Protocol development for donor liver function assessment during end-ischaemic normothermic machine perfusion as a platform for transplant viability testing and therapeutics evaluation

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Declaration

This thesis was submitted to the University of Birmingham for the degree of Doctor of Medicine.

Dedication

This thesis is dedicated to Alexia, my parents, my brother, Thomas, and my sister, Sophie. I am forever grateful for your constant understanding and support.

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Contents

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ACR	acute cellular rejection
ADMA	asymmetric dimethylarginine
ADP	adenosine diphosphate
ALT	alanine aminotransferase
AST	aspartate aminotransferase
ATP	adenosine triphosphate
BMI	Body Mass Index
CA	cholic acid
CDCA	chenodeoxycholic acid
CE	capillary electrophoresis
CEAD	coulometric electrochemical array detection
CHA	common hepatic artery
CIT	cold ischaemia time
DBD	donation after brainstem death
DCD	donation after cardiac death
DRI	donor risk index
EAD	early allograft dysfunction
ECD	extended criteria liver
ETC	electron transport chain
FDR	false discovery rate
FTCIR	Fourier transform infrared spectroscopy
fWIT	functional warm ischaemia time
GABA	gamma-aminobutyric acid

GC	gas chromatography
GC	glycocholic acid
GCDCA	glycochenodeoxycholic acid
GDCA	glycodeoxycholic acid
GPC	glycophosphocholine
HILIC	Hydrophilic Action Liquid Chromatography
HPLC	high-performance liquid chromatography
HR-MAS	high-resolution magic angle spectroscopy
INR	international standardized ratio
IPA	propan-2-ol
IPF	initial poor function
IRI	ischaemia reperfusion injury
LC	liquid chromatography
LPV	left portal vein
m/z	mass to charge ratio
MELD	Model of End-Stage Liver Disease
MHV	middle hepatic vein
MMA	monomethylarginine
MS	Mass Spectrometry
NAD	nicotinamide adenine dinucleotide
NHSBT	National Health Service Blood and Transplant
NMP-L	normothermic machine perfusion of the liver
NMR	primarily Nuclear Magnetic Resonance Spectroscopy
NO	nitric oxide
PC	phosphocholine

PCA	Principal Components Analysis
PGD	primary graft dysfunction
PNF	primary non-function
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-analyses
PRS	post reperfusion syndrome
PT	Prothrombin time
PV	portal vein
QC	quality control
RHA	right hepatic artery
ROS	reactive oxygen species
RPLC	Reverse Phase Liquid Chromatography
RPV	right portal vein
RSD	Relative Standard Deviation
SCS	static cold storage
t0.25	sample obtained within first 15 minutes of perfusion
t2	sample obtained at two hours of perfusion
t4	sample obtained at four hours of perfusion
TCA	taurocholic acid
TCBA	taurine-conjugated bile acids
TCDCA	taurochenodeoxycholic acid
TDCA	taurodeoxycholic acid
UHPLC	Ultra-High Performance Liquid Chromatography and Mass Spectrometry
UPLC	ultra performance liquidchromatography

UW	University of Wisconsin Solution
VITTAL	Viability Testing and Transplantation of Marginal Livers

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Abstract

Donor organ shortage has increased reliance on extended criteria donor livers for transplantation. Simultaneously, the development of machine perfusion technology has caused a paradigm shift in organ preservation practices from cold storage metabolic suppression to dynamic organ functional support. This platform has provided opportunities to assess the transplant viability of these extended criteria organs based on functional parameters rather than static donor variables. Machine perfusion has therefore been shown to provide superior organ preservation than static cold storage. This has in turn led to an increase in basic science and clinical research with the development of perfusion protocols designed to optimise liver function in various ways. However, this research is dominated by small series of liver perfusions due to the scarcity of donor organs for research. This, coupled with the singularity of individual livers, has restricted translation into clinical medicine. The VITTAL clinical trial indicated that the viability assessment potential of normothermic machine perfusion increases the utilisation of extended criteria grafts. Using high throughput analytical technologies, a metabolomic study was conducted in tandem with the clinical trial to elucidate the molecular pathways that influence extended criteria donor metabolic behaviour and organ viability during perfusion. Key metabolic pathways were discovered, relating lipid pathways, fatty acid metabolism and amino acid metabolism with links to anti-inflammatory and gluconeogenic pathways. A second study was a proof-of-concept experiment which sought to tackle the aforementioned issues surrounding research perfusions by exploring the basic science implications of normothermic split liver perfusion. In addition to doubling the number of perfusion experiments; metabolic, perfusion and histological characteristics were comparable between split lobes from the same liver. This demonstrated the perfusion protocol's potential

in providing internal controls for the assessment of therapeutics, perfusion techniques and optimisation protocols.

Chapter 1 Introduction: A Historical Perspective of Human Orthotopic Liver Transplantation, present challenges, and the development of dynamic organ preservation

1.1 A Historical Perspective of Liver Transplantation

The success story of liver transplantation is rooted in a series of landmark events that have shaped the landscape of this life-saving procedure over the last 60 years. Initial research into liver transplantation began with the work of Thomas Starzl, who experimented with liver transplant techniques in dogs(1). In 1963, Starzl et al. published the first three attempts at human liver transplantation, a surgical procedure involving the removal of a diseased liver and replacing it with a healthier one from a human donor(2). Unfortunately, the first patient died on the operating table secondary to haemorrhage, and the remaining two patients survived for 22 and seven days respectively(2). It was not until 1968, when a further series of seven cases was published, that the procedure demonstrated extended survival, with the case of a 19-month-old girl diagnosed with hepatocellular carcinoma who survived for 13 months after surgery before dying from metastatic disease(3).

