2	0.76	0.66	1.34E-032
SC+AD+ACLF+ALI+ALF	144	SOFA	2	0.64	0.42	1.38E-012
SC+AD+ACLF	110	Child-Pugh	2	0.69	0.52	4.82E-016
SC+AD+ACLF	110	CLIF-SOFA	2	0.66	0.49	2.51E-013
SC+AD+ACLF	110	UKELD	2	0.66	0.52	8.49E-016

Table 27. Summary of PLS regression models from positive ESI UPLC-MS lipid data

N= number of patients; No components – number of Y-predictive components; GCS – Glasgow coma scale; HE – hepatic encephalopathy; INR – international normalised ratio; AST – aspartate transaminase; ALT – alanine aminotransferase; MELD -model for end stage liver disease; SOFA – sequential organ failure assessment; CLIF-SOFA – chronic liver failure- sequential organ failure assessment; UKELD – united kingdom model for end stage liver disease;

Classes	N=	Y- variable	Components	R ² Y	Q ² Y	CV-ANOVA p-value
HC+SC+AD+ACLF+ALI+ALF	142	FiO ₂	2	0.44	0.28	2.54E-009
HC+SC+AD+ACLF+ALI+ALF	142	GCS	3	0.74	0.60	2.31E-024
HC+SC+AD+ACLF+ALI+ALF	141	HE grade	3	0.60	0.41	9.58E-013
SC+AD+ACLF+ALI+ALF	117	CRP	2	0.60	0.45	4.19E-014
SC+AD+ACLF+ALI+ALF	142	Neutrophil	1	0.30	0.25	3.39E-009

SC+AD+ACLF+ALI+ALF	141	INR	2	0.58	0.50	1.23E-019
SC+AD+ACLF+ALI+ALF	84	Fibrinogen	1	0.56	0.38	3.68E-009
SC+AD+ACLF+ALI+ALF	142	Creatinine	3	0.60	0.41	9.23E-014
SC+AD+ACLF+ALI+ALF	142	Bilirubin	2	0.62	0.55	6.53E-023
SC+AD+ACLF+ALI+ALF	120	Conjugated Bilirubin	2	0.59	0.48	7.86E-016
SC+AD+ACLF+ALI+ALF	142	AST	2	0.59	0.49	4.53E-019
SC+AD+ACLF+ALI+ALF	102	ALT	2	0.71	0.67	3.05E-022
SC+AD+ACLF+ALI+ALF	142	Total Protein	2	0.43	0.31	1.90E-010
SC+AD+ACLF+ALI+ALF	110	Cholesterol	2	0.65	0.54	5.77E-017
SC+AD+ACLF+ALI+ALF	127	Lactate	2	0.44	0.32	7.24E-010
SC+AD+ACLF+ALI+ALF	141	MELD	2	0.75	0.71	9.03E-036
SC+AD+ACLF+ALI+ALF	141	SOFA	2	0.62	0.51	3.61E-020
SC+AD+ACLF	107	UKELD	2	0.67	0.57	2.75E-018
SC+AD+ACLF	107	Child Pugh Score	2	0.65	0.54	1.49E-016
SC+AD+ACLF	107	CLIF-SOFA	2	0.62	0.52	1.30E-015

Table 28. Summary of PLS regression models from negative ESI- UPLC-MS lipid data

See Table 8 for abbreviations. Additional abbreviation includes CRP – C-reactive protein;

5.4.5 Metabolite ID from ESI+ UPLC-MS data

Following multivariate analysis, 34 discriminating features (See Table 29) selected from S-plots and VIP plots for AD vs ACLF and ALF vs ACLF were subjected to tandem mass spectrometry. Furthermore, 70 features with the highest overall intensities were also subjected to tandem mass spectrometry (see Table 30). Fragmentation data generated by tandem mass spectrometry was used to confirm the tentative IDs matched to database searches. The identity of 4 of the discriminating features (LysoPC(14:0) [M+H]⁺, LysoPC(18:1) [M+H+H₂O]⁺, LysoPC(16:1) [M+H+H₂O]⁺ at 1.52 minutes and 0.74 minutes see (Table 29) and 37 high intensity features were confirmed by tandem mass spectrometry (Table 30). The remaining 30 discriminating features and 35 high intensity features were either not successfully fragmented or the fragments generated were not identifiable and require further investigation or are unidentifiable from existing MS/MS data. See Chapter 2 for further detail on metabolite identification from tandem mass spectrometry data. Univariate analysis was subsequently employed to confirm class-specific differences from discriminant features identified from multivariate OPLS-DA models and analyse for differences across classes for high intensity features.

Retention Time (mins)	m/z	Putative ID	Adduct
0.69	450.2	no ID match	
0.73	457.334	LPA(18:0)	[M+CH ₃ OH+H] ⁺

0.74	512.334	LysoPC(16:1)	[M+H+H ₂ O] ⁺
0.76	589.328	PA(12:0/13:0)	[M+K] ⁺
0.78	576.361	no ID match	
1.07	540.365	LysoPC(18:1)	[M+H-H ₂ O] ⁺
1.30	341.186	EPA	[M+K] ⁺
1.30	626.308	Leukotriene C ₄	[M+H] ⁺
1.38	468.308	LysoPC(14:0)	[M+H] ⁺
1.52	512.335	LysoPC(16:1)	[M+H+H ₂ O] ⁺
1.89	991.674	Thyroxine glucuronide	[M+K] ⁺
4.61	328.257	no ID match	
4.61	494.354	no ID match	
4.61	495.357	no ID match	
4.61	509.325	Bassic acid	[M+Na] ⁺
4.62	488.395	2,3-bis(Acetyloxy)propyl icosanoate	[M+NH ₄] ⁺
5.35	355.284	MG(18:2)	[M+H] ⁺
5.36	327.253	no ID match	
5.36	328.257	Clavepictine B	[M+Na] ⁺
5.36	522.385	no ID match	
5.36	523.387	no ID match	
5.36	537.356	PA(12:0/12:0)	[M+H] ⁺
5.37	523.382	LysoPC(P-18:1)	[M+NH ₄] ⁺
6.08	355.284	MG(18:2)	[M+H] ⁺
6.08	545.461	DG(14:0/18:3)	[M+H-H ₂ O] ⁺
6.18	599.505	DG(18:2/18:2)	[M+H-H ₂ O] ⁺
6.18	740.524	PE(16:0/20:4)	[M+H] ⁺
6.18	741.528	no ID match	
6.18	742.528	PE(18:1/18:2)	[M+H] ⁺
6.19	716.526	PE(16:0/18:2)	[M+H] ⁺
6.19	718.531	PE(16:0/18:1)	[M+H] ⁺

Table 29. Discriminant features subjected to tandem mass spectrometry

Features that were identified from fragmentation data are highlighted in green.

Retention Time (mins)	m/z	Putative ID	Adduct
0.40	235.223	myristic acid	[M+Li] ⁺
0.88	415.211	no ID match	
0.88	473.263	Chenodeoxycholic acid 3-sulfate	[M+H] ⁺
1.30	585.269	Bilirubin	[M+H] ⁺
1.30	299.138	no ID match	
1.53	433.232	LPA(16:0)	[M+Na] ⁺
1.68	520.340	LysoPC(18:2)	[M+H] ⁺
1.76	544.339	LysoPC(20:4)	[M+H] ⁺
1.89	496.341	LysoPC(16:0)	[M+H] ⁺
2.13	522.356	LysoPC(18:1)	[M+H] ⁺
2.42	524.372	LysoPC(18:0)	[M+H] ⁺
3.13	594.413	LysoPC(22:2)	[M+H+H ₂ O] ⁺
3.17	354.409	1-Tetracosene	[M+NH ₄] ⁺
3.26	430.380	no ID match	
3.41	410.472	no ID match	
3.65	413.266	Nutriacholic acid	[M+Na] ⁺
3.80	385.245	no ID match	
4.70	396.352	N-oleoyl isoleucine	[M+H] ⁺
4.75	509.325	polyphenol-type compound	[M+Na] ⁺
4.75	488.395	2,3-bis(Acetyloxy)propyl icosanoate	[M+NH ₄] ⁺
4.77	327.253	HETE	[M+Li] ⁺

4.96	523.472	DG(14:0/16:0)	[M+H-H ₂ O] ⁺
4.96	717.467	no ID match	
5.20	675.543	SM(d18:1/14:0)	[M+H] ⁺
5.33	701.559	SM(d16:1/18:1)	[M+H] ⁺
5.53	516.426	no ID match	
5.53	730.538	PC(16:1/16:1)	[M+H] ⁺
5.76	780.555	PC(16:0/20:5)	[M+H] ⁺
5.76	756.554	PC(16:0/18:3)	[M+H] ⁺
5.92	703.575	SM(d18:1/16:0)	[M+H] ⁺
5.92	782.569	PC(18:2/18:2)	[M+H] ⁺
6.05	806.570	PC(16:0/22:6)	[M+H] ⁺
6.09	732.553	PC(16:0/16:1)	[M+H] ⁺
6.24	758.575	PC(16:0/18:2)	[M+H] ⁺
6.24	808.584	PC(18:1/20:4)	[M+H] ⁺
6.30	740.524	PE(16:0/20:4)	[M+H] ⁺
6.38	784.585	PC(16:0/20:3)	[M+H] ⁺
6.38	740.522	PE(18:2/18:2)	[M+H] ⁺
6.45	784.585	PC(16:0/20:3)	[M+H] ⁺
6.45	806.566	PC(18:1/20:5)	[M+H] ⁺
6.60	734.569	PC(14:0/18:0)	[M+H] ⁺
6.60	742.574	PC(P-16:0/18:2)	[M+H] ⁺
6.76	794.605	PC(P-18:1/20:3)	[M+H] ⁺
6.76	760.589	PC(16:0/18:1)	[M+H] ⁺
6.93	810.599	PC(18:2/20:4)	[M+H] ⁺
6.93	786.602	PC(18:0/18:2)	[M+H] ⁺
7.12	746.604	PC(O-18:1/16:0)	[M+H] ⁺
7.12	812.616	PC(18:0/20:3)	[M+H] ⁺
7.42	785.652	SM(d16:1/24:1)	[M+H] ⁺
7.42	788.615	PC(18:0/18:1)	[M+H] ⁺
7.50	811.667	SM(d18:2/24:1)	[M+H] ⁺
7.50	785.653	SM(d18:1/22:1)	[M+H] ⁺
7.98	813.683	SM(d18:1/24:1)	[M+H] ⁺
8.60	815.700	SM(d18:1/24:0)	[M+H] ⁺
9.91	894.755	TG(18:2/18:2/18:3)	[M+NH ₄] ⁺
9.91	721.507	PG(16:0/16:1)	[M+H] ⁺
10.12	844.739	SM(d18:1/26:0)	[M+H] ⁺
10.19	896.771	TG(18:1/18:2/18:3)	[M+NH ₄] ⁺
10.19	870.755	TG(16:0/18:2/18:3)	[M+NH ₄] ⁺
10.36	820.738	TG(16:0/16:1/16:1)	[M+NH ₄] ⁺
10.40	846.753	TG(16:1/18:1/16:1)	[M+NH ₄] ⁺
10.44	872.772	TG(16:0/18:2/18:2)	[M+NH ₄] ⁺
10.67	848.770	TG(16:1/16:0/18:1)	[M+NH ₄] ⁺
10.67	874.787	TG(16:0/18:1/18:2)	[M+NH ₄] ⁺
10.73	900.802	TG(16:0/18:1/20:3)	[M+NH ₄] ⁺
10.83	862.785	TG(16:0/17:1/18:1)	[M+NH ₄] ⁺
10.96	876.803	TG(16:0/18:0/18:2)	[M+NH ₄] ⁺
10.99	902.817	TG(18:1/18:1/18:1)	[M+NH ₄] ⁺
11.15	852.801	TG(16:0/16:0/18:1)	[M+NH ₄] ⁺
11.23	904.832	TG(18:0/18:1/18:1)	[M+NH ₄] ⁺

Table 30. High intensity features subjected to tandem mass spectrometry
Features that were identified from fragmentation data are highlighted in green.

5.4.6 Univariate analysis of identified ESI+ lipid metabolites

All lipid metabolites from the positive ESI UPLC-MS analysis were identified from tandem mass spectrometry fragmentation data are summarised in Table 31. The major groups of metabolites putatively identified included the lysophosphatidylcholines (LPC), phosphatidylcholines (PC), sphingomyelins (SM), triglycerides (TG), lysophosphatidic acid (LPA) and phosphatidylethanolamine (PE). Univariate analyses of these metabolites compared class differences between HC, SC, AD, ACLF, ALI and ALF groups (see Figure 47) and also differences between survivors and non-survivors from the AD and ACLF groups at 30 days post admission (Figure 48).

5.4.6.1. Metabolic differences in AD, ACLF, ALI and ALF relative to HC

Pairwise comparisons of AD vs HC, ACLF vs HC, ALF vs HC and ALI vs HC show a decrease in LysoPC(14:0), LysoPC(18:0), LysoPC(18:1), LysoPC(18:2), PC(18:0/20:3), PC(18:1/20:5), SM(d16:1/18:1), SM(d16:1/24:1), SM(d18:1/16:0), SM(d18:1/22:1) and SM(d18:1/24:0) and TG(18:2/18:2/18:3) and an increase in PC(14:1/18:0), PC(16:1/18:2) and LPA(18:0e/0:0) in AD, ACLF, ALF and ALI patients relative to HC. Furthermore, a decrease in LysoPC(20:4), SM(d18:1/16:0) and SM(d18:2/24:1) was seen in ACLF, ALI and ALF patients relative to HC. Whilst a decrease in TG(16:0/18:2/18:3), PC(16:0/20:3) and PC(16:0/22:6) and a rise in PC O(18:1/16:0) was seen in AD, ACLF and ALF patients relative to HC. A decrease in PC(18:0/20:4) and PC(P-18:1/20:3) was common to ALF and ACLF relative to HC. A decrease in LysoPC(16:0) was common to SC, AD and ACLF relative to HC. Finally a rise in PE(18:2/18:2) and a decrease in LysoPC(16:1) were common to ALI and ALF relative to HC.

5.4.6.2. Metabolic differences discriminating between AD and ACLF

Metabolite changes discriminant for AD vs ACLF included a decrease of LysoPC(14:0), LysoPC(18:0), LysoPC(16:1), LysoPC(18:1), LysoPC(18:2), LysoPC(20:4), PC(16:0/22:6), PC(18:0/18:2), PC(18:0/20:3), PC(18:0/20:4), PC-P(18:1/20:3), PC(18:1/20:5), SM(d18:1/16:0), SM(d18:1/22:1), SM(d18:1/24:0) and SM(d18:1/24:1) in ACLF compared to AD. Furthermore, a rise in LysoPC(16:0) and LPA(18:0e/0:0) in ACLF compared to AD was also discriminant for AD vs ACLF.

5.4.6.3 Metabolic differences discriminating between ALF and ACLF

Metabolic differences discriminant for ALF vs ACLF included a decrease of LysoPC(16:0), LysoPC(16:1), LysoPC(18:1), PC(18:2/18:2) and SM(d18:1/24:1) in ALF compared to ACLF. Also an increase of PC(16:0/22:6) and PE(18:2/18:2) in ALF compared to ACLF.

5.4.6.4 Metabolic differences discriminating between survivors and non-survivors at 30 days post admission

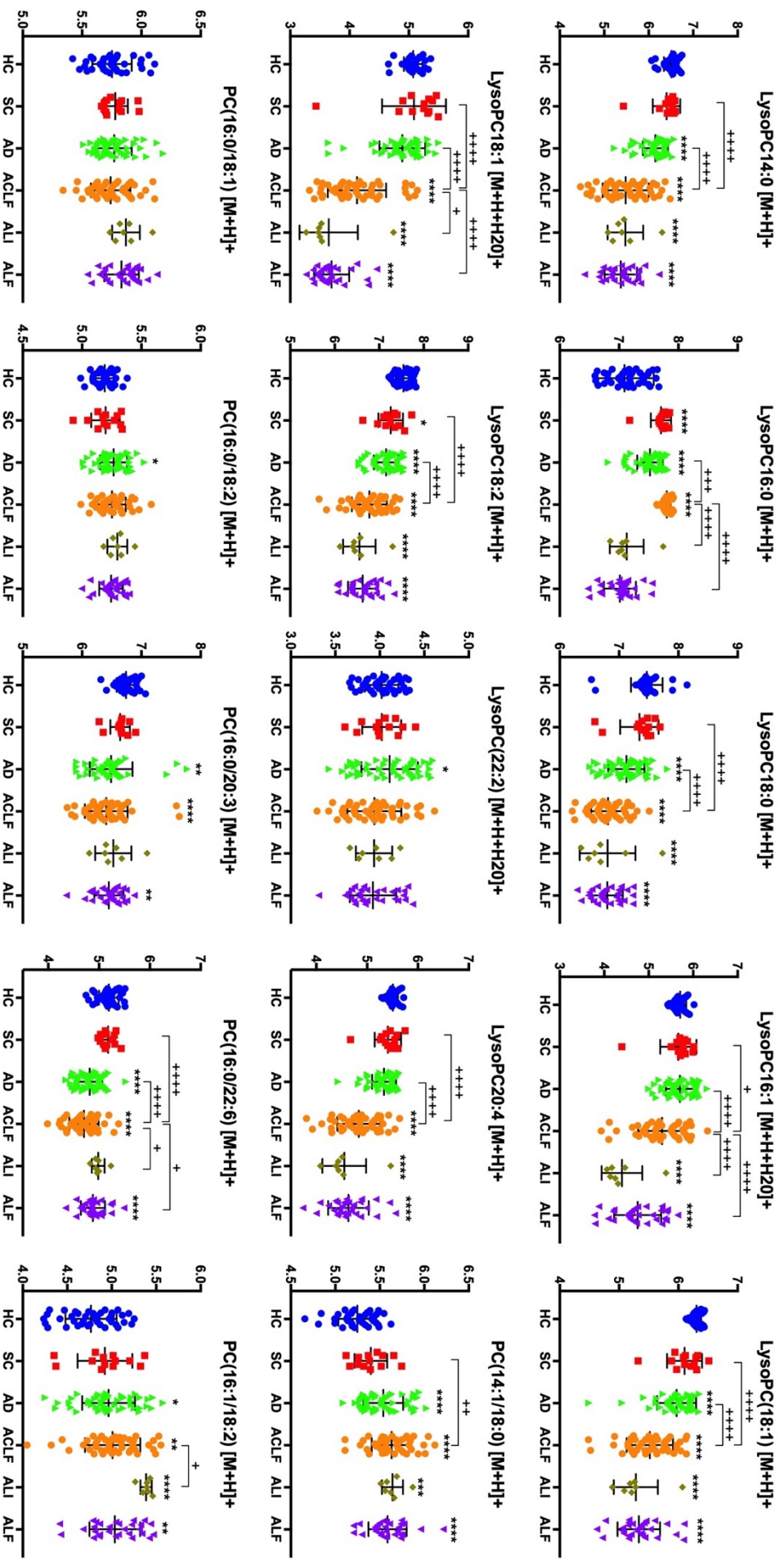
Pairwise comparisons of the identified positive ESI lipid metabolites in survivors vs non-survivors at 30 days post admission from the AD and ACLF groups are shown in Figure 48. Overall no changes were seen in TGs and PE, with the main groups of metabolites showing changes according to survival were the PCs, LysoPCs, LPA and the SMs. Pairwise comparisons of all of the LysoPCs identified (Table 31) revealed they were decreased in non-survivors compared to survivors, whilst comparisons of the PCs revealed a decrease in PC(18:0/18:2), PC(18:0/20:3), PC(P-18:1/20:3) and PC(18:1/20:5) in non-survivors compared to survivors. LPA(18:0e/0:0) showed an increase in non-survivors compared to survivors. Comparisons of SMs revealed a decrease in SM d16:1/24:1, SM d18:1/16:0, SM d18/22:1, SM d18:1/24:0, SM d18:1/24:1 and SM d18:2/24:1 in non-survivors compared to survivors.

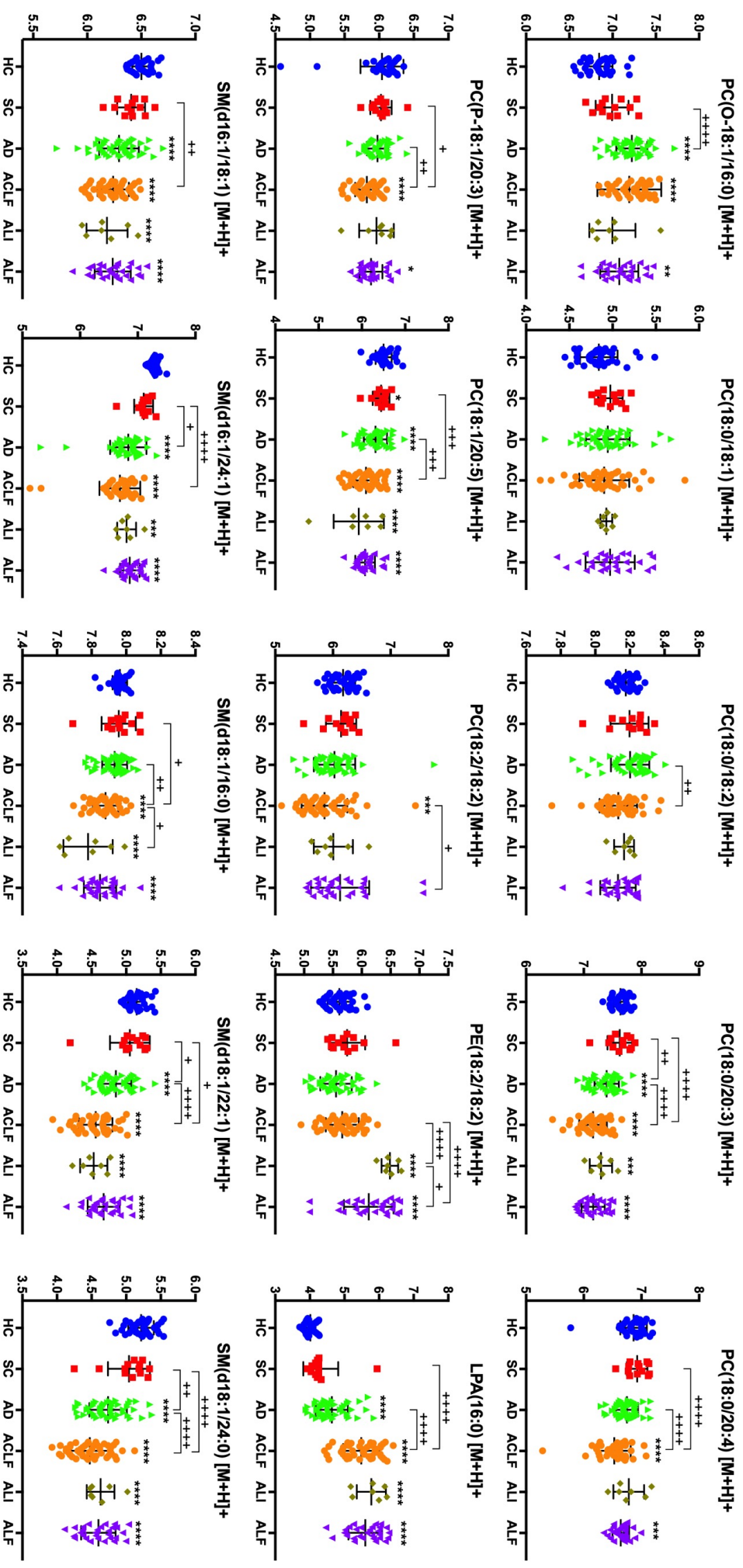
Putative ID	Adduct	Molecular formula	Fragments	Retention Time (mins)	Observed m/z	Theoretical m/z	Δ ppm	Class Comparisons		30-day survival	
								ANOVA p-value	FDR corrected p-value	t-test p-value	FDR corrected p-value
LysoPC(14:0)	[M+H] ⁺	C ₂₂ H ₄₆ NO ₂ P	4	1.38	468.3084	468.3085	-0.21	2.00E-16	8.40E-15	2.72E-08	1.91E-07
LPA(16:0)	[M+Na] ⁺	C ₂₁ H ₄₅ NO ₆ P	2	1.53	433.2326	433.2326	0.00	2.00E-16	5.60E-16	8.99E-03	2.36E-02
LysoPC(16:0)	[M+H] ⁺	C ₂₄ H ₅₀ NO ₂ P	3	1.89	496.3397	496.3398	-0.20	2.00E-16	4.20E-15	2.72E-08	1.63E-07
LysoPC(18:2)	[M+H] ⁺	C ₂₆ H ₅₀ NO ₂ P	3	1.68	520.3401	520.3398	0.58	2.00E-16	2.80E-15	8.37E-06	4.40E-05
LysoPC(18:1)	[M+H] ⁺	C ₂₆ H ₅₂ NO ₂ P	3	2.04	522.3557	522.3554	0.57	2.00E-16	9.33E-16	3.09E-10	6.48E-09
LysoPC(18:0)	[M+H] ⁺	C ₂₆ H ₅₄ NO ₂ P	3	2.43	524.3715	524.3711	0.76	2.00E-16	2.10E-15	2.87E-03	1.00E-02
LysoPC(18:1)	[M+H+H2O] ⁺	C ₂₆ H ₅₂ NO ₃ P	3	1.07	540.3653	540.3659	-1.11	2.00E-16	1.68E-15	2.47E-10	1.04E-08
LysoPC(22:2)	[M+H+H2O] ⁺	C ₃₀ H ₅₈ NO ₂ P	2	3.01	594.4137	594.4129	1.35	2.00E-16	1.40E-15	3.51E-09	3.68E-08
LysoPC(16:1)	[M+H+H2O] ⁺	C ₂₄ H ₄₈ NO ₂ P	4	1.52	512.3349	512.3346	0.59	2.17E-02	2.60E-02	8.99E-03	2.22E-02
LysoPC(16:1)	[M+H+H2O] ⁺	C ₂₄ H ₄₈ NO ₂ P	4	0.74	512.3340	512.3349	-1.76	2.00E-16	1.20E-15	3.51E-09	2.94E-08
LysoPC(20:4)	[M+H] ⁺	C ₂₈ H ₅₀ NO ₂ P	3	1.76	544.3386	544.3397	-2.02	2.00E-16	1.05E-15	2.02E-09	2.83E-08
PC(14:1,18:0)	[M+H] ⁺	C ₄₀ H ₇₈ NO ₈ P	3	6.09	732.5525	732.5537	-1.64	1.07E-10	1.87E-10	7.49E-01	7.87E-01
PC(O-18:1/16:0)	[M+H] ⁺	C ₄₂ H ₈₄ NO ₂ P	4	7.12	746.6041	746.6058	-2.28	1.31E-10	2.20E-10	3.20E-01	4.48E-01
PC(16:1,18:2)	[M+H] ⁺	C ₄₂ H ₇₈ NO ₈ P	3	5.76	756.5540	756.5537	0.40	1.11E-05	1.73E-05	2.42E-01	3.63E-01
PC(16:0/18:2)	[M+H] ⁺	C ₄₂ H ₈₀ NO ₈ P	3	6.24	758.5754	758.5694	7.91	9.20E-03	1.14E-02	1.18E-01	1.98E-01
PC(16:0/18:1)	[M+H] ⁺	C ₄₂ H ₈₂ NO ₈ P	4	6.76	760.5887	760.5851	4.73	7.93E-02	9.25E-02	6.69E-01	7.40E-01
PC(18:2,18:2)	[M+H] ⁺	C ₄₄ H ₈₀ NO ₈ P	4	5.92	782.5692	782.5694	-0.26	1.63E-03	2.28E-03	9.27E-01	9.27E-01
PC(16:0/20:3)	[M+H] ⁺	C ₄₄ H ₈₂ NO ₈ P	3	6.38	784.5851	784.5851	0.00	1.44E-05	2.16E-05	3.55E-02	7.09E-02
PC(18:0/18:2)	[M+H] ⁺	C ₄₄ H ₈₄ NO ₈ P	3	6.93	786.6024	786.6007	2.16	4.41E+03	4.41E+03	1.61E-02	3.56E-02
PC(18:0/18:1)	[M+H] ⁺	C ₄₄ H ₈₆ NO ₈ P	3	7.42	788.6152	788.6164	-1.52	2.79E-01	2.93E-01	6.07E-01	6.89E-01
PC(P-18:1/20:3)	[M+H] ⁺	C ₄₆ H ₈₄ NO ₂ P	2	6.76	794.6046	794.6046	0.00	2.40E-05	3.48E-05	9.40E-04	3.95E-03
PC(18:1/20:5)	[M+H] ⁺	C ₄₆ H ₈₀ NO ₈ P	4	6.45	806.5660	806.5694	-4.22	1.33E-13	2.79E-13	1.45E-02	3.39E-02
PC(16:0/22:6)	[M+H] ⁺	C ₄₆ H ₈₀ NO ₈ P	4	6.05	806.5699	806.5694	0.62	2.00E-16	5.25E-16	4.84E-01	6.35E-01
PC(18:0/20:4)	[M+H] ⁺	C ₄₆ H ₈₄ NO ₈ P	4	6.93	810.5991	810.6007	-1.97	6.04E-11	1.10E-10	9.40E-04	4.39E-03
PC(18:0/20:3)	[M+H] ⁺	C ₄₆ H ₈₆ NO ₈ P	3	7.12	812.6156	812.6164	-0.98	2.00E-16	8.40E-16	5.35E-03	1.61E-02
PE(18:2,18:2)	[M+H] ⁺	C ₄₁ H ₇₄ NO ₈ P	3	6.38	740.5216	740.5225	-1.22	2.00E-16	7.64E-16	2.44E-01	3.53E-01

SM(d16:1/18:1)	[M+H] ⁺	C ₃₉ H ₇₇ N ₂ O ₆ P	1	5.33	701.5586	701.5592	-0.86	1.91E-14	4.46E-14	7.19E-02	1.37E-01
SM(d18:1/16:0)	[M+H] ⁺	C ₃₉ H ₇₉ N ₂ O ₆ P	2	5.92	703.5750	703.5749	0.14	3.65E-12	7.30E-12	3.36E-02	7.05E-02
SM(d16:1/24:1)	[M+H] ⁺	C ₄₅ H ₈₈ N ₂ O ₆ P	2	7.42	785.6520	785.6531	-1.40	2.00E-16	7.00E-16	7.84E-02	1.43E-01
SM(d18:1/22:1)	[M+H] ⁺	C ₄₅ H ₈₈ N ₂ O ₆ P	2	7.50	785.6529	785.6531	-0.25	2.00E-16	6.46E-16	3.09E-03	9.99E-03
SM(d18:2/24:1)	[M+H] ⁺	C ₄₇ H ₉₁ N ₂ O ₆ P	2	7.50	811.6669	811.6688	-2.34	4.46E-03	6.04E-03	8.75E-02	1.53E-01
SM(d18:1/24:1)	[M+H] ⁺	C ₄₇ H ₉₃ N ₂ O ₆ P	3	7.98	813.6831	813.6844	-1.60	1.07E-13	2.37E-13	5.35E-03	1.50E-02
SM(d18:1/24:0)	[M+H] ⁺	C ₄₇ H ₉₅ N ₂ O ₆ P	2	8.60	815.7004	815.7001	0.37	2.00E-16	6.00E-16	2.06E-03	7.86E-03
TG(16:1/16:0/18:1)	[M+NH ₄] ⁺	C ₅₃ H ₉₈ O ₆	3	10.67	848.7698	848.7696	0.24	1.37E-01	1.48E-01	7.49E-01	7.68E-01
TG(16:0/17:1/18:1)	[M+NH ₄] ⁺	C ₅₄ H ₁₀₀ O ₆	4	10.83	862.7850	862.7858	-0.93	1.30E-01	1.44E-01	4.81E-01	6.51E-01
TG(16:0/18:2/18:3)	[M+NH ₄] ⁺	C ₅₅ H ₉₆ O ₆	4	10.19	870.7546	870.7545	0.11	1.15E-14	2.84E-14	1.81E-01	2.82E-01
TG(16:0/18:1/18:2)	[M+NH ₄] ⁺	C ₅₅ H ₁₀₀ O ₆	3	10.67	874.7870	874.7858	1.37	8.68E-03	1.10E-02	6.07E-01	7.08E-01
TG(16:0/18:0/18:2)	[M+NH ₄] ⁺	C ₅₅ H ₁₀₂ O ₆	3	10.96	876.8027	876.8009	2.05	7.85E-03	1.03E-02	6.02E-01	7.43E-01
TG(18:2/18:2/18:3)	[M+NH ₄] ⁺	C ₅₇ H ₉₆ O ₆	3	9.91	894.7550	894.7540	1.12	2.56E-11	4.89E-11	6.07E-01	7.29E-01
TG(18:1/18:2/18:3)	[M+NH ₄] ⁺	C ₅₇ H ₉₈ O ₆	3	10.19	896.7712	896.7696	1.78	1.07E-07	1.73E-07	4.88E-01	6.21E-01
TG(18:0/18:1/18:1)	[M+NH ₄] ⁺	C ₅₇ H ₁₀₂ O ₆	4	10.73	900.8022	900.8009	1.44	1.11E-01	1.26E-01	1.58E-01	2.55E-01