In 1968, the publication of a report entitled "A Definition of Irreversible Coma" by the Ad Hoc Committee of the Harvard Medical School led the acceptance of irreversible coma secondary to brainstem death as a legal definition of death(4). This was a landmark event which led to a more controlled procedure for procurement of the liver for transplantation from a human donor(4). This allowed organ procurement under ideal physiological conditions while the donor's heart was still beating and circulation was intact, thereby improving graft quality and recipient survival. Despite this, organ rejection by the recipient's immunological defences remained an issue(5). From 1968 until the late 1970s, immunosuppressive regimens were based mainly on steroids and azathioprine, and the overall one-year survival rate for liver transplantation did not exceed 30%. This changed with the introduction of the calcineurin inhibitor ciclosporin in 1979 by Calne, which helped achieve clinically superior

immunosuppression(6-8). Along with improved recipient selection and surgical techniques, this development resulted in one-year survival rates of 70%(5). However, it was not until 1983 that the US National Institutes of Health established, by consensus, that liver transplantation was to be considered clinically as definitive therapy for end-stage liver disease(9). In 1988, the development of the University of Wisconsin cold storage solution provided an organ preservation medium that safely extended *ex vivo* organ preservation time, thereby permitting organ sharing between centres, increasing procurement of suitable grafts and allowing a smoother surgical procedure(10). In 1989, five-year survival rates post-liver transplant reached as high as 64%(5). In the late eighties and early nighties, the immunosuppressant tacrolimus was introduced. It was shown to be more effective than ciclosporin at reducing acute cellular rejection and improving graft and patient survival(11, 12). It has subsequently become the gold standard immunosuppressive regimen for liver transplant patients(5).

Current one-year and five-year survival rates for liver transplantation are as high as 85% and 72% respectively(13). This is in sharp contrast to end-stage liver disease if left untreated, with a five-year survival rate of 50% in the advanced stages of liver cirrhosis(14). The procedure's success has led to the establishment of hundreds of liver transplant centres worldwide. Nevertheless, liver transplantation has become a victim of its success. With the gradual expansion of indications, more and more patients are being referred to centres for consideration of transplantation. Consequently, the demand for donor livers has exceeded its supply(15). In the UK alone, the number of patients on the transplant waiting list has almost doubled in the last decade(13). Many countries are witnessing high waiting list mortality rates, prompting centres worldwide to develop alternative organ procurement, preservation, and allocation strategies, which are all prominent areas of research in liver transplant surgery today(16).

1.2 Indications for Liver Transplantation

The history of liver transplantation lays bare its multifaceted success as a surgical procedure. Advances in immunosuppression regimens, organ preservation, surgical technique and intensive care management have all transformed it into the gold-standard curative treatment for liver conditions which otherwise cannot be treated with alternative therapies(5). These include end-stage liver disease secondary to liver-based metabolic defects and cirrhosis, acute fulminant hepatic failure, and early-stage hepatocellular carcinoma(17). Worldwide, the leading aetiology for liver transplantation is infection with hepatitis C(18). However, the development of highly effective antiviral therapies and the declining prevalence of infection has caused a steep decline in waitlist registrations and transplants for this condition(19). In developed countries, with shifting lifestyles, dietary habits, and alarming increases in obesity rates, non-alcoholic fatty liver disease has become the most common aetiology of chronic liver disease and is fast becoming a major indication for liver transplantation(20).

The timing of referral for transplantation has also evolved over the years. Originally, waiting time was essential to a patient's position on the transplant list(21). In 2002, the Model of End-Stage Liver Disease (MELD) was implemented by the United Network for Organ Sharing in the United States for determining donor liver allocation. The MELD score is a validated mathematical scoring system determined from a patient's serum bilirubin, serum creatinine and international standardised ratio (INR), shown to accurately predict a patient's risk of three-month mortality from liver failure without access to a liver transplant(22). At the time of its introduction, this score was viewed as an objective and standardised measure of a transplant candidate's disease severity, irrespective of aetiology or waiting time, enabling donor organs to be allocated according to clinical urgency(23). In 2008, the UKELD score was developed

for use in the United Kingdom. Its formula was similar to MELD with the addition of the candidate's serum sodium and is still used to predict survival for patients listed for liver transplantation today. These models, however, have their limitations. Most notably, they were developed with the sole purpose of predicting death from liver failure(24). They do not accurately represent the risk of death from conditions such as primary liver cancer and score exceptions are granted to these patients.

1.3 Donor Organ Procurement

Presently, deceased patients are the principal source of donor livers. This may occur under two circumstances – donation after brainstem death (DBD) or after cessation of circulation and cardiac death (DCD)(25). DBDs are considered the ideal source, as procurement is allowed to proceed while the donor's heart is still beating. Therefore, the liver remains perfused with warm oxygenated blood until the organ is disconnected from the donor's circulation(25). In DCD, circulatory arrest must be confirmed before donation can proceed, exposing the liver to a period of warm ischaemia until surgically removed(26). Following procurement, the organ is stored in cold preservation fluid and ice (static cold storage, SCS) for delivery to the implanting centre. The hypothermic temperatures (0 - 4 ^{o}C) drastically reduce the metabolic requirements of the liver, thus lessening the impact of ischaemic damage before implantation(25).

1.4 Organ Donor Characteristics

The pioneering cases of liver transplantation were performed with donors following cardiac death, during which confirmation of circulatory arrest had to be given before donor organ

procurement could proceed. The landscape of liver transplantation was permanently altered by the acceptance of brainstem death as a legal definition of natural death.

Catastrophic brain injury, due to spontaneous or traumatic events, causes irreversible brain injury. However, it spares the organs of the rest of the body, such as the heart, for some time while being supported by intensive care-based medical management. These organs cease to function if this support is discontinued, and patients progress to natural death. Neurological death confirmed by brainstem death, was therefore considered to be a natural death. Confirmation of death in this scenario allowed for organs to be procured while circulation was intact, altering the landscape of organ donation practice(25).

For a time, most DBDs were victims of catastrophic traumatic neurological events. These were commonly young victims of road traffic accidents who were confirmed to be brain-dead on arrival at the hospital. Being young, often healthy, individuals without significant comorbidity, their liver was considered optimal quality and, therefore, ideal to ensure the best clinical outcome for the recipient. Subsequent improvements in road and vehicle safety measures over the last few decades have significantly reduced the number of deaths attributed to motor vehicle accidents. In the Annual Transplant Activity Report 2017-2018, the National Health Service Blood and Transplant (NHSBT) stated that donors have become older, more obese and die more frequently from non-traumatic causes(27). In the United Kingdom, between 2008 and 2018, there was an eight per cent increase in the proportion of donors with a body mass index (BMI) greater than 30 kg/m^2 and a nine per cent increase in donors older than 69 years. The effect has been a shortage of ‘ideal’ donor livers available for transplantation(27).

1.5 The Extended Criteria Donor Liver

In order to tackle the discrepancy between donor organ supply and demand, many centres have now turned to extended-criteria donor (ECD) liver procurement for transplantation to expand the donor pool(28). An ECD liver is associated with an increased risk of poor function or failure that may expose the recipient to a greater risk of morbidity and mortality. Donor organs with steatosis, organs exposed to prolonged preservation times or organs from elderly donors are more vulnerable to preservation and ischaemia-reperfusion injury (IRI) and, therefore, are referred to as ECD organs(28). There is no specific consensus on the factors defining an ECD liver, which are heavily influenced by the local population demographics and transplant centre expertise. However, commonly accepted factors which have been shown to negatively impact graft performance include donor age of 60 years or older, cold preservation times longer than 10 hours, macrovesicular steatosis, donation after cardiac death, donors with an infection risk or a history of previous malignancy and split liver grafts(28, 29). The latter is a well-recognised surgical approach in transplantation to increase organ availability by dividing the liver into two separate independently-functioning units, thus enabling two recipients to "share" the same organ. Although a split liver may be obtained from a standard or "ideal" criteria donor, splitting it creates two smaller-volume ECD grafts, with a subsequently higher risk of dysfunction than the parent graft(29). A study of 611 liver transplant recipients revealed that age greater than 65 years, macrovesicular steatosis on biopsy of more than 40% and cold preservation time longer than 14 hours were identified as significant risk factors of inferior patient and graft survival(30). However, ECD livers have also been recognised as essential to fill the gap between organ supply and demand, especially due to evolving changes in donor demographics.

1.6 Ischaemia-Reperfusion Injury: An Overview

A central focus of donor liver selection for transplantation is the organ's perceived ability to tolerate a period of hypoxia once deprived of its blood supply and withstand reperfusion when implanted into the recipient. This is a consequence of IRI, the paradoxical tissue damage on organ reperfusion following a period of ischaemia(31). Indeed, developments in effective organ preservation methods pre-transplantation have roots in strategies to attenuate IRI-induced cellular injury(31). It is a pathophysiological phenomenon that targets hepatocytes, sinusoidal cells and cholangiocytes, impacting liver function and predisposing to graft dysfunction. The mechanisms involved are highly complex, with many cellular components, factors and mediators(31). Despite their clinical relevance, the pathophysiology has yet to be fully understood(32, 33).

The traditionally accepted model of liver IRI is represented in two stages, a local ischaemic insult followed by inflammation-mediated reperfusion injury. The first stage, or ischaemic injury, is a localised metabolic disturbance initiated by 'cold' injury which develops *ex vivo* and occurs because of damage to the hepatic sinusoidal endothelium and disruption of the hepatic microcirculation during procurement and cold preservation(32). 'Warm' injury, the second stage, occurs during the implantation process when the liver reaches physiological temperatures and is triggered *in vivo* by hepatocellular damage. Ischaemia and hypoxia interrupt the mitochondrial electron transport chain (ETC), the main ATP and energy production line of the cell under aerobic conditions. This halts respiration which therefore results in a depletion of available ATP. There is also an acceleration of anaerobic glycolysis, lactate production and changes in H^+, Na^+ and Ca^{2+} homeostasis, compromising hepatocyte integrity(32). The second stage occurs on completion of the surgical anastomoses with the restoration of blood flow to the liver, hence the term "reperfusion injury"(32).

As a consequence of the first stage, subsequent rewarming and reintroduction of oxygen kickstart the ETC with undirected electron transfer, resulting in the generation of reactive oxygen species(31, 32). This leads to an accumulation of cellular metabolic disturbances with oxidative stress and parenchymal cell death. Furthermore, the sudden availability of oxygen further accentuates oxidative stress on restoring blood flow, and the rush of blood generates an intense sterile inflammatory response consisting of both direct and indirect cytotoxic mechanisms(31, 32). This includes leukocyte and platelet sequestration to the endothelium, activation of liver Kupffer cells, transmigration of neutrophils, the release of endogenous inflammatory mediators, and several other factors. These, in turn, are permeate throughout the circulation with systemic consequences(31, 32) (**Figure 1**).

IRI effects are influenced by the integrity of the liver's cellular compartments, which may be compromised by donor age, trauma, infection, and pre-existing medical conditions. The liver's ability to attenuate IRI determines its ability to function in the recipient. IRI that overwhelms the donor liver will lead to early allograft dysfunction (EAD) or, worse, primary non-function(31, 32). Experiments with ECD livers have demonstrated the higher production of reactive oxygen species (ROS) in these organs and, therefore, their increased susceptibility to oxidative stress(20). Furthermore, IRI severity following reperfusion has been shown to correlate directly with the duration of ischaemia. This pathophysiological event has consequently generated a considerable body of research to elucidate its effects on organ viability for transplantation(32).