Table 31. Metabolites identified from ESI+ UPLC-MS data

Metabolites identification was based on: m/z matching to online databases, in-source fragmentation and MS^E mode spectrum. Univariate analysis included a 1-way ANOVA to compare all classes and a paired t-test to compare survival at 30 days post admission. LPA -lysophosphatidic acid; LysoPC -lysophosphatidylcholine; PC -phosphatidylcholine; SM -sphingomyelin; TG -triglyceride





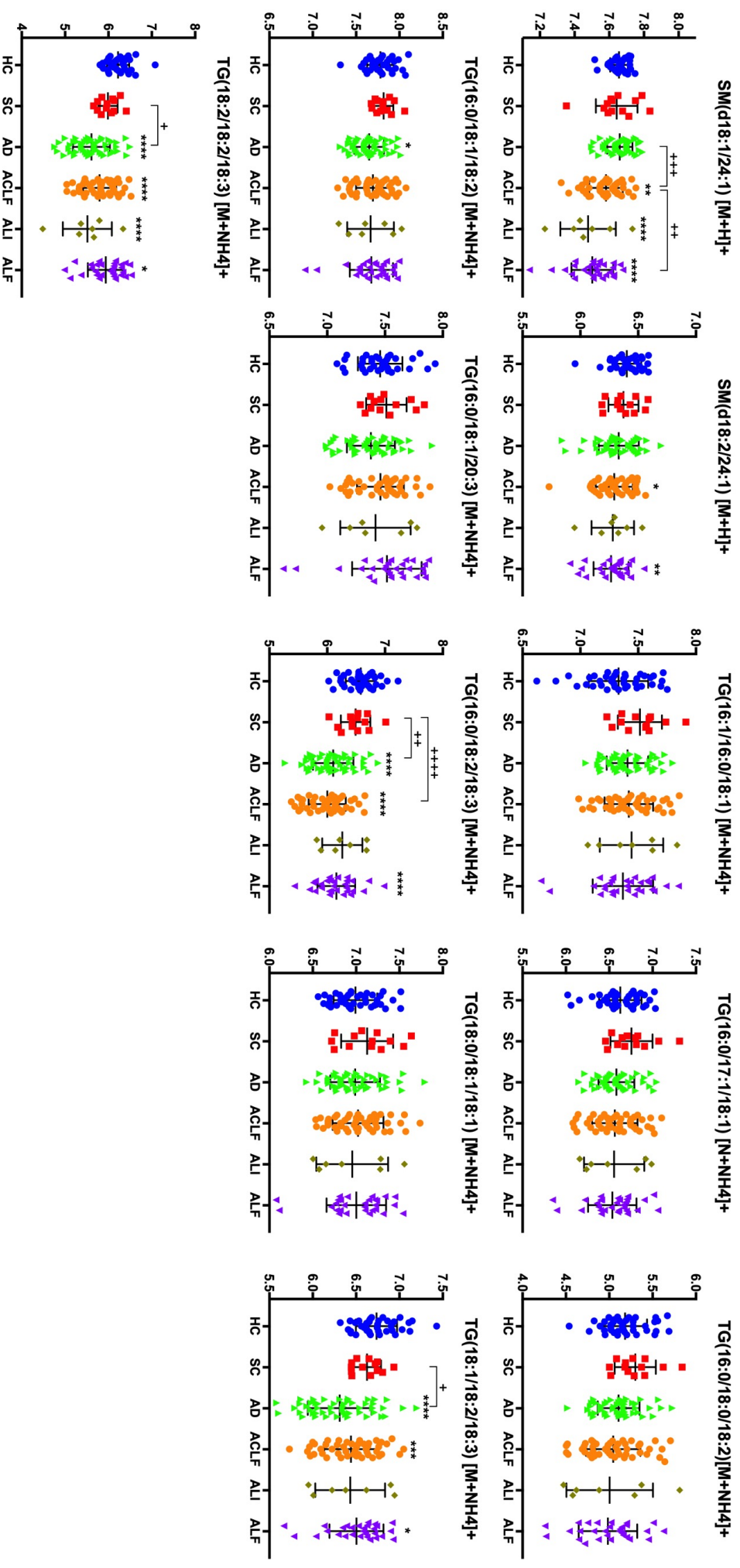
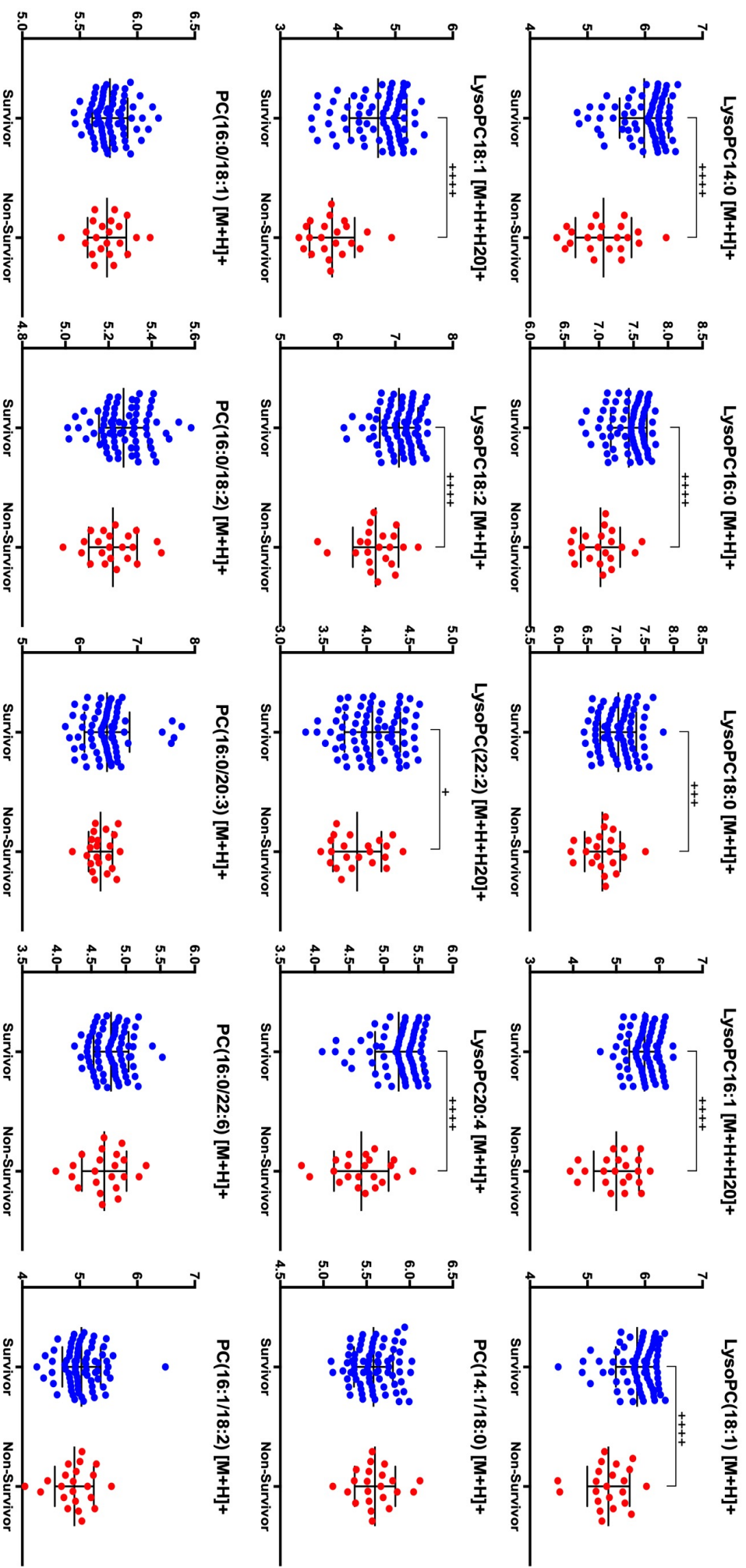
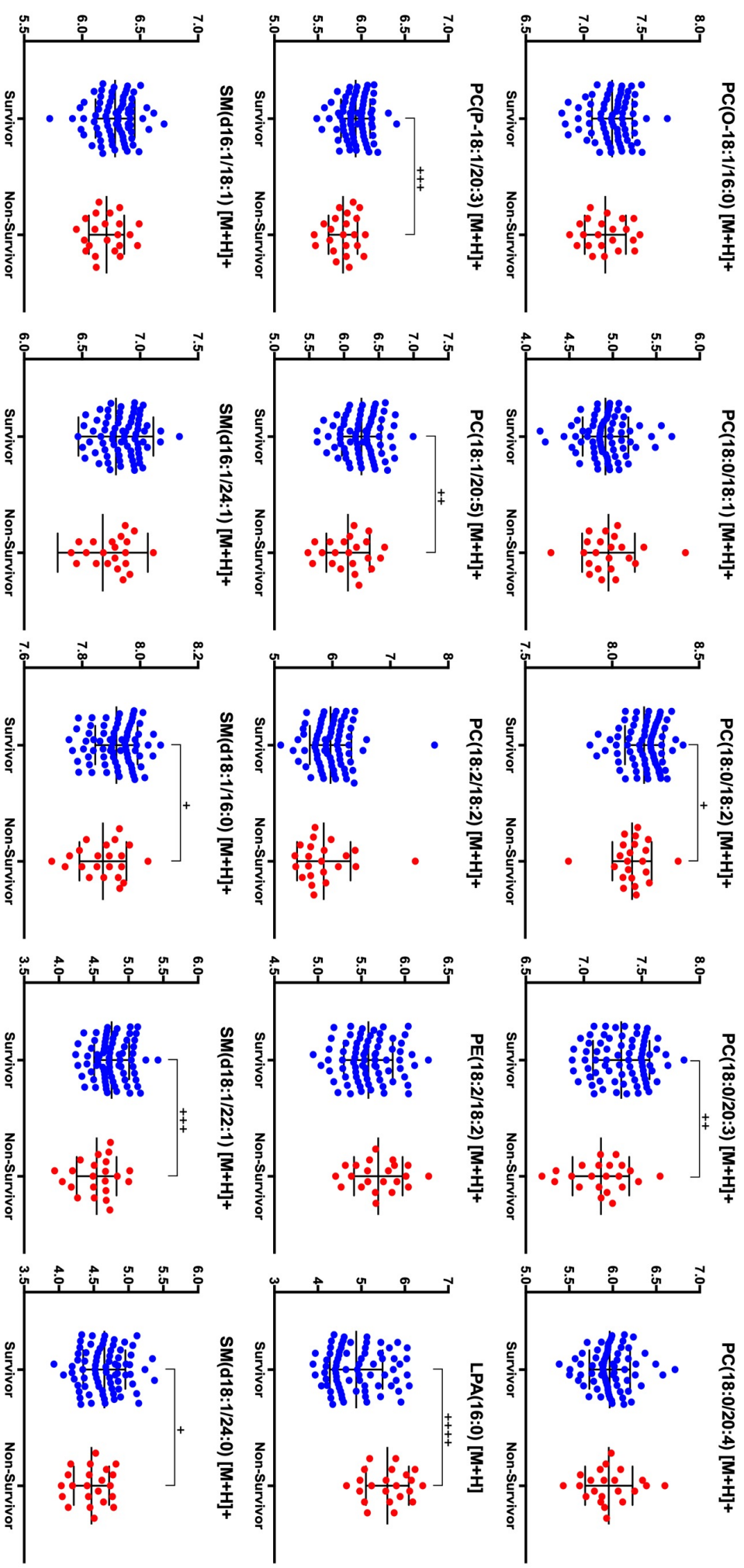


Figure 47. Univariate analysis of class differences between identified ESI+ UPLC-MS lipids

These plots display metabolite changes relative to HC (*) and between liver disease groups (+). A 1-way ANOVA was performed on log transformed data log₁₀(X) followed by a post-hoc Sidak test. Error bars representing the standard deviation and indicating the mean are shown per group. Multiplicity adjusted p-values denoted by asterisks (*) show significance relative to HC and crosses (+) show significance within disease groups, specified by brackets; ****<0.0001, ***<0.001, **<0.01, *<0.05;





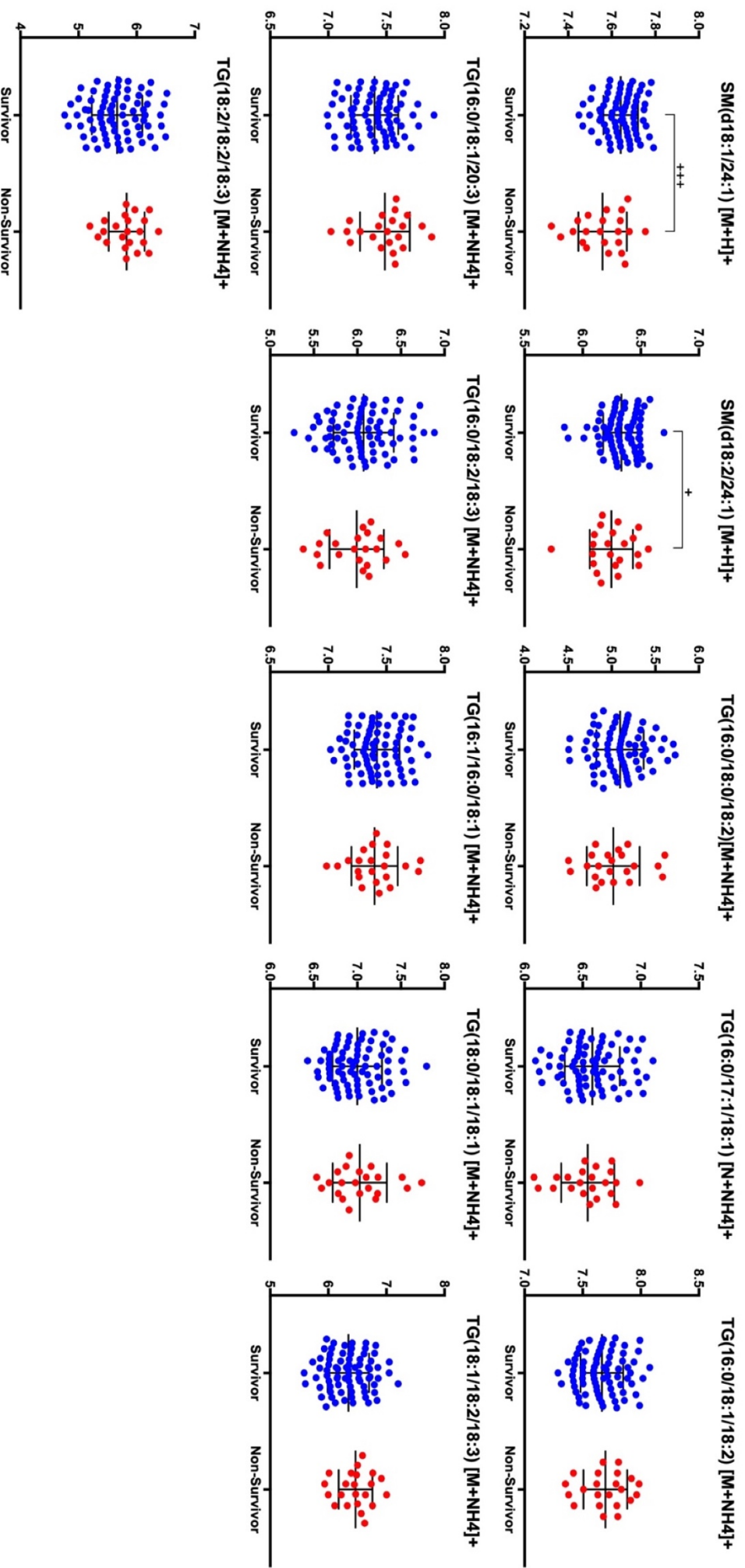


Figure 48. Univariate analysis of survival at 30 days post admission between identified ESI+ UPLC-MS lipids

These plots display metabolite changes between survivors (blue points) and non-survivors (red points). An unpaired t-test was performed on log transformed data log₁₀(X) followed by a post-hoc Sidak test. Error bars representing the standard deviation and indicating the mean are shown per group. Statistically significant p-values denoted by asterisks (*) and brackets indicate differences between survivors and non-survivors, ***<0.0001, **<0.001, *<0.01, *<0.05

5.4.7 Hierarchical cluster analysis of lipids identified from ESI+ UPLC-MS data

The lipids detected in positive ESI were further assessed to determine correlation patterns in acute (ALI and ALF) and chronic liver disease (SC, AD and ACLF), as performed previously for the ^1H NMR spectral data and the UPLC-MS-detected amines. Correlation heatmaps were computed from the identified positive ESI lipid $\log_{10}$ transformed data, using Pearson correlation r^2 colour scale of -0.5-1.0, separately for HC (Figure 49A), acute liver disease (Figure 49B) and cirrhosis (Figure 49C).