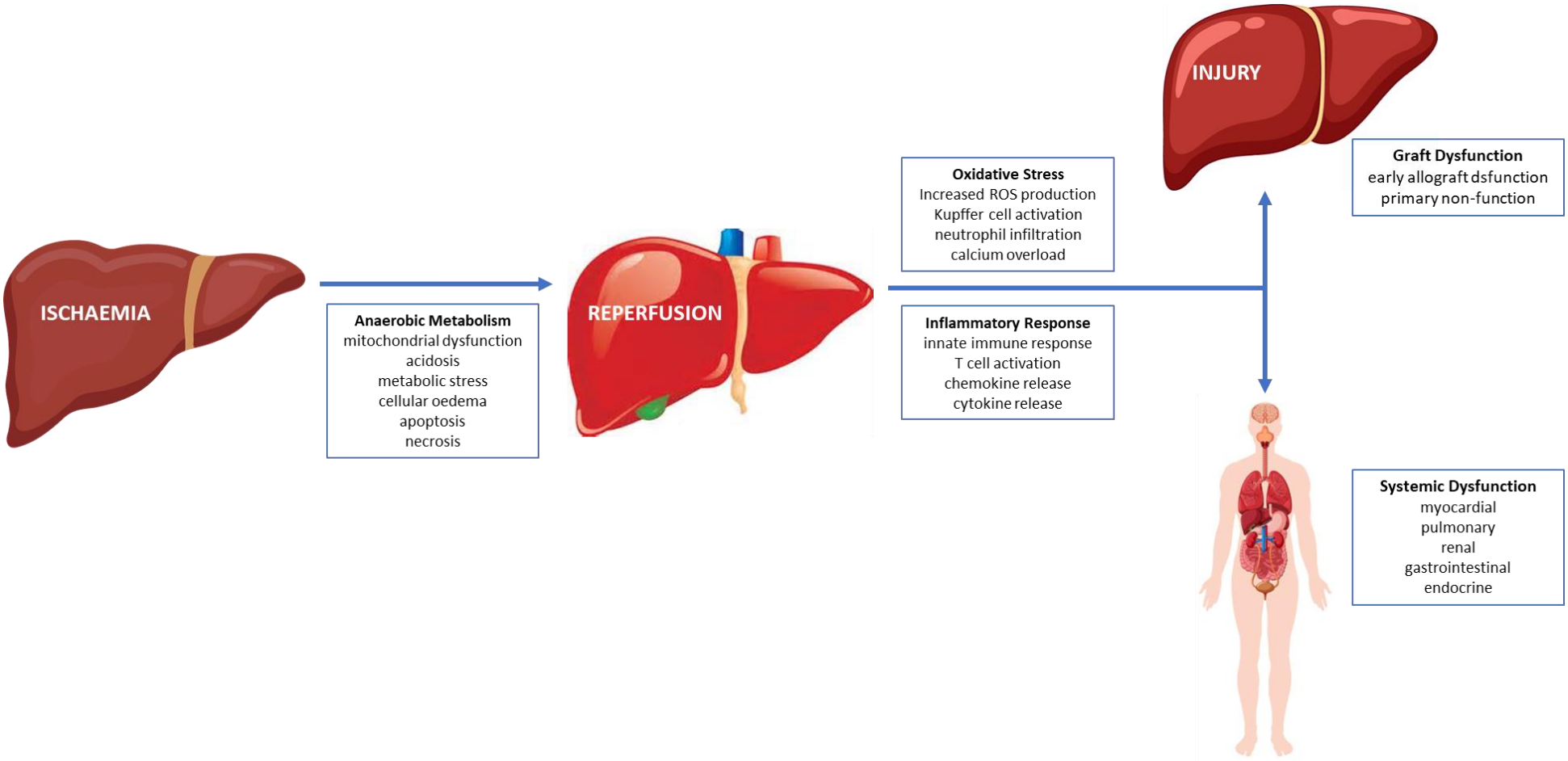

Figure 1. IRI in the liver. Ischaemia results in mitochondrial dysfunction and the accumulation of toxic metabolic products in hepatocytes that are released into the circulation on reperfusion, accelerating oxidative stress and generating an inflammatory response with local and systemic consequences.

1.7 Organ Viability Assessment for Transplantation

In its broadest, when applied to a cell or organism, viability may refer to that which is alive or capable of living. In biological experiments, viability indicates a treated sample's ability to exhibit a specific function expressed as a proportion of the same function exhibited by the same sample before treatment. Cell viability refers to the number of live, healthy cells in a sample, commonly expressed as a percentage(34). Hence, in transplantation, organ viability represents the ability of a functioning donor organ to reproduce its function in the recipient(35).

In basic science research, viability assays determine the ability of cells or tissues to maintain or recover a state of survival. Various markers are employed to measure cells' physical and physiological health in response to external stimuli, chemical agents, or therapeutic treatments or when determining optimal growth conditions in cell culture(34). Likewise, several markers, found to correlate with peri-operative clinical events, have been put forward in organ transplantation to assess organ viability and, therefore, suitability for transplantation(35).

As outlined previously, assessment of donor liver viability for transplantation traditionally involves the appraisal of donor history and biochemistry prior to procurement, followed by visual inspection of the organ *in situ(36)*. If needed, this may be supported by histological assessment of tissue biopsies, most commonly employed to determine the degree of steatosis(37). The decision to procure and transplant is aided by prognostic indices developed from multivariate analyses of extensive donor datasets and transplantation outcomes to estimate the risk of graft failure. These indices exist to help reduce the level of uncertainty about the suitability of a particular graft for transplantation(36). However, they do not eliminate it entirely. Moreover, their use has increasingly been shown to be unreliable and is responsible

for the underutilisation of potential donors(36). Donor liver viability assessment for transplantation remains a hot topic in the field and has been recognised as the key to improving the utilisation of ECD and, therefore, riskier livers.

1.8 Strategies to Improve Extended Criteria Donor Liver Utilisation

1.8.1 Evidence-based Prognostic Scores

The increased use of ECD livers initially resulted in significantly reduced graft and patient survival for unfavourable donor-to-recipient matching categories. However, several studies demonstrated that careful ECD graft selection and recipient allocation provide comparable results to so-called “ideal” donor grafts. This cannot be ignored, especially since changing donor characteristics, such as the progressive ageing of the population, are increasing the proportion of ECD livers, making them a significant resource in the donor pool. This has prompted the development of several clinical prognostic indices to balance the risk of allocating high-risk livers to recipients on the waiting list.

The donor risk index (DRI), developed by Feng et al. in 2006, considered donor variables known to adversely affect transplant outcomes and scored livers based on the cumulative effect of these parameters(38). Notable limitations of this score were that it relied heavily on donor age and only considered data at the time of procurement. Additionally, it did not assess the interaction of donor parameters with recipient characteristics(39). Research into combined donor-recipient prognostic indices produced scores such as the D-MELD, which uses donor age and MELD(40), and the balance-of-risk (BAR), which considers MELD, recipient age, re-transplantation, life support dependence prior to liver transplantation, donor age and duration of cold storage(41). More recently, the UK DCD score has been developed. This score uses

donor age, donor BMI, functional donor warm ischaemia, duration of cold storage, recipient age, recipient MELD and re-transplantation to detect high-risk and futile combinations of donor-and-recipient factors in DCD liver transplantation and improve the utilisation of these livers(42).

It is important to note that these prognostic indices mainly focus on the risk of graft loss and recipient death while on the waiting list rather than the recipient's physiological reserve to recover during the post-operative period following a liver transplant. This adds another layer of complexity to the decision to transplant ECD livers in sicker patients. Unfortunately, there is little consensus on how this should be approached. Moreover, to offset risk, many centres may allocate riskier livers to "less" sick patients, and this raises another ethical dilemma as it may expose these individuals unnecessarily to post-operative complications.