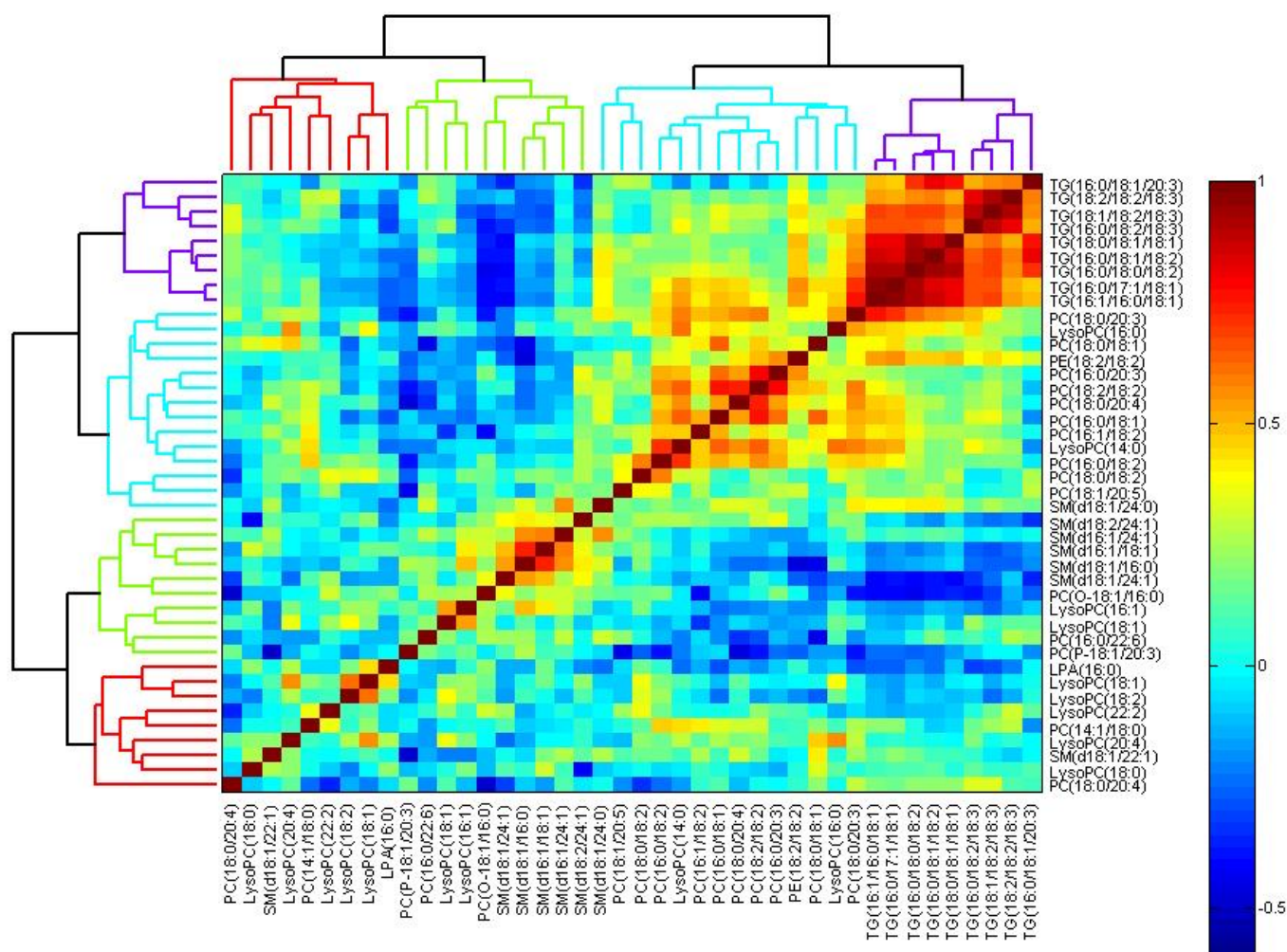
Four main clusters of lipids were determined from metabolite-metabolite correlation analysis of the HC group. In the purple cluster TG(18:2/18:2/18:3), TG(18:1/18:2/18:3), TG(16:0/18:2/18:3), TG(16:0/18:1/18:2), TG(18:0/18:1/18:1), TG(16:0/18:0/18:2), TG(16:0/17:1/18:1) and TG(16:1/16:0/18:1) showed a strong intercorrelation ($r^2 > 0.7$). In the blue cluster there were additionally, there was a strong correlation between PC(16:0/20:3), PC(18:2/18:2), PC(16:0/18:1), LysoPC(14:0) and PC(16:0/18:2) ($r^2 > 0.6$). Furthermore, PC(16:0/18:1), LysoPC(14:0), PC(16:0/18:2) were correlated with PC(18:0/20:3), TG(16:1/16:0/18:1) and TG(16:0/17:1/18:1) ($r^2 > 0.7$). Within the green cluster there was a strong correlation between SMs including SM(d16:1/24:1), SM(d16:1/18:1), SM(d18:1/24:1) ($r^2 > 0.6$).

Two main clusters were determined from the correlation heatmap from the acute liver disease (ALI and ALF) groups. In the red cluster TG(16:0/18:0/18:2), TG(16:1/16:0/18:1), TG(16:0/17:1/18:1), TG(18:0/18:1/18:1), TG(16:0/18:1/18:2) and TG(18:1/18:2/18:3) were correlated ($r^2 > 0.8$). Also, in the red cluster, SM(d18:1/24:1), SM(d18:2/24:1), SM(d16:1/18:1), SM(d18:1/16:0) were correlated ($r^2 > 0.7$). In addition, SM(d16:1/24:1), SM(d18:1/24:0), PC(18:0/20:3) and PC(16:0/20:3) also from the red cluster were correlated ($r^2 > 0.8$). LysoPC(18:1), LysoPC(20:4), LysoPC(18:2), LysoPC(14:0), LysoPC(18:1), LysoPC(18:0) and LysoPC(16:0) in the blue cluster were correlated ($r^2 > 0.8$).

Three main clusters were determined from the correlation heatmap from the cirrhosis (SC, AD and ACLF) groups. TG(16:0/18:1/20:3), TG(18:0/18:1/18:1), TG(16:0/18:0/18:2), TG(16:0/17:1/18:1), TG(16:1/16:0/18:1), TG(16:0/18:1/18:2), TG(18:1/18:2/18:3) and TG(18:2/18:2/18:3) were correlated ($r^2 > 0.8$). Furthermore, LysoPC(18:0), LysoPC(18:1), LysoPC(16:0), LysoPC(14:0), LysoPC(20:4), LysoPC(18:1) and LysoPC(18:2) were correlated ($r^2 > 0.8$). Furthermore, PC(18:0/20:3), SM(d16:1/24:1), SM(d18:1/24:0), SM(d18:1/22:1) and SM(d18:1/16:0) were highly correlated ($r^2 > 0.8$) and SM(d18:1/16:0), SM(d18:1/24:1), SM(d16:1/18:1) and SM(d18:2/24:1) were highly correlated ($r^2 > 0.8$).

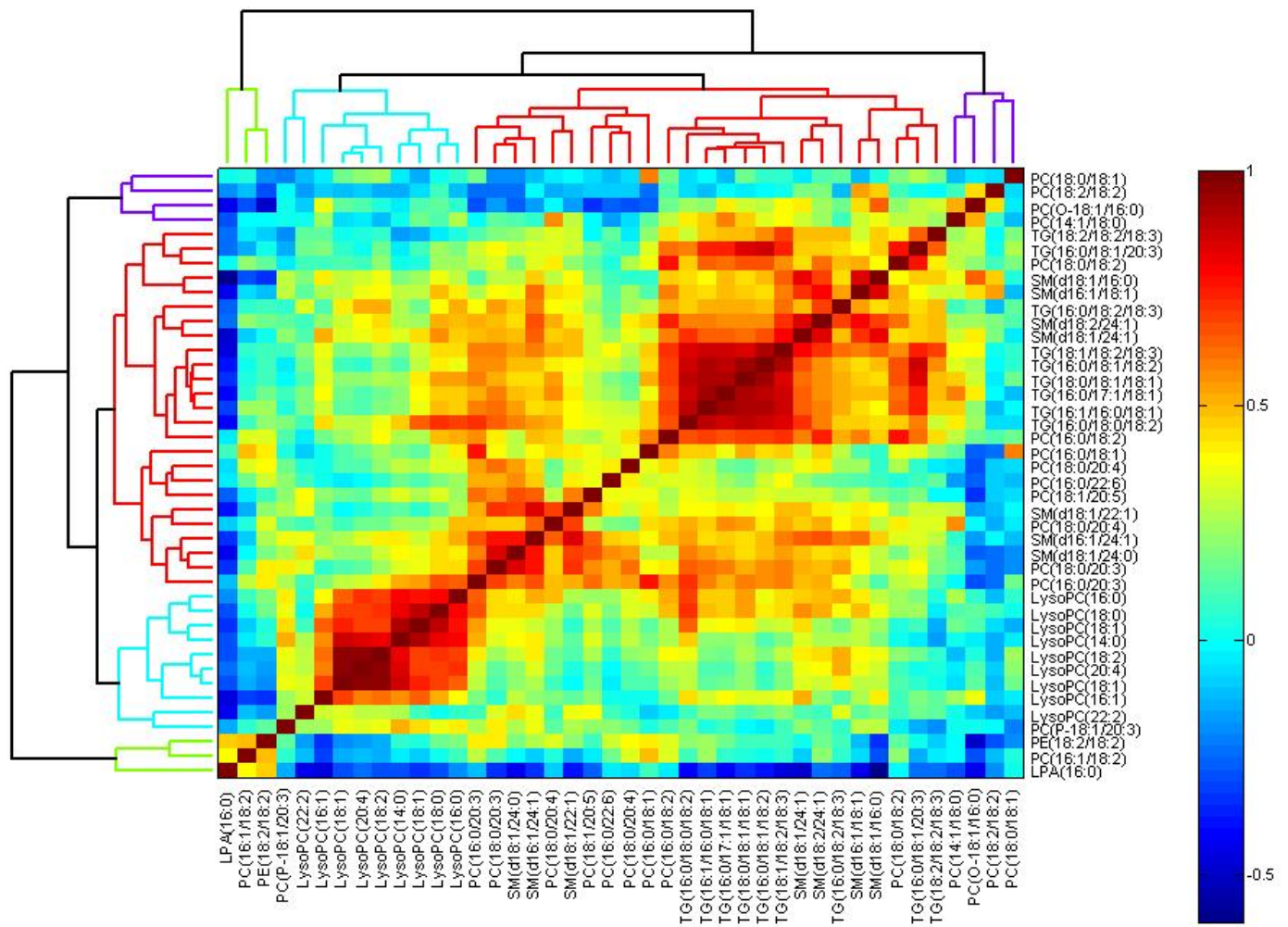
Overall, metabolite correlations within the LysoPC and SM groups intensified in both cirrhosis and acute liver disease compared to HCs.

A) Healthy Controls



B)

Acute Liver Disease



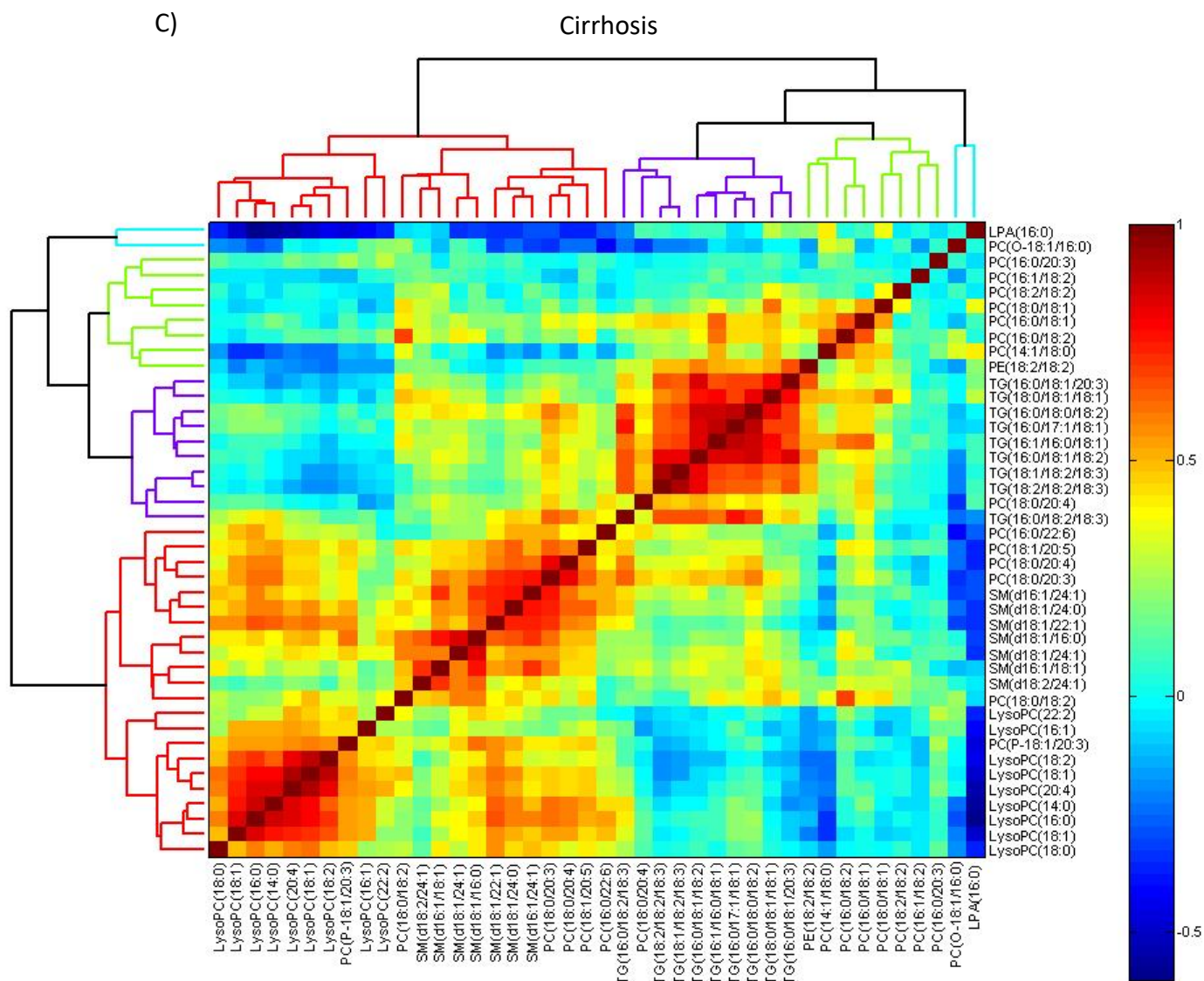


Figure 49. Heat map visualisation of metabolite-metabolite correlations from ESI+ UPLCMS data

Correlations determined from identified lipids from healthy controls, chronic liver disease patients (SC, AD & ACLF) and acute liver disease patients (ALI and ALF) detected using positive ESI – Coloured squares represent Pearson's correlation coefficients (r^2).