1.8.2 Donor Liver Machine Perfusion and Dynamic Organ Preservation

Over the last decade, machine perfusion of the donor organ has become a promising alternative strategy for organ preservation and utilisation(43). The first *ex situ* perfusion experiments were carried out in the 1930s by Alex Carrell and Charles Lindenberg who demonstrated the viability of various organs for several days while being perfused with normothermic oxygenated serum(44, 45). Further experiments were carried out in animal models in the 1960s, most notably by Thomas Starzl. However, this technology was put on the back burner due to the logistics involved in developing it further for clinical application. Later in the 1980s, University of Wisconsin Solution (UW) was introduced, a preservation fluid shown to attenuate liver injury and improve outcomes in cold-stored livers. This facilitated the safe storage and transport of these organs and, subsequently, SCS became the organ preservation gold standard.

Research into dynamic organ preservation technology was revived in the late 1990s with the recognised need to investigate alternative sources of donor organs and the increasing use of ECD livers for transplantation(45). In 2001, a noteworthy study by Schon et al, using a porcine model of transplantation, demonstrated the feasibility of ex-situ liver preservation and successful transplantation using normothermic machine perfusion of the liver (NMP-L) after these livers were exposed to one hour of warm ischaemia(46). NMP-L, conducted by Imber et al, was shown to enhance the preservation of pig livers and improve bile production, glucose metabolism, galactose clearance and factor V production compared to SCS controls(47). Brockmann et al, also using a porcine perfusion model, reported factors predictive of a successful outcome, namely bile flow, threshold values of transaminases, portal flow and resistance(48). The benefits of NMP-L were found to be threefold. Firstly, it provided superior organ preservation to conventional SCS as it shortened the period of ischaemia and appeared to alleviate the damaging effects of IRI. Secondly, it enabled the assessment of liver function in real-time under near-physiological conditions. Lastly, it provided a platform for the resuscitation and reconditioning of these organs(48, 49).

Since its reintroduction into liver transplantation, machine perfusion technology has evolved significantly to include various perfusion devices, perfusate temperatures, media, modalities, and protocols. It can be performed *in vivo* during procurement or *ex vivo* once the organs have been harvested. Furthermore, it has been employed to completely replace SCS as an organ preservation method or following a period of SCS after the organ is transported to the implanting centre for transplantation, also known as the end-ischaemic method. *Ex vivo* experimentation with different perfusion temperatures has generated three main perfusion platforms: hypothermic machine perfusion (4 – 10^{o}C), NMP-L (35 – 37 ^{o}C) and sub-normothermic machine perfusion (21^{o}C)(35, 43, 50).

1.9 Normothermic Machine Perfusion

NMP-L is a dynamic organ preservation platform whereby the liver is perfused with a warm oxygenated perfusion fluid at physiological temperatures(49). It is generally performed using a perfusate based on packed red blood cells as an oxygen carrier, enriched with nutrient substrate allowing the liver to recover and maintain its metabolic capacity in an extracorporeal manner. The perfusate also requires components to maintain physiological oncotic pressure and osmolarity. These have included fresh frozen plasma, gelofusine or Steen solution(49). Other additives are anti-thrombotic agents, such as low molecular weight heparin, and acid-base agents to reduce cellular swelling, cholestasis, microvascular injury, and ROS effects. Several NMP-L devices are available, which mainly consist of the following components: a sterile containment unit for housing the liver, a perfusate reservoir, oxygenators, physiological pressure pumps, sensors monitoring physiological parameters and tubing systems to connect to the vessels(49).

There is a multitude of published perfusion protocols, but the fundamental principle of NMP-L has been the ability to assess donor organ function in real time at physiological temperatures during *ex vivo* preservation. It has shifted organ viability assessment for transplantation from relying solely on static donor and procurement variables to the appraisal of dynamic organ metabolism. This is proving to be an invaluable tool for increased ECD liver utilisation, as risk could be determined based on continuous monitoring of objective markers of the organ's functional capacity. The advantages of NMP-L, however, are not just restricted to viability assessment(20). An extensive body of research is developing with regard to the administration of pharmacological agents with the aim of organ reconditioning and possibly improving organ quality, such as in IRI mitigation or defatting therapies to mobilise lipid stores and reduce

steatosis. Recently published work has also demonstrated its potential in facilitating transplant logistics by extending organ preservation time beyond what was traditionally acceptable. Nevertheless, the platform does have its drawbacks. As physiological temperatures are maintained during NMP-L, technical problems disrupting perfusion would expose the liver to the detrimental effects of warm ischaemia *ex vivo*. Furthermore, normothermic perfusion has been shown to simulate the conditions of IRI in the liver, exposing it to ROS and activation of the inflammatory cascade(35).

1.10 Machine Perfusion as a Tool for Viability and Therapeutics Assessment

1.10.1 Viability Assessment

As a dynamic organ preservation technique, machine perfusion technology has gained momentum in part due to its ability to operate as a platform for the viability assessment of donor livers based on the monitoring of metabolic and, thus, functional parameters in real time. This has been most evident with NMP-L, where organ assessment occurs at physiological temperatures(36).

To date, various viability markers have been investigated and proposed in the context of liver transplantation(32, 35, 36). These include metabolic parameters such as in blood gas analysis, bile analysis, measurement of vascular flows and transaminase levels. It is important to note, however, that most of these parameters, and their suggested thresholds, have only been examined in comparatively small clinical series of liver transplantations and have, therefore, yet to be validated in larger cohorts(35). Consequently, a limited number of events obscures the correlation of biomarkers with post-operative outcomes(35).

Furthermore, the current published evidence lacks uniformity between studies, in terms of patient recruitment, perfusion protocols and viability criteria. Another confounder is the different perfusion modalities employed in centres: hypothermic, sub-normothermic and normothermic. While the evidence points towards all techniques benefitting ECD livers in different ways, controversy remains as to which is the best platform(35). Moreover, the liver is among the most complex organs in the human body, with many physiological functions as it oversees thousands of metabolic reactions across different tissues. The intricate nature of liver physiology, further compounded by organ preservation and transplantation, has made evaluating potential viability markers challenging(32).

In the context of liver transplantation, the ideal marker would predict the extent of EAD, PNF or ischaemic cholangiopathy in the liver. It is most likely that a combination of markers will be able to satisfy such a demanding requirement rather than one in isolation(32). These would generate a composite score similar to that provided by currently employed prognostic indices based on quantifying different parameters. This score would allow the surgeon to match the risk of graft failure to the risk of mortality in the recipient should they not proceed with transplantation, thus supporting the decision-making process between the patient and clinician(32). For this reason, clear parameter thresholds linked to clinically relevant and standardised outcomes must be established.