5.5 Discussion

In this study, multivariate analysis of serum lipid profiles generated by UPLC-MS using positive and negative ESI, found that lipid metabolism is discriminant both for the progression of AD to ACLF and between ALF vs ACLF as well as being predictive of survival at 30 days post admission in AD and ACLF (see Figure 44 & 45). Following metabolite ID, the main groups of lipids identified including the lysophosphatidylcholines (LysoPCs), phosphatidylcholines (PC), sphingomyelins (SM) and a small number of triglycerides (TG) were found to be largely depleted in acute liver disease and chronic liver disease relative to HC. In addition, the univariate statistical analysis of survival the TGs showed no change, whilst most LysoPCs (aside

from LysoPC(16:0) and LysoPC(22:2)), a number of PCs and all SMs were depleted in non-survivors (See Figure 50).

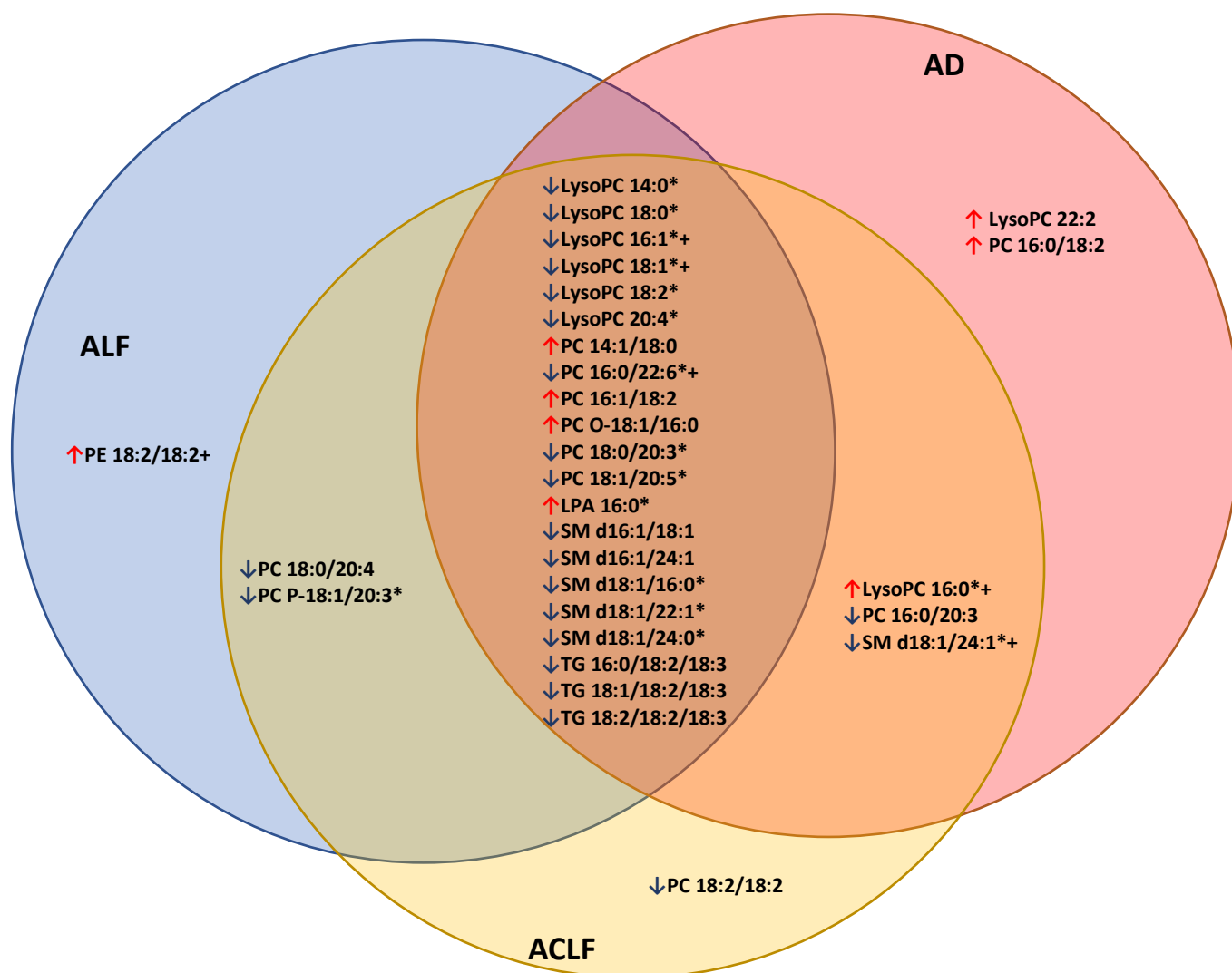


Figure 50. Venn diagram summarising metabolic differences in AD, ACLF and ALF

The diagram summarises the metabolic changes observed in AD, ACLF and ALF relative to HC as well as differences between them. Statistical significance was derived from earlier univariate analyses (see Figure 6). Differences that were significant between AD vs ACLF are marked with an asterisk (*) and significant changes between ALF and ACLF are marked with a cross (+).

5.5.1 Statistical approach for metabolite identification

The data in this study demonstrated that utilizing a multivariate approach to identify discriminate lipid features from comparisons of interest has significant limitations. The most discriminate features selected using this approach were difficult to identify by tandem mass spectrometry as they were of low intensity and fragments generated from these were also low intensity and indistinguishable from noise. Instead selecting the most intense features for metabolite ID and identifying features showing significant differences between groups of

interest using a univariate approach was most effective for identifying discriminant features by tandem mass spectrometry. As the fragments generated from these features were higher intensity and therefore more distinguishable from surround noise. These findings indicate that the feasibility of a multivariate approach for selecting discriminate metabolites is data dependent. In this data low intensity features were more influential in the multivariate models which complicated metabolite identification. This suggests further exploration of scaling approaches and the incorporation of a high signal intensity threshold should be investigated prior to multivariate modelling of such datasets. However, it is also possible to introduce bias to the data by excessive filtering and selective removal of features which may cause unintended clustering. A further alternative is to implement a well characterised targeted lipid method which, although, would limit the number of metabolites profiled would overcome the analytical challenges associated with identifying discriminant metabolites in these patients.

5.5.2 LysoPCs and SMs are markers of disease severity and can discriminate survival in AD and ACLF patients

UPLC-MS serum lipid profiles from AD and ACLF patients can predict survival at 30 days post admission (Figure 45) and the univariate analysis of identified lipids revealed LPCs and SMs were depleted in non-survivors at 30 days post-admission, confirming previous work.⁸⁸ Systemic lipid depletion, or hypolipidemia, has been identified in a number of chronic liver diseases of various aetiologies such as hepatitis B, alcoholic hepatitis and NAFLD.^{211,212,219} Such studies have concluded that lipid derangements in cirrhosis are independent predictors of survival in these patients following comparison with clinical scores such as MELD and Child-Pugh. Our data confirms this and also shows survival prediction is best at 30 days compared to 90 days post admission in contrast to previous findings.¹⁵⁶

More specifically, depleted LysoPCs and PCs have been associated with mortality risk in a number of studies on patients with cirrhosis which correlated changes in these lipids to disease severity.^{211,220} However, it is currently unknown whether saturated vs unsaturated phospholipids show a difference in predicting mortality, whilst the data presented here shows that LysoPCs and SMs containing both saturated and unsaturated FAs were depleted to a greater degree in non-survivors (Figure 48). Previous studies found that a specific depletion of saturated LysoPCs was associated with mortality risk in cirrhosis.²²¹ Furthermore, a reduction in LysoPC (16:0) and (18:0) were the most commonly observed in cirrhosis,^{220,222,223} whilst the data presented here confirms this change in LysoPC(18:0), it also showed that

LysoPC(16:0) and LysoPC(22:2) were increased in AD and ACLF, although the mechanistic reason for this increase is currently unclear. Overall, however, these data show a decrease in circulating LysoPCs and also identify a number of changes in LysoPC species. These changes in SMs are important for predicting survival in AD and ACLF patients and have previously been negatively correlated with the MELD score and disease severity.^{221,224}

5.5.3 Dyslipidemia and impaired lipoprotein synthesis

The most striking observation from the lipid profile data was the significant depletion of lipids in the serum of patients with acute and chronic liver disease relative to HCs. The depletion of circulating lipids has been observed in patients with varying aetiologies of liver disease and occurs, in part, as a result of the liver's declining capacity for lipoprotein synthesis via the glycerol-3-phosphate pathway²²⁵ (see Figure 51). In this pathway, fatty acids (FA) are esterified to acetyl-CoA, a process catalysed by G3P acyltransferase enzymes. Acetyl-CoA is subsequently further esterified to lysophosphatidic acid (LPA) and then phosphatidic acid (PA), which is converted to diacylglycerol (DAG), which itself is a precursor for the synthesis of LysoPCs, PCs and TGs.²²⁵ In acute and chronic liver disease, increased rates of *de novo* FA lipogenesis and decreased rates of FA oxidation to acetyl-CoA lead to an accumulation of FAs in the hepatocyte.^{226,227} Intrahepatic FAs are used for the synthesis of TGs which are either stored as lipid droplets within the hepatocyte or assembled into lipoproteins where apolipoproteins, PCs, cholesterol form a monolayer around the hydrophobic core of the TG.²²⁵ During dyslipidemia, accumulating FAs and reduced intracellular PC lead to impaired secretion of vLDLs and increased intrahepatic storage of TGs leading to a reduction in levels circulating TGs.²²⁸

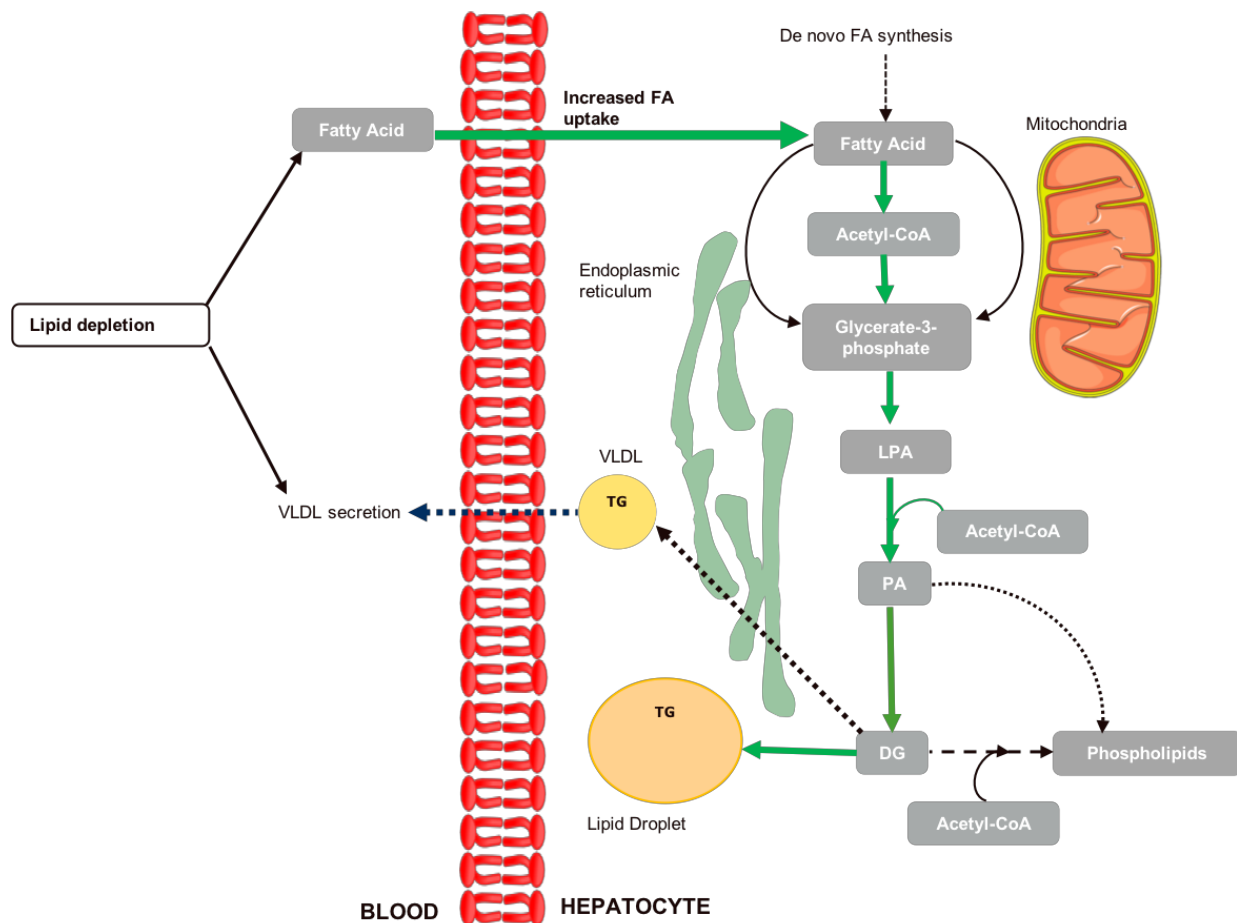


Figure 51. Increased FA uptake and dyslipidemia in acute and chronic liver disease

These data show that there is no clear direction of change in serum PCs; some PCs show an increase with worsening liver disease and some show a decrease, which suggests that in addition to dyslipidemia, changes in PC metabolism also have other mechanistic fates. Intrahepatic PC is synthesised by reacylation of LysoPCs by LysoPC acyltransferases (LPCAT). These enzymes are upregulated in hepatocytes in murine NASH²²⁹ models and it is possible they are also upregulated in the livers of acute and chronic liver disease patients. Approximately 30% of all the PCs synthesised by the liver is by methylation of PE catalysed by the enzyme PEMT.²³⁰ Interestingly, the lipidomics data shows that the PE is selectively raised in ALF plasma, which may indicate a selective inhibition of PEMT in ALF leading to elevated levels of PE released from the hepatocyte; this hypothesis requires further investigation.

Excess hepatic concentrations of LysoPC and PC and the depletion of circulating LysoPC and PC concentrations are also reported in models of NASH^{231,232} and, as a result of impaired export of these lipids into the blood stream, are thought to contribute to hepatic dyslipidemia.²³³ This mechanism may explain the depleted circulating concentrations of LysoPC in AD, ACLF and ALF, particularly, as the liver is known to synthesise and secrete large amounts of LysoPC into the circulation, reportedly up to twice as much compared to PCs.²³⁴

This is largely dependent on the presence of circulating albumin which is depleted in worsening cirrhosis and liver failure and seen in the clinical metadata (see Table 2).²³⁵ Furthermore, the majority of LysoPCs synthesised from PCs by the liver contain unsaturated FAs (mostly 18:2 and 20:4), the production of which are catalysed by PLA-1 (phospholipase-1), whilst approximately 25-37% of LysoPCs are made up of saturated FAs (predominantly 16:0 and 18:0) which is catalysed by PLA-2.^{234,236} In the profiles generated here the majority of LysoPCs identified were made up of unsaturated FAs which agrees with the literature. Furthermore, according to the literature, in the presence of higher concentrations of saturated FAs in hepatocytes, LysoPC production from DGs occurs at a greater rate compared to TG production from DG,²³⁷ which explains the greater magnitude of perturbation seen in LysoPCs compared to TGs.