Watson et al. illustrated that viability assessment must incorporate various compartments: hepatocellular, cholangiocyte, vascular and immune cells(36). Currently, the most prominent parameter employed to test viability during NMP-L in the literature is perfusate lactate, a by-product of anaerobic metabolism during ischaemia. Periportal hepatocytes are the main

contributors to lactate metabolism, and their location in zone 1 of the liver means that they are the last cells to suffer hypoxia. High perfusate lactate would therefore imply global ischaemia and liver hypoperfusion(36). For this reason, perfusate lactate has been described as evidence of severe hepatocellular dysfunction, as lactate metabolism is an ATP-dependent process(35). Hepatocyte-function-based criteria have also focused on the regulation and maintenance of acid-base balance(36). This not only takes into account the metabolism of organic acid anions, such as lactate, but also the metabolism of amino acids, such as glutamine, by glutaminase, a pH-dependent enzyme, to produce ammonia fed into the urea cycle. Urea synthesis occurs in all zones in the liver, and dysregulation of ammonia metabolism impairs the liver's ability to regulate pH, leading to acidosis(36). Proposed cholangiocyte viability criteria have included the assessment of bile flow, composition and pH. It is hypothesised that cholangiocyte physiological processes will predict the risk of ischaemic cholangiopathy. Bile is a cumulative result of these processes(36). Vascular resistance is another parameter frequently quoted in clinical studies. Disruption of the hepatic endothelium increases vascular resistance(36). It is well known that vascular resistance is initially high when a liver is removed from SCS and reperfused but rapidly falls as flow is established. Consequently, persistently high vascular pressures during machine perfusion may imply significant endothelial disruption and translate into global hypoperfusion(36).

Hypothermic machine perfusion maintains the organ at a low metabolic rate, so assessing function using this perfusion technique poses a challenge(35). However, studies of hypothermic machine perfused livers have demonstrated high tissue ATP content post-perfusion(35). Subsequently, recent viability assessment during hypothermic machine perfusion has focused on mitochondrial function and proposed flavin mononucleotide, a molecule in mitochondrion complex 1, as a potential marker of viability. Flavin

mononucleotide is released during organ perfusion from the same area as reactive oxygen species. It may therefore be linked to the severity of IRI and mitochondrial dysfunction, which itself may reflect the risk of EAD(35). This link, however, has yet to be established in the rigor of a clinical trial.

1.10.2 Therapeutics Assessment

Machine perfusion has been recognised as an effective method of liver resuscitation, optimisation and viability assessment for transplantation. However, the higher risk of graft dysfunction and post-operative complications in ECD livers has highlighted the need for improved methods to ameliorate IRI in these organs. Subsequently, machine perfusion has also demonstrated its potential as a platform for basic scientific research into hepatic IRI and the effect of pharmacological agents and therapeutic interventions on graft function and viability(51). This will undoubtedly continue to expand its applications as well as its acceptance as a more reliable method of organ preservation for liver transplantation.

The use of administered therapeutics during machine liver perfusion has shown promising results in many basic science studies. Unfortunately, many of these remain limited to animal-based experiments(51). Notable areas of therapeutics research in animal models include vasodilator administration impact on IRI, manipulation of RNA interference pathways to silence specific genes implicated in IRI and employing defatting cocktails to reduce steatosis and mobilise lipid stores for energy utilisation(51). The latter has also shown encouraging results in studies involving discarded human donor livers(52).

It is clear however that, while novel therapeutic approaches are being developed through basic science research, their use in clinical medicine and treatment in patients for liver transplantation has yet to be fully explored. While these studies can be used to optimise perfusion protocols, discarded human donor liver research is frequently criticised due to the heterogeneity between donors and small sample size, making satisfactory comparisons difficult, and thus limiting application to human clinical trials and translation to clinical medicine(51).

1.11 Organ Viability Testing and Metabolomics

1.11.1 Omics

Another area of research that has gained traction in recent years in liver transplantation is the application of omics-based high-throughput bioinformatics tools, primarily Nuclear Magnetic Resonance Spectroscopy (NMR) and Mass Spectrometry (MS), to donor liver metabolism during organ procurement, preservation and implantation(53, 54). This has been studied extensively in relation to transplant organ viability with the principal aim of identifying markers of graft function and dysfunction that may be applied as objective clinical tests to predict graft and recipient outcomes before transplantation(53, 54). As previously outlined, while routine clinical chemistry and biochemistry diagnostic tests in transplantation do exist, they rely on specific markers that are often only one parameter related to one functional aspect of the liver and have been shown to be unreliable when predicting graft outcomes. The complex physiology of the transplanted liver means that there is unlikely to be one single marker that is up to the task. For this reason, bioanalytical multi-omics technologies have been increasingly employed in analysing donor liver samples(53, 54). These platforms can be applied to a biological system of interest to obtain a high-resolution snapshot of the underlying biology and

have enabled the simultaneous analysis of thousands of cellular markers during organ transplantation.

Many areas of research can be classified as omics. Examples include proteomics, transcriptomics, genomics, metabolomics, lipidomics, and epigenomics which correspond to the global analyses of proteins, RNA, genes, metabolites, lipids, and methylated DNA or modified histone proteins in chromosomes, respectively(53). There are many motivations for conducting omics research. One common reason is to comprehensively understand the biological system under study(55). Another goal of omics studies is to use this understanding to associate omics-based molecular measurements with a clinical outcome of interest(55). The rationale is that by taking advantage of omics-based measurements, there is the potential to develop a more accurate predictive or prognostic model of a particular condition or disease. Metabolomics in particular has featured prominently in this regard in liver transplantation(56).

1.11.2 Metabolomics

Metabolomics is the global study of metabolites in human biofluids and tissues through high-throughput analytical technologies(55, 57). Metabolites are low molecular weight compounds (less than 2000 Da in size) that comprise sugars, amino acids, lipids, organic acids, and nucleotides, all of which drive cellular metabolism. The quantitative collection of all metabolites in a system is known as the metabolome. The human metabolome itself is currently known to consist of more than 100,000 metabolites and counting. Its composition is the result of upstream influence from higher levels of cellular function: proteins in the proteome, mRNA in the transcriptome, and DNA in the genome, as well as external stimuli(55, 57). Starting at the cell's genetic constitution (or genotype), interactions between all these levels encode

cellular structure and function. Additional influence from external stimuli produces its biological phenotype or expressed physical traits(55, 57). **Figure 2** illustrates the different levels of function and how they interact. Study of the metabolome has become a key feature in understanding and deciphering disease pathophysiology. Metabolite turnover in a cell is such that changes in composition occur over a matter of seconds, as opposed to days or hours, with proteins and messenger RNA(55, 57). Therefore, metabolite analysis could provide a snapshot of alterations in cellular metabolism as they occur.

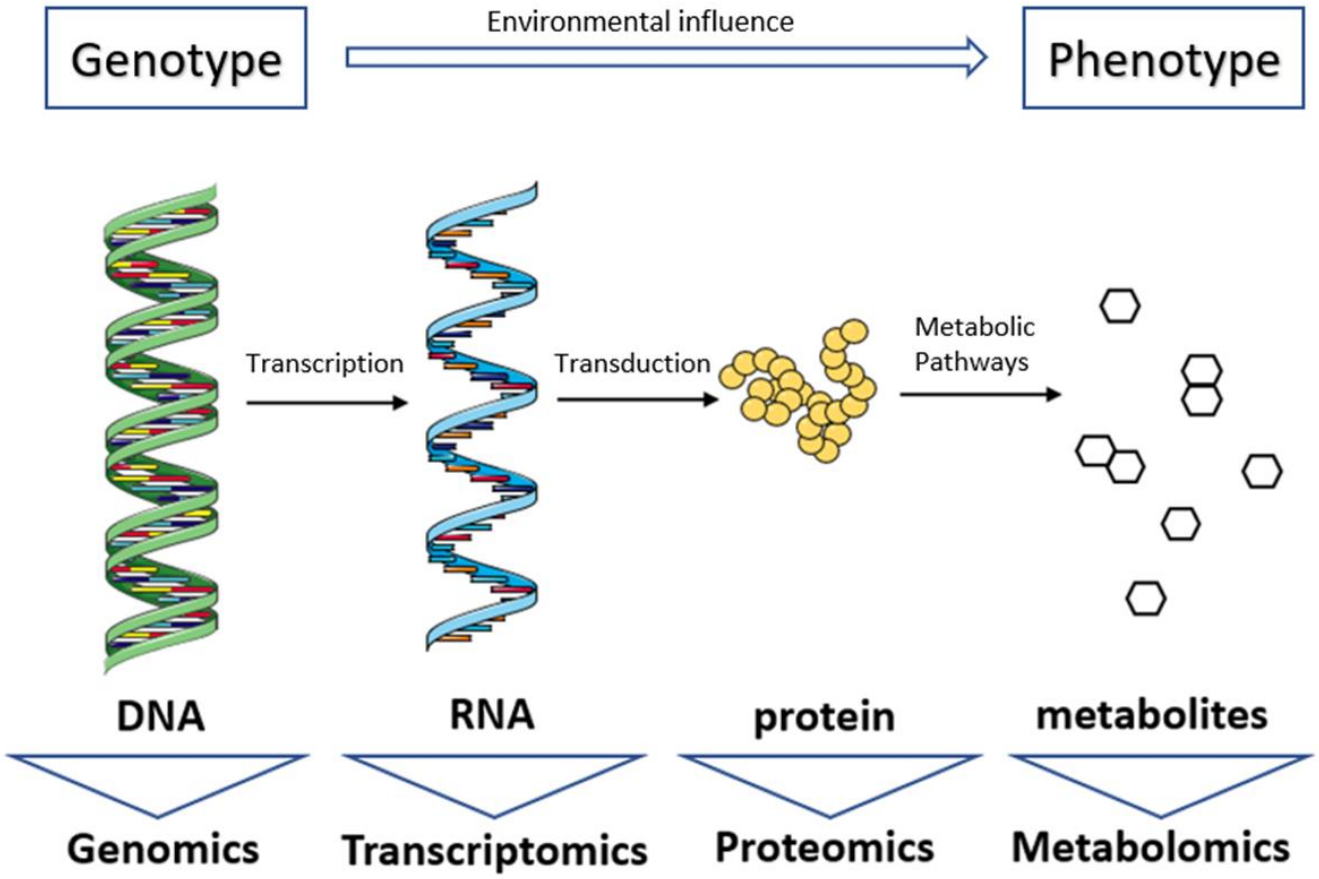

Figure 2. The 'Omics' Cascade illustrating the different levels of cell function and their relationship to the 'Omics' analytical sciences. Further down the cascade influence from environmental factors (external stimuli) increases. Therefore, the metabolome is more closely related to the biological phenotype than the genome

1.11.3 The Application of Metabolomics to Organ Transplantation

Metabolomics has been shown to be an invaluable tool in characterising the metabolic changes that occur in donor organs(54). A search of the published literature will reveal many metabolomic studies in all manners of solid organ transplantation, including kidney, liver, heart, pancreas, and lungs(54). These studies have mainly focused on specific circumstances: post-reperfusion damage, graft rejection and graft dysfunction(54). Interestingly, various metabolic perturbations that have been identified in these situations have been linked to the most basic and common of cellular pathways, such as glycolysis, gluconeogenesis, lipid metabolism and amino acid metabolism(54). Changes in these metabolites, such as glucose, citrate, lactate, and ATP, reflect alterations in cell viability and homeostasis in general. These molecules provide information about cell function or cell stress and, therefore, about organ function. Moreover, clinical assays for many of these metabolites are widely available.

1.11.4 Metabolomics of Donor Liver Transplantation

The link between donor liver metabolism during organ procurement, cold preservation and transplantation and post-transplant graft dysfunction has been extensively studied. These studies have identified intermediates in several critical metabolic pathways, including amino acid and nitrogen metabolism, anaerobic glycolysis, lipid breakdown products, purine biosynthesis, energy metabolism and bile acid composition as potential markers of graft dysfunction. These will be covered in a systematic review of the literature in Chapter 2.