5.5.4 The impact of dyslipidemia on mechanisms of cell death

Hepatocyte apoptosis and necrosis are described in both acute and chronic liver disease³⁷⁻³⁹ and have been linked mechanistically to perturbed phospholipid metabolism in acute decompensation,¹⁵⁶ and may also be present in ALF (where necrosis was previously thought to predominate). Circulating LysoPC has been reported to induce apoptosis (lipoapoptosis) of endothelial cells in experimental models of sepsis and is also linked to apoptosis of hepatocytes.²³⁷ This may occur through mitochondrial caspase signaling or by the activation of G-protein coupled receptors.²³⁷

Necrosis of hepatocytes in acute and chronic liver disease contributes to loss of hepatic capacity and therefore hepatic lipid biosynthesis leading to depletion of circulating lipids.²²⁵ Liver necrosis has also been associated with higher hepatic glycerophosphocholine (GPC) in a rat model of NASH. Furthermore, in a rat model of acetaminophen induced hepatic necrosis there is a notable reduction in serum concentrations of GPC²³⁸ which may indicate that necrosis and hepatic dyslipidemia are mechanistically linked.

Furthermore, SMs which are sphingolipids made up of ceramide and either PC or PE were depleted in AD, ACLF and ALF serum (Figure 47). These changes have been observed previously in hepatitis B induced cirrhosis – specifically SM(d18:1/24:0),²²⁴ which is also seen in this study. However, the majority of reported studies have focused on ceramides, which are precursors to SM, where long chain ceramides are depleted in AD serum and are thought to switch cells from a proliferative state to an apoptotic one.²³⁹ In addition, a recent study on a knockout mouse model of Cer-synthase-2, an enzyme which is expressed in the liver and

produces long chained ceramides, demonstrated increased rates of hepatocellular apoptosis and liver dysfunction, indicating sphingolipids have a hepatoprotective role against cell death.²⁴⁰ A reduction in long-chain ceramides in the mouse knockout model leads to increased mitochondrial oxidative stress which further augments apoptotic signaling.²⁴⁰ Further studies focused on understanding the mechanistic implications of abnormal sphingolipid metabolism in worsening cirrhosis, leading to AD or ACLF as well as its role in ALF, are required.

5.5.5 Immune dysfunction is linked to LPC and PC dysregulation

As outlined in Chapter 1, mechanisms of systemic immune dysfunction are driven by high rates of hepatocyte death in ALF and ACLF and these drive pathogenesis leading to multi organ failure and death.⁸ Mechanisms of immune dysregulation observed in ALF and ACLF include monocyte and neutrophil dysfunction and heightened anti and pro-inflammatory signaling,^{51–53,241} the cytokine array data presented in Chapter 3 Figure 9 confirms the elevation of pro and anti-inflammatory signalling in ALF and ACLF. Furthermore, PLS regression has revealed associations between the UPLC-MS data and IL-12p70, IL-13, IL-1 β and IL-2 suggesting that changes in lipid metabolism are linked to the immune dysfunction seen in ALF and ACLF (Table 28). Perturbation of phospholipid metabolism in ALF and ACLF may function as a driver of systemic immune dysfunction and explain abnormalities in immune function observed in these patients.

Phospholipids, which are a major component of cell membranes, undergo significant remodelling in immune cells such as monocytes, neutrophils and platelets during infection and injury as part of the innate immune response³⁶. In particular, membrane phospholipids become bioactive when oxidised and play a crucial role in immune cell signalling including mechanisms such as chemotaxis, stimulating phagocytosis, upregulating expression of adhesion molecules and generating the cytokine cascade during systemic inflammatory response.^{36,242,243} The main mechanism for breakdown of membrane phospholipids is via phospholipase mediated hydrolysis which generates fatty acid substrates, these are subsequently oxidised to eicosanoids and partake in inflammatory and immune mediated signalling.²⁴⁴ *In vitro* studies have demonstrated that LysoPC concentrations decrease in pro-inflammatory conditions.²⁴⁵ This mechanism would explain the global depletion of circulating LysoPCs, PCs and SMs observed here in AD, ACLF and ALF patients.

Finally, circulating levels of LPA (lysophosphatidic acid) which is a breakdown product of LPC is mediated by the enzyme autotaxin (ATX),²⁴⁶ was significantly elevated in AD, ACLF and ALF in contrast to LysoPC (Figure 47). The LysoPC-ATX-LPA pathway is immunoregulatory^{246,247} and is implicated in chronic inflammatory diseases including hepatitis, multiple sclerosis, rheumatoid arthritis and pulmonary fibrosis.²⁴⁸ In addition, a clinical study on patients with chronic hepatitis C reported an increase in circulating LPA concentrations as well as an increase serum ATX activity.²⁴⁹ Data from this study indicates the LysoPC-ATX-LPA is also perturbed in AD, ACLF and patients and linked to mechanisms of immune dysfunction. Further work investigating the LysoPC-ATX-LPA pathway and its link to monocyte dysfunction in ALF and ACLF patients is currently ongoing.

5.6 Study Limitations

The main limitation was metabolite identification, representing a significant analytical bottleneck in many studies of this type. Further lipid identification work is outstanding from both positive and negative ESI datasets.

In addition, compared to other groups of metabolites such as amino acids and carbohydrates the metabolic pathways for lipids largely remain the least characterised and the difference in metabolism according to FA chain length and saturation and downstream effects of this are currently poorly characterised. Hence, pathway analysis of majority of the lipids detected in this project was not possible as a large number of lipids lack a KEGG or HMDB ID which are required for pathway analysis software such as metaboanalyst or IPA. In addition, a lot of lipids have the same generic ID for their class such as TGs which also limits this type of analysis.

5.7 Conclusions

The main findings from the untargeted UPLC-MS profiling of lipids from the serum of the patient cohort in this project were that changes in serum lipid profiles are discriminant for AD to ACLF and ALF vs ACLF. Furthermore, serum lipid profiles of AD and ACLF patients were able to discriminate survival at 30 days post admission and were superior to standard clinical scoring systems including MELD, CLIF-SOFA, UKELD and Child-Pugh.

Furthermore, whilst, metabolite identification was only performed from positive mode MS/MS data, negative mode MS/MS data also exists, and discriminant features

selected from the negative mode data also require identification. Hence, all subsequent univariate analyses were performed on lipids identified from the positive mode UPLC-MS data.

The depletion of lipids observed in AD, ACLF and ALF patients was associated with disease severity and survival. Furthermore, it was hypothesised lipid depletion was mechanistically linked to hepatic dyslipidemia and cell death which requires further investigation in liver tissue. Whilst the depletion of LysoPCs and SMs was specific to AD and ACLF and greater in non-survivors was associated with cell death and immune dysregulation. In addition, the elevation of LPA implicated the immunoregulatory LysoPC-ATX-LPA pathway which is subject to further investigation through *in vitro* work on monocytes isolated from ALF and ACLF patients and discussed in Chapter 6.



Chapter 6

CONCLUSIONS AND FUTURE WORK

In this thesis metabolic profiling techniques were applied to characterise global changes in plasma and serum from a unique cohort of healthy controls and patients with acute and chronic liver disease. Phenotypic differences were compared between groups, with a focus on discrimination of AD and ACLF and ALF and ACLF.

The main objective of this chapter is to distil the key findings of the research presented in this thesis and discuss how they enhance our understanding of the mechanistic differences between acute and chronic liver disease and the translational implications of these findings. In addition, the main limitations of the findings are also summarised together with future work plans.

6.1 Key Findings

Metabolic changes characterised by ^1H NMR spectroscopy and UPLCMS analysis identified changes in lipids and AAs between AD and ACLF and ALF vs ACLF. Analysis of all datasets revealed a core metabolic phenotype for ALF and ACLF, as well as, metabolic changes that were discriminant for ALF or ACLF. Overall a greater metabolic perturbation was observed for ALF compared to ACLF from the ^1H NMR spectroscopic data and quantitative UPLCMS amine data as shown by greater elevations of amino acids and energy metabolites. However, the UPLC-MS lipid data did not show this trend, instead it showed there were minor differences in metabolic perturbations of lipid species between ALF vs ACLF and an overall depletion of lipids between AD and ACLF. Moreover, a depletion of LPC and SM but not TGs was associated with survival at 30 days post admission in AD and ACLF patients, this was not observed in ALF.

6.1.1 A core metabolic phenotype of ACLF and ALF

Changes in metabolites linked to the TCA cycle, urea cycle, transsulfuration pathway, methionine metabolism and lipid metabolism were common to both ACLF and ALF. These pathways are vital for mitochondrial function and immune function and mechanisms of detoxication

An elevation of pyruvate, lactate, citrate and acetate concentrations in ACLF and ALF was likely indicative of TCA cycle perturbation and is likely associated with an upregulation of

downstream enzymes: pyruvate dehydrogenase, lactate dehydrogenase or acetyl-coA synthase. Whilst, disruption of the urea cycle was characterized by elevated amounts of ornithine and citrulline and was also linked to hyperammonemia as a result of impaired nitrogen clearance, resulting in elevated glutamine and glycine concentrations. Perturbation of the methionine cycle and the transsulfuration pathways were indicated by a significant rise in pyruvate, methionine, cystine and cystathionine in ALF and ACLF and implicated a number of enzymes that might repay further study. A final common perturbation of amino acid metabolism was a rise in 4-hydroxyproline, the mechanistic implications of this change in ALF and ACLF remain unclear.

Finally, a global depletion of circulating lipid concentrations including the triglycerides (TG), sphingomyelins (SM) and lysophosphatidylcholines (LysoPC), was also a core phenotype of ALF and ACLF. A rise in LPA (lysophosphatidic acid) was also observed in ALF and ACLF and hypothesized to be linked to cell death and immune dysfunction. Alongside a systemic depletion of LPC, a rise in LPA in both ALF and ACLF indicated an upregulation of the enzyme autotaxin, which has been observed in a previous study of liver disease.²⁴⁹

6.1.2 Metabolic changes discriminant for ALF vs ACLF

The only unique discriminatory metabolite in ACLF was serine, which was depleted. This metabolic change has been observed previously and linked to progressive fibrosis and depletion of glutathione.^{177,178} Metabolites and enzymes linked to serine metabolism which require further investigation have been identified.

In ALF a number of amino acids were selectively elevated, these included alanine, aspartic acid (aspartate), histidine and proline. The elevation of alanine and aspartic acid was linked to and disruption of the glucose-alanine cycle and urea cycle. A rise in histidine was suggested to arise from impairment of the enzyme histidine ammonia lyase in the histidine pathway. A rise in proline was associated with perturbation of the arginine and proline pathway and associated with hyperlactatemia mediated inhibition of proline breakdown to glutamate.

Unique changes in lipid metabolism included a selective rise in PE(18:2/18:2) in ALF was hypothesized to result from an inhibition of the enzyme PEMT which methylates PE to form PCs. However, this trend was only identified in one PE species and requires further

investigation across other PE species to validate this hypothesis. The precise mechanistic basis for this observation remains unclear.

6.2 Limitations

The main limitations of this project overall were the low numbers per class, including ALI, SC and ALF groups this limited analysis reducing the power of statistical testing. A further limitation was the time of sampling, where day one of admission was unlikely to be the precise point of disease development or consistently at the same point of disease development across patients. A further limitation was the variety of drugs the patients were taking which would vary according to any pre-existing medical conditions and disease aetiology of the individual. In addition, a further source of inter-individual variability are environmental and genetic influences. These limitations are unavoidable therefore strongly support a replication of the study in a larger validation cohort to reduce the effects of interindividual variability and heterogeneity. A further main limitation of the study was the lack of a non-liver disease control, which limits current interpretation of the data as some metabolic perturbations observed might be caused by mechanisms unrelated to liver disease such as systemic inflammation from sterile injury including trauma or non-sterile sepsis which is a key reason for decompensation in sepsis. Other limitations specific to the technique applied are summarised at the end of each chapter (Chapter 3, 4 & 5).

6.3 Considerations for Future Work

6.3.1 Further metabonomics analyses and validation for survival prediction

Further analysis of the UPLC-MS lipid data presented in this thesis is required including completion of metabolite identification of features targeted by tandem MS for both ESI+ and ESI- datasets and analysis of all identified features. Also required is confirmation of the metabolite IDs by carrying out experiments where stable labelled compounds are added in known concentrations because the lipid mixes do not include all relevant lipids.

In addition, analysis of additional metabonomics data generated from the same cohort of patients and volunteers requires analysis. Data generated include untargeted UPLC-MS HILIC data and targeted UPLC-MS datasets including eicosanoids, acylcarnitines and bile acids.

Analyses of these data and comparison to data presented in this thesis will broaden our understanding of metabolic perturbation in acute and chronic liver disease and. Finally, to confirm all significant metabolic changes identified in the patient classes, a comparative analysis needs to be generated on an independent validation cohort.

6.3.2 Translational application of metabolic phenotyping

To make the metabolic phenotyping technologies applied in this thesis translational, collaborations between multiple hospitals offering liver transplantation would be required.⁸⁸ Furthermore, standardized protocols for data acquisition, processing and modelling would need to be implemented to allow for comparisons between hospitals. As well as, a global approach to personalized medicine, the metabolites identified from this study and following metabolomics analysis on a validation cohort could be used to develop a new biomarker panel for clinical scoring in AD and ACLF, to either form a new clinical scoring system or to improve the current CLIF-SOFA.

6.3.3 Multi-omics approach to phenotyping ALF and ACLF

Comparison of all metabolomics data generated from this cohort can also be integrated with other data generated from these patients which including cytokine array data and circulating microRNA data. These data can then be analysed and compared simultaneously in a cell network analysis in IPA which correlates data identifies associations between specific metabolites, microRNAs or cytokines based on published data accessed by the software from the software literature database.²⁵⁰ Which will allow a more comprehensive analysis of mechanistic changes in ALF and ACLF. Network analysis summarises the data into a cell network which can be mapped to cellular locations at the membrane, cytosol and nucleus level. Molecules at these cellular locations can be mechanistically linked to changes in omics data and therefore identify the impact of global changes on downstream cell signalling molecules such as membrane bound receptors, signalling kinases and nuclear receptors.²⁵⁰

6.3.4 Approaches for further pathway characterisation

A number of enzymes hypothesised to be perturbed based on the metabolic profiling performed during the course of this research can be linked to key metabolic changes characterised and require further characterisation. These can be addressed by measuring metabolic enzyme concentrations of targets from discriminate pathways identified in this thesis from serum and liver tissue and also quantify enzyme activity of these targets from hepatocyte cell fractions. Furthermore, targeted methods to characterise and quantify changes in neighbouring metabolites from discriminate pathways identified in this thesis will further enhance pathway characterisation.

Furthermore, metabolic phenotyping of additional biofluids including ascitic fluid, faecal water, and urine will enhance our understanding of changes in metabolite pathways identified in this study in ALF and ACLF patients across biocompartments.

6.3.5 Development of monocyte cell culture protocol for metabolic phenotyping in ALF and ACLF

As we noted above LPC and LPA are immunoregulatory lipids involved in monocyte chemotaxis and activation. Furthermore, a study on a mouse model of sepsis found that LPC pre-treatment reduced pro-inflammatory cytokine production and mortality.²⁵¹ There as a prelude to comprehensive characterisation of immune metabolic interactions in these cells I have developed an SOP for ¹H NMR spectroscopic profiling of monocytes isolated from blood derived PBMCs. Based on these findings, monocyte cell culture experiments with LPC pre-treatment are being conducted in the group. In addition, I have also optimised a method, as part of this project for extraction of intracellular and extracellular metabolites from monocytes isolated from ACLF and ALF patients for metabolomics analysis.

6.4 Conclusion

This thesis sheds light on new metabolic mechanisms in ALF and ACLF. Furthermore, discriminant multivariate models for AD vs ACLF and ALF vs ACLF are computed from multiple metabonomics data generated in this thesis for the first time. Furthermore, key findings from this thesis highlight multiple metabolic pathways perturbed in ALF and ACLF, which are

identified for the first time. These are currently undergoing further investigation and enzymes mechanistically linked to these perturbations are subject to future experiments. Furthermore, findings from this thesis also have the potential for identification of novel prognostic method and therapeutic targets in liver failure.



Appendix

Clinical Score Formulae or Parameters

The following formulae or parameters were used to manually calculate the clinical scores presented in this thesis.

MELD Score = $3.78 \times \ln[\text{serum bilirubin(mg/dL)}] + 11.2 \times \ln[\text{serum INR}] + 9.57 \times \ln[\text{serum creatinine (mg/dL)}] + 6.43$ (equation accessed from²⁵²)

UKELD Score = $(5.395 \times \ln[\text{serum INR}]) + (1.485 \times \ln[\text{serum creatinine (mg/dL)}]) + (3.13 \times \ln[\text{serum bilirubin(mg/dL)}]) - 81.565 \times \ln[\text{serum Na}] + 435$ (equation accessed from¹⁵⁷)

Child Pugh Score			
Measure	1 Point	2 Points	3 Points
Total Bilirubin, mg/dL	<2	2-3	>3
Serum Albumin, g/dL	>3.5	2.8-3.5	<2.8
INR	<1.7	1.7-2.3	>2.3
Ascites	None	Mild (or medically controlled)	Moderate to severe
Hepatic Encephalopathy	None	Grade I-II	Grade III-IV

Table 32. Child Pugh Score Parameters

Adapted from (²⁵³)

SOFA Score					
Organ System	Measure	0 Point	1 Point	2 Points	3 Points
Respiratory	PaO ₂ /FiO ₂ (mmHg)	≥ 400	< 400	< 300	< 200 and mechanically ventilated
Cerebral	GCS	15	13 - 14	10 - 12	6 - 9
Cardiovascular	MAP or administration of vasopressors required	MAP ≥ 70mmHg	MAP < 70mmHg	Dopamine ≤ 5µg/kg/min	Dopamine < 5µg/kg/min; OR Epinephrine ≤ 0.1µg/kg/min OR Norepinephrine ≤ 0.1µg/kg/min
Hepatic	Bilirubin, mg/dL	< 1.2	1.2 - 1.9	2.0 - 5.9	6.0 - 11.9
Coagulation	Platelets x 10 ³ /µL	≥ 150	< 150	< 100	< 50
Renal	Creatinine, mg/dL	< 1.2	1.2 - 1.9	2.0 - 3.4	3.5 - 4.9
					> 12.0
					< 20
					> 5.0

Table 33. SOFA Score Parameters

Adapted from ⁽²⁵⁴⁾, parameters highlighted in bold are classified as organ failures; GCS – Glasgow Comma Scale; MAP - mean arterial pressure; PaO₂ – partial pressure or arterial oxygen; FiO₂ – fraction of inspired oxygen; SpO₂ – pulse oximetric saturation

CLIF-SOFA Score						
Organ System	Measure	0 Point	1 Point	2 Points	3 Points	4 Points
Respiratory	PaO ₂ /FIO ₂ (mmHg)	> 400	> 300 to ≤ 400	> 200 to ≤ 300	> 100 to ≤ 200	≤ 100
Respiratory	SpO ₂ /FIO ₂	> 512	> 357 to ≤ 512	> 214 to ≤ 357	> 89 to ≤ 214	≤ 89
Cerebral	HE-grade	No HE	I	II	III	IV
Cardiovascular	MAP or administration of vasopressors required	≥ 70	< 70	Dopamine ≤ 5 or Dobutamine or Terlipressin	Dopamine > 5 OR Epinephrine ≤ 0.1µg/kg/min OR Norepinephrine ≤ 0.1µg/kg/min	Dopamine > 15 OR Epinephrine > 0.1µg/kg/min OR Norepinephrine > 0.1µg/kg/min
Hepatic	Bilirubin, mg/dL	< 1.2	≥ 1.2 to ≤ 2.0	≤ 2.0 to < 6.0	≥ 6.0 to < 12.0	≥ 12.0
Coagulation	INR or platelet count x 10 ³ /µL	< 1.1	≥ 1.1 to < 1.25	≥ 1.25 to < 1.5	≥ 1.5 to < 2.5	≥ 2.5 or platelet count ≤ 20 x 10 ⁹ /L
Renal	Creatinine, mg/dL	< 1.2	≥ 1.2 to < 2.0	≥ 2.0 to < 3.5 / or use of RRT	≥ 3.5 to < 5.0 / or use of RRT	≥ 5.0 / or use of RRT

Table 34. CLIF-SOFA Score Parameters

Adapted from (²⁸), the CLIF-SOFA score; parameters highlighted in bold are classified as organ failures.

Abbreviations: RRT – renal replacement therapy; HE grade – hepatic encephalopathy grade; INR – international normalised ratio; MAP – mean arterial pressure;

CLIF-SOFA classification	Definition
AD	No organ failure OR Single organ failure if serum creatinine <1.5 mg/dL and no HE OR HE with serum creatinine <1.5mg/dL
ACLF grade 1	Single kidney failure OR Single liver, coagulation, circulatory or lung failure associated with serum creatinine of 1.5-1.9mg/dL and/or cerebral failure: HE grade 1-2 OR Single cerebral failure with a serum creatinine of 1.5-1.9mg/dL
ACLF grade 2	Two organ failures
ACLF grade 3	Three organ failures or more

Table 35. CLIF-SOFA criteria for AD diagnosis and ACLF grading
Adapted from (28)

¹H NMR spectroscopic analysis

¹ H Shift (δ)	Multiplicity	Molecule	CV (%)	Method of ID	Integrated Region	
					Lower ppm	Upper ppm
0.84	m	vLDL	-	literature ¹	-	-
0.87	m	LDL	-	literature ¹	-	-
0.93	t	Isoleucine	-	literature ¹	-	-
0.97	d	Valine	-	literature ¹	-	-
1.00	d	Isoleucine	2	literature ¹	1.00	1.01
1.02	d	Valine	3	literature ¹	1.04	1.05
1.29	m	LDL	-	literature ¹	-	-
1.30	m	vLDL	-	literature ¹	-	-
1.33	d	Lactate	-	literature ¹	-	-
1.48	d	Alanine	2	literature ¹	1.47	1.48
1.57	m	vLDL	-	literature ¹	-	-
1.75	m	Lysine	2	literature ¹	1.72	1.73
1.91	s	Acetate	2	literature ¹	1.91	1.92
2.03	s	Nac-g	-	literature ²	-	-
2.12	m	Glutamine	-	literature ¹	-	-
2.13	s	Methionine	-	literature ¹	-	-
2.22	m	CH ₂ CO Lipid	-	literature ¹	-	-
2.37	s	Pyruvate	1	literature ¹	2.36	2.37
2.42	m	Glutamine	3	literature ¹	2.46	2.47
2.52	dd	Citrate	4	literature ¹	2.54	2.55
2.64	t	Methionine	4	literature ¹	2.64	2.64
2.90	m	Unknown	-	-	-	-
3.03	s	Creatine	1	literature ¹	-	-
3.05	s	Creatinine	-	literature ¹	-	-
3.11	dd	Histidine	-	Spike in	-	-
3.15	dd	1-methylhistidine	-	Spike in	-	-
3.16	dd	3-methylhistidine	-	Spike in	-	-
3.22	s	Phosphatidylcholine	-	literature ²	-	-
3.25	s	Unknown	-	-	7.66	7.67
3.56	s	Glycine	4	literature ¹	3.55	3.56
3.68	s	3-methylhistidine	-	Spike in	-	-
3.98	dd	Histidine	-	Spike in	-	-
4.05	s	Creatinine	-	literature ¹	-	-
4.11	q	Lactate	2	literature ¹	4.11	4.12
4.97	s	Unknown	-	-	-	-
5.8	m	Urea	-	literature ³	-	-
5.23	d	Glucose	2	literature ¹	5.22	5.24
5.28	m	CH=CH Lipid	-	literature ¹	5.24	5.39
6.89	d	Tyrosine	-	literature ¹	-	-
6.98	s	3-methylhistidine	-	Spike in	-	-
7.00	s	1-methylhistidine	-	Spike in	-	-
7.07	s	Histidine	4	Spike in	7.05	7.07
7.18	d	Tyrosine	2	literature ¹	7.17	7.20
7.32	d	Phenylalanine	4	literature ¹	7.20	7.31
7.38	m	Phenylalanine	-	literature ¹	-	-

7.41	m	Phenylalanine	-	literature ¹	-	-
7.62	s	1-methylhistidine	-	Spike in	-	-
7.66	s	Unknown	-	literature ²	7.66	7.67
7.77	s	3-methylhistidine	-	Spike in	-	-
7.79	s	Histidine	-	Spike in	-	-
8.45	s	Formate	9	literature ⁴	8.45	8.46

Table 36. Assigned metabolites from plasma ¹H NMR spectroscopic profiles

A summary of all metabolites detectable in plasma by 1H NMR spectroscopy. Chemical shift is denoted by δ ; Multiplicity refers to type of peak detected: s- singlet; d-doublet; dd-doublet of doublets; q- quartet; m-multiplet; CV – coefficient of variation calculated from QC samples (see Chapter 3 Figure 3) ppm - parts per million;

literature¹ - Nicholson, J.K. and Foxall, P.J.D. (1995) 750 MHz ¹H and ¹H-¹³C NMR Spectroscopy of Human Blood Plasma. Analytical Chemistry;

literature² - Sands, C. J. *et al.* (2014) Metabolic Phenotyping for Enhanced Mechanistic Stratification of Chronic Hepatitis C-Induced Liver Fibrosis. American Journal of Gastroenterology;

literature³ – McPhail, M. J. W. *et al.* (2016) Multivariate metabotyping of plasma predicts survival in patients with decompensated cirrhosis. Journal of Hepatology;

literature⁴ - Saric, J., Wang, Y., Li, J. *et al.* (2007) Species Variation in the Fecal Metabolome Gives Insight into Differential Gastrointestinal Function. Journal of proteome research;

Metabolite Integral	ANOVA p-value	FDR ANOVA q-value	Post hoc Sidak p-value										
			HC vs SC	HC vs AD	HC vs ACLF	HC vs ALF	HC vs ALI	SC vs AD	SC vs ACLF	AD vs ACLF	ALF vs ALI	ACLF vs ALF	ACLF vs ALI
3-hydroxybutyrate	1.35E-07	1.60E-07	0.9954	0.0184	<0.0001	<0.0001	0.4653	0.9304	0.0938	0.2565	0.9735	0.9373	>0.9999
Unknown 6.67ppm	2.56E-07	2.86E-05	0.9494	0.0892	<0.0001	>0.9999	0.9944	>0.9999	0.2418	0.0811	0.9990	<0.0001	0.0134
Acetate	1.52E-12	2.63E-12	>0.9998	0.1179	0.0110	<0.0001	0.0019	0.2018	0.0467	0.9934	>0.9999	<0.0001	0.2148
Alanine	9.93E-05	1.05E-04	>0.9999	0.2012	0.3202	0.3766	0.9282	0.9346	0.9676	>0.9999	>0.9999	0.0009	0.2449
CH=CH lipid	2.00E-16	3.80E-15	0.9934	<0.0001	<0.0001	<0.0001	0.0442	0.0066	<0.0001	0.0003	0.9300	0.6449	0.2504
Citrate	2.00E-16	1.90E-15	0.2054	<0.0001	<0.0001	<0.0001	<0.0001	0.0004	<0.0001	0.4626	>0.9999	0.0445	0.7592
Formate	2.00E-16	1.27E-15	0.6254	<0.0001	<0.0001	<0.0001	0.0214	0.0002	0.0009	0.9999	>0.9999	0.6150	0.9825
Glucose	6.10E-02	6.10E-02	0.9903	0.0742	0.8377	0.9997	0.9997	0.9961	>0.9999	0.9004	0.9948	0.9975	0.8967
Glutamine	9.02E-14	1.90E-13	>0.9999	>0.9999	0.8095	<0.0001	0.0070	>0.9999	0.9835	0.3662	>0.9999	<0.0001	0.0581
Glycine	2.00E-16	9.50E-16	>0.9999	0.1021	<0.0001	<0.0001	0.0006	0.5331	0.0010	0.0073	0.9962	0.0001	0.8193
Histidine	4.66E-10	5.90E-10	0.9993	0.9241	0.9844	<0.0001	0.6823	>0.9999	0.8622	0.1756	0.8486	<0.0001	0.9441
Isoleucine	2.07E-10	3.03E-10	0.1292	<0.0001	<0.0001	0.0039	0.7165	0.2377	0.6404	0.9962	>0.9999	0.4504	0.8979
Lactate	1.08E-12	2.05E-12	>0.9999	0.0551	<0.0001	<0.0001	0.0017	0.7957	0.0217	0.1027	>0.9999	0.0056	0.8126
Lysine	2.00E-16	7.60E-16	0.8024	>0.9999	0.0006	<0.0001	<0.0001	0.8634	0.0003	<0.0001	>0.9999	<0.0001	0.0170
Methionine	1.44E-15	3.42E-15	>0.9999	0.9252	0.0001	<0.0001	0.0027	0.9664	0.0106	0.0053	>0.9999	0.0066	0.6827
Phenylalanine	2.00E-16	6.33E-16	0.9226	<0.0001	<0.0001	<0.0001	<0.0001	0.2308	<0.0001	0.0020	>0.9999	0.0001	0.0862
Pyruvate	7.46E-12	1.18E-11	>0.9999	0.0884	<0.0001	<0.0001	0.0758	0.7403	0.0019	0.0048	0.9900	0.5345	>0.9999
Tyrosine	2.00E-16	5.43E-16	0.7491	<0.0001	<0.0001	<0.0001	<0.0001	0.0853	0.1629	>0.9999	>0.9999	0.0001	0.5291
Valine	2.32E-10	3.15E-10	0.1561	<0.0001	<0.0001	0.0798	0.9981	0.2822	0.8212	0.9671	0.9997	0.1409	0.4588

Table 37. Summary of the statistics from 1-way ANOVA analyses of ¹H NMR integrals
 Healthy Controls (HC), Stable Cirrhosis (SC), Acute decompensation (AD), Acute-on-Chronic Liver Failure (ACLF) and Acute Liver Failure (ALF) Acute Liver Injury (ALI).
 FDR (false discovery rate) correction of ANOVA p-value was performed using the Benjamin Hochberg method, p-values reported in bold reached the significance threshold p<0.05;

Metabolite	Fold Change											
	SC vs HC	AD vs HC	ACLF vs HC	ALF vs HC	ALI vs HC	AD vs SC	ACLF vs SC	ACLF vs AD	ALF vs ALI	ACLF vs ALF	ACLF vs ALI	
3-hydroxybutyrate	1.22	1.61	2.18	2.33	1.79	1.32	1.79	1.35	1.30	0.94	1.22	
Unknown 6.67ppm	1.15	1.25	1.58	1.06	0.88	1.09	1.37	1.26	1.21	1.49	1.79	
Acetate	0.94	1.55	1.47	2.33	2.32	1.65	1.56	0.95	1.00	0.63	0.63	
Alanine	1.00	0.86	0.89	1.40	1.62	0.86	0.89	1.03	0.86	0.63	0.55	
CH=CH lipid	0.91	0.65	0.50	0.57	0.69	0.72	0.55	0.77	0.83	0.88	0.73	
Citrate	1.33	2.18	2.55	3.40	3.36	1.64	1.92	1.17	1.01	0.75	0.76	
Formate	1.37	3.11	2.73	1.99	2.00	2.27	1.99	0.88	1.00	1.37	1.37	
Glucose	1.28	1.25	1.13	1.05	0.93	0.98	0.89	0.91	1.13	1.08	1.22	
Glutamine	1.00	1.05	1.29	2.42	2.59	1.05	1.29	1.23	0.93	0.53	0.50	
Glycine	0.98	1.19	1.49	2.09	1.97	1.21	1.52	1.25	1.06	0.71	0.76	
Histidine	0.95	0.93	1.14	1.63	1.45	0.99	1.20	1.22	1.13	0.70	0.78	
Isoleucine	0.81	0.61	0.67	0.75	0.78	0.74	0.83	1.11	0.96	0.89	0.86	
Lactate	1.03	1.25	1.65	2.41	2.25	1.21	1.59	1.32	1.07	0.69	0.73	
Lysine	0.88	0.99	1.31	1.87	2.14	1.13	1.49	1.32	0.87	0.70	0.61	
Methionine	1.03	1.32	2.03	2.86	2.62	1.28	1.96	1.53	1.09	0.71	0.78	
Phenylalanine	1.20	1.53	2.15	3.24	3.60	1.28	1.79	1.40	0.90	0.66	0.60	
Pyruvate	1.04	1.30	1.79	2.20	1.86	1.24	1.72	1.38	1.18	0.81	0.96	
Tyrosine	1.24	1.71	1.66	2.58	2.29	1.37	1.34	0.97	1.12	0.64	0.72	
Valine	0.80	0.61	0.68	0.84	0.99	0.76	0.85	1.13	0.85	0.81	0.69	

Table 38. Fold change differences between classes from ¹H NMR integral data

Comparisons for fold change values reported in bold were found to be statistically significant (p<0.05) from univariate analyses on ¹H NMR integrals (Figure 2); CH=CH Lipid – determined from resonance at 5.28ppm

Targeted UPLC-MS amine analysis

Metabolite	ANOVA p-value	FDR Adjusted p-value	Post hoc Sidak p-value											
			HC vs SC	HC vs AD	HC vs ACLF	HC vs ALF	HC vs ALI	SC vs AD	SC vs ACLF	AD vs ACLF	ALI vs ALF	ACLF vs ALF	ACLF vs ALI	
4-hydroxyproline	7.92E-11	2.38E-10	7.28E-01	<1.00E-04	<1.00E-04	<1.00E-04	1.50E-03	8.89E-01	2.43E-01	7.86E-01	>9.99E-01	9.84E-01	9.22E-01	
Alanine	3.10E-10	7.61E-10	9.98E-01	9.90E-01	9.99E-01	<1.00E-04	1.06E-02	9.70E-01	9.87E-01	>9.99E-01	9.99E-01	<1.00E-04	3.80E-03	
AABA	4.63E-02	5.00E-02	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	
Arginine	5.05E-01	5.05E-01	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	
Asparagine	5.60E-13	3.02E-12	9.52E-01	2.48E-01	1.10E-02	<1.00E-04	4.00E-04	>9.99E-01	9.96E-01	9.90E-01	4.21E-01	<1.00E-04	6.67E-01	
Aspartic Acid	1.83E-05	2.91E-05	5.74E-01	9.99E-01	7.40E-02	<1.00E-04	1.53E-01	8.69E-01	>9.99E-01	5.21E-01	9.44E-01	3.42E-01	>9.99E-01	
BAIBA	1.53E-06	2.75E-06	>9.99E-01	7.68E-02	<1.00E-04	<1.00E-04	4.00E-03	9.84E-01	2.44E-01	3.62E-01	>9.99E-01	>9.99E-01	>9.99E-01	
Citrulline	3.44E-05	5.16E-05	>9.99E-01	1.34E-01	3.53E-02	<1.00E-04	3.00E-01	9.77E-01	9.06E-01	>9.99E-01	9.71E-01	8.84E-01	>9.99E-01	
Cystathionine	2.00E-16	5.40E-15	9.97E-01	<1.00E-04	<1.00E-04	<1.00E-04	<1.00E-04	5.38E-01	1.72E-01	9.91E-01	>9.99E-01	1.90E-02	3.53E-01	
Ethanolamine	2.00E-16	2.70E-15	7.33E-01	<1.00E-04	<1.00E-04	<1.00E-04	<1.00E-04	3.67E-01	1.00E-02	2.87E-01	9.48E-01	7.88E-01	>9.99E-01	
Glutamic Acid	1.38E-02	1.55E-02	1.43E-01	>9.99E-01	>9.99E-01	2.84E-01	4.05E-01	3.50E-01	8.57E-02	9.88E-01	9.99E-01	2.35E-01	1.66E-01	
Glutamine	2.13E-07	4.42E-07	9.45E-01	9.99E-01	9.99E-01	<1.00E-04	2.39E-01	7.81E-01	8.05E-01	>9.99E-01	5.79E-01	1.00E-04	6.49E-01	
Glycine	2.63E-11	8.88E-11	>9.99E-01	5.07E-01	2.61E-01	<1.00E-04	2.00E-04	9.39E-01	8.59E-01	>9.99E-01	9.75E-01	<1.00E-04	1.09E-01	
Histidine	2.50E-10	6.75E-10	9.91E-01	>9.99E-01	9.31E-01	<1.00E-04	4.86E-02	9.99E-01	>9.99E-01	9.98E-01	2.35E-01	<1.00E-04	5.34E-01	
Isoleucine	6.58E-05	8.88E-05	9.99E-01	<1.00E-04	3.25E-02	1.07E-02	1.11E-01	3.85E-01	9.90E-01	5.56E-01	>9.99E-01	>9.99E-01	>9.99E-01	
Leucine	1.05 E-03	1.35E-03	>9.99E-01	3.00E-04	7.91E-02	8.39E-01	9.96E-01	2.38E-01	8.72E-01	7.97E-01	>9.99E-01	9.26E-01	9.72E-01	
Lysine	1.06E-15	9.54E-15	9.16E-01	>9.99E-01	4.49E-01	<1.00E-04	<1.00E-04	9.58E-01	2.35E-01	5.24E-01	9.87E-01	<1.00E-04	9.40E-03	
Methionine	2.77E-12	1.07E-11	9.99E-01	6.15E-02	<1.00E-04	<1.00E-04	9.00E-04	9.92E-01	3.57E-01	4.84E-01	4.99E-01	4.52E-02	>9.99E-01	
Ornithine	3.76E-06	6.35E-06	9.99E-01	1.06E-01	3.60E-03	<1.00E-04	2.91E-01	9.97E-01	8.68E-01	9.92E-01	5.04E-01	6.10E-01	>9.99E-01	
Phenylalanine	2.45E-12	1.10E-11	6.70E-01	8.40E-03	<1.00E-04	<1.00E-04	<1.00E-04	>9.99E-01	9.15E-01	6.52E-01	9.93E-01	1.68E-01	9.92E-01	
Proline	5.16E-07	9.95E-07	>9.99E-01	6.78E-01	7.18E-01	<1.00E-04	1.46E-02	9.99E-01	>9.99E-01	>9.99E-01	9.83E-01	1.50E-03	4.50E-01	
Sarcosine	1.10E-01	1.14E-01	9.94E-01	8.77E-01	9.94E-01	8.26E-01	9.77E-01	>9.99E-01	>9.99E-01	>9.99E-01	4.84E-01	3.26E-01	>9.99E-01	
Serine	3.61E-03	4.24E-03	7.84E-01	9.97E-01	2.81E-02	5.20E-01	>9.99E-01	9.83E-01	2.50E-02	8.30E-03	8.34E-02	2.00E-04	4.37E-01	

Threonine	5.19E-13	3.50E-12	>9.99E-01	9.68E-01	>9.99E-01	<1.00E-04	1.40E-03	9.99E-01	>9.99E-01	9.99E-01	6.09E-01	<1.00E-04	7.70E-03
Tryptophan	1.15E-03	1.41E-03	>9.99E-01	1.50E-02	3.00E-04	4.48E-02	2.44E-02	4.74E-01	1.40E-01	9.90E-01	9.84E-01	8.01E-01	>0.9999
Tyrosine	4.94E-09	1.11E-08	8.50E-01	2.13E-02	3.90E-03	<1.00E-04	1.20E-01	>9.99E-01	9.99E-01	>9.99E-01	9.70E-02	1.67E-02	>0.9999
Valine	4.91E-05	6.98E-05	>9.99E-01	1.00E-04	9.00E-04	1.06E-01	7.44E-01	8.30E-02	1.73E-01	>9.99E-01	>9.99E-01	8.06E-01	8.76E-01

Table 39. Summary of univariate analysis of quantified and semi-quantified amino compound concentrations

A 1-way ANOVA was performed on log transformed data ($\log_{10}(X) + 1$), followed by a post-hoc Sidak test. Summarised in the table are all the p-values determined for all the ANOVA analyses per compound and the FDR adjusted q-value determined using the Benjamin Hochberg method. The significance threshold was <0.05; these are highlighted in bold font. N.D. – not determined.

Amino Compound	Fold Change										
	SC vs HC	AD vs HC	ACLF vs HC	ALF vs HC	ALI vs HC	AD vs SC	ACLF vs SC	ACLF vs AD	ALF vs ALI	ACLF vs ALF	ACLF vs ALI
4-hydroxyproline	1.7	2.9	4.0	3.6	3.4	1.8	2.4	1.4	1.1	1.1	1.2
Alanine	1.2	0.9	1.0	2.5	2.4	0.8	0.9	1.1	1.0	0.4	0.4
AABA	1.2	1.6	1.4	1.6	2.3	0.8	1.1	1.4	0.7	0.8	0.6
Arginine	0.8	0.9	1.0	1.5	1.1	1.1	1.2	1.1	1.4	0.7	1.0
Asparagine	1.3	1.4	1.7	3.4	2.8	1.1	1.3	1.2	1.2	0.5	0.6
Aspartic Acid	1.6	1.1	1.6	2.0	1.6	0.7	1.0	1.4	1.2	0.8	1.0
BAIBA	1.4	7.3	8.6	4.3	4.3	5.2	6.2	1.2	1.0	2.0	2.0
Citrulline	1.2	1.6	1.7	2.1	2.0	1.3	1.3	1.0	1.0	0.8	0.8
Cystathionine	2.4	8.4	10.4	25.8	21.8	3.5	4.3	1.2	1.2	0.4	0.5
Ethanolamine	1.9	4.1	5.7	7.4	7.7	2.2	3.0	1.4	1.0	0.8	0.7
Glutamic Acid	2.3	1.1	1.1	1.3	1.6	0.5	0.5	0.9	0.8	0.8	0.7
Glutamine	0.8	1.2	1.2	2.9	2.1	1.5	1.6	1.1	1.4	0.4	0.6
Glycine	0.9	1.3	1.4	2.5	2.2	1.3	1.4	1.1	1.1	0.6	0.6
Histidine	1.2	1.1	1.2	2.6	1.9	0.9	1.0	1.1	1.4	0.5	0.7
Isoleucine	0.9	0.6	0.8	0.7	0.7	0.6	0.9	1.4	1.0	1.1	1.1
Leucine	1.0	0.6	0.8	0.9	1.0	0.6	0.8	1.4	1.0	0.9	0.9
Lysine	0.8	1.0	1.4	2.5	2.4	1.3	1.7	0.6	1.1	0.5	0.6
Methionine	1.3	2.6	4.6	6.9	5.1	2.1	3.6	1.8	1.3	0.7	0.9
Ornithine	1.3	1.5	2.2	2.7	2.1	1.2	1.8	1.5	1.3	0.8	1.1
Phenylalanine	1.6	1.8	2.5	3.7	3.4	1.1	1.6	1.4	1.1	0.7	0.7
Proline	1.1	1.3	1.3	2.3	2.4	1.2	1.2	1.0	1.0	0.6	0.5
Serine	1.3	1.1	0.8	1.3	1.1	0.9	0.6	0.7	1.2	0.6	0.8
Sarcosine	0.6	0.7	0.8	1.5	0.6	1.1	1.3	1.2	2.4	0.5	1.3
Threonine	1.0	1.2	1.2	2.8	2.4	1.2	1.2	1.0	1.2	0.4	0.5
Tryptophan	1.1	0.7	0.6	0.8	0.6	0.7	0.6	0.9	1.3	0.8	1.1
Tyrosine	1.6	2.0	2.0	3.5	2.2	1.3	1.3	1.0	1.6	0.6	0.9
Valine	1.1	0.6	0.7	0.8	0.9	0.6	0.6	1.1	0.9	0.9	0.8
1-methylhistidine	2.1	3.0	4.0	1.6	3.2	1.4	1.9	1.3	0.5	2.4	1.2
3-methylhistidine	0.4	2.0	0.9	0.2	0.0	5.0	2.4	0.5	11.6	5.2	59.8
Aminoadipic acid	0.9	1.2	1.0	1.9	2.0	1.3	1.1	0.8	0.9	0.6	0.5
Beta-alanine	1.05	1.12	1.06	0.99	1.02	1.07	1.01	0.94	0.97	1.07	1.04
Cystine	2.0	2.1	1.8	2.0	2.5	1.1	0.9	0.8	0.8	0.9	0.7
GABA	4.0	2.8	3.5	3.2	3.9	0.7	0.9	1.2	0.8	1.1	0.9

Homoserine	1.7	2.3	3.1	3.3	2.8	1.4	1.8	1.3	1.2	0.9	1.1
Hydroxylysine	1.1	1.3	1.3	2.2	2.3	1.2	1.2	1.0	1.0	1.2	0.6
Taurine	1.0	1.2	0.8	1.4	1.6	1.2	0.9	0.7	0.9	0.6	0.5

Table 40. Fold change differences between classes per quantified amino compound

Comparisons for fold change values reported in bold were found to be statistically significant (p<0.05) from univariate analyses on amino compound concentrations (SI Table 5) and peak areas (SI Figure 1); Semi-quantified compounds are indicated in the table in italic.

Amino Compound	Detectable	Fold Change											
		SC vs HC	AD vs HC	ACLIF vs HC	ALF vs HC	ALI vs HC	AD vs SC	ACLIF vs SC	ACLIF vs AD	ALF vs ALI	ACLIF vs ALF	ACLIF vs ALI	
5-aminovaleric acid	Yes	1.7	10.2	24.7	14.2	6.6	6.0	14.4	2.4	2.2	1.7	3.7	
Allantoin	Yes	1.3	2.3	3.3	1.1	1.4	1.7	1.70	1.0	0.8	1.4	2.3	
Creatine	Yes	0.9	1.0	1.0	1.0	1.0	1.0	1.1	1.1	1.0	1.0	1.0	
Creatinine	Yes	1.0	0.9	1.0	1.0	1.0	0.9	1.0	1.1	1.0	1.0	1.0	
Cysteine	Yes	1.0	1.0	0.9	0.9	1.0	1.1	0.9	0.8	0.9	0.9	0.8	
Glycylglycine	Yes	0.9	2.7	2.5	2.51	2.1	2.9	2.8	1.0	1.2	1.0	1.2	
Methylamine	Yes	0.8	0.8	1.1	1.2	1.2	1.0	1.4	1.4	1.0	0.9	0.9	
N-methyl-phenylalanine	Yes	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	
o-phosphoethanolamine	Yes	0.9	0.9	0.9	0.9	0.9	1.0	1.0	1.0	1.1	1.0	1.0	
Tryptamine	Yes	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	
3-hydroxyanthranilic acid	No	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	
3-hydroxykynurenic acid	No	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	
3-hydroxytyramine	No	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	
4-aminohippuric acid	No	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	
Anserine	No	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	
Epinephrine	No	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	
Galactosamine	No	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	
Glutathione	No	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	
Homocysteine	No	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	
Homocystine	No	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	
N-methyl-L-valine	No	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	
Octopamine	No	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	
Ophthalmic acid	No	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	
Putrescine	No	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	

Table 41. Fold change differences between monitored amino compounds

Fold change values reported in bold were determined as statistically significant ($p < 0.05$) from univariate analyses on amino compound peak areas; Compounds not detected are indicated in the table at the bottom in italic. N.D. – not determined.

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