Experimental NMP-L research has identified critical metabolic changes in steatotic livers compared to their non-steatotic counterparts, namely impaired glutathione synthesis, increased

fatty acid oxidation and increased ketone body production(58, 59). Most clinical NMP-L series have focused on perfusate lactate clearance, pH maintenance, glucose metabolism, and several other functional parameters(36). Perfusion studies have indicated that higher liver ATP content and recovery before transplantation correlate with favourable transplant outcomes(36). However, the metabolic changes in ECD livers during NMP-L in the clinical setting remain under-explored(60). Metabolomics may provide an in-depth analysis of the metabolic changes that influence organ viability during the perfusion process and may contribute to developing other objective markers to increase confidence in these livers while providing safer care for patients.

1.12 Aims of this Thesis and Thesis Outline

The objective of the research described in this thesis was to explore protocol development for the assessment of donor liver function during end-ischaemic NMP-L. The first part of this thesis focuses on the application of metabolomics to donor liver viability testing. A systematic review examines metabolomics studies that have thus far been performed in the context of liver transplantation. Critical appraisal of these studies is accompanied by a comprehensive description of the putative metabolic markers of graft function identified, their involvement in critical metabolic pathways and their link to clinical outcomes. Avenues for future research are also identified. This is followed by original work relating to the UHPLC-MS-based metabolomic analysis of the 31 ECD livers included in the VITTAL trial, a peer-reviewed phase 2 liver transplant trial which demonstrated that viability testing of ECD livers with NMP-L is feasible and enables the objective assessment of these organs in a functional capacity pre-transplantation.

The second part of this thesis describes a novel NMP-L proof-of-concept study protocol using split liver grafts to address the limitations of current NMP-L studies when assessing perfusion techniques and therapeutic interventions. It was developed following lessons learnt from the VITTAL trial, with the aim of addressing the limitations of current machine perfusion research and establishing an alternative methodology for providing suitable comparative controls in the evaluation of liver metabolic function and therapeutic interventions during NMP-L.

Chapter 2 Systematic Review of the Literature: Liver Transplantation, Metabolomics, its Role in Clinical Research and Prospective Future

The work outlined in this chapter represents original research work performed by the author, which has been peer-reviewed and published:

OMICS. 2019 Oct;23(10):463-476. doi: 10.1089/omi.2019.0086.

Figures and tables have been adapted from the original manuscript.

2.1 Introduction

2.1.1 The Metabolomic Experiment Workflow

The principal aim of metabolomics is translating metabolite data from biological samples to biological knowledge(55, 57). However, as metabolomics provides a global view of this data, the latter is heavily influenced by several factors: the definition of groups in the population under study from which the samples will be derived (e.g., disease versus control), the sample type, the analytical instrument set-up, and the method of sample quenching, that is the procedure employed for halting metabolic processes in the samples to be analysed(55, 57, 61).

The initial analysis is usually untargeted, a broad sweep of all low molecular weight compounds in the biological sample(55). Any patterns that emerge in the data help generate hypotheses to explain the observed variance in the proportions of various metabolites. These hypotheses can subsequently be tested using a targeted analysis, which, as the name implies, focuses on a specific set of metabolites(55, 62). **Table 1** summarises the main differences between the untargeted and targeted analysis. Complex bioinformatics tools isolate single or groups of metabolites that demonstrate the most abundance variation between samples(63). The points of maximal variation are termed principal components, and the process is a principal component analysis (PCA). It is important to note that this analysis scrutinises the data irrespective of the group of origin of the sample. If the investigator wishes to examine the data against predefined groups of samples, a partial least squares discrimination analysis is performed(63). **Figure 3** provides an illustrated overview of the metabolomic experiment.

Table 1. Main features of Untargeted and Targeted Metabolomic Analyses

UNTARGETED ANALYSIS	**TARGETED ANALYSIS**
Global sample screening for a large number of metabolites, relative and not absolute concentrations determined	Identification and quantification of a small number of metabolites
Hypothesis-generating	Hypothesis-testing
Experimental design appropriate for global detection of metabolites in a sample	Experimental design adapted for optimal detection of a subset of metabolites in the metabolome
Metabolite identity not known prior to data acquisition; the data acquired is used to identify unknown metabolites	Metabolite identity is known prior to data acquisition, the data acquired is used to confirm the identity

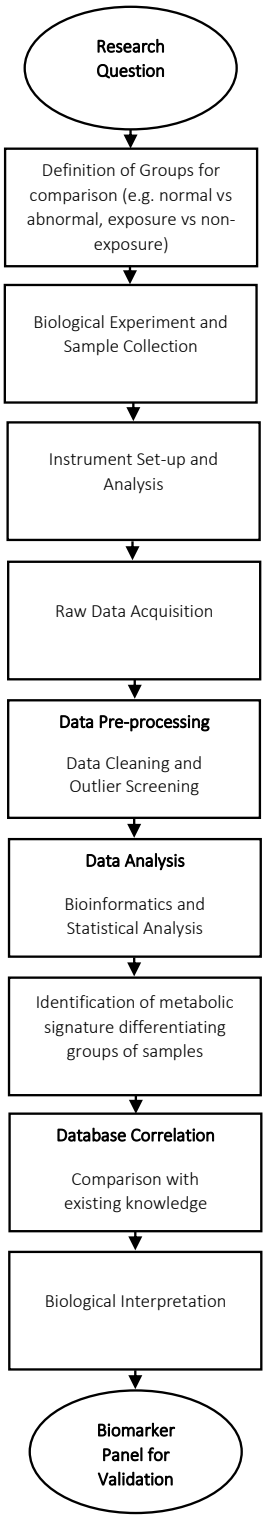

Figure 3. The metabolomic workflow, illustrating the process of the metabolomic experiment from experimental design to data analysis and interpretation. The high sensitivity of metabolomic analytical instruments in detecting metabolites means each step prior to raw data acquisition must be carefully orchestrated to eliminate bias in the results.

2.1.2 Analytical Technologies

Metabolomics research has been driven forward by advances in two analytical platforms—nuclear magnetic resonance spectroscopy and mass spectrometry (55, 57). A summary of the main features of these platforms is provided in **Table 2**. MS is being increasingly employed in metabolomic investigations due to its higher sensitivity, ability to detect thousands of metabolites simultaneously, and its enhanced chemical identification capabilities when coupled with chromatographic separation(55).

Table 2. Main differences between the two major metabolomic analytical platforms

	NMR	**MS**
Sample Preparation	Minimal	Extensive
Reproducibility	High	Low
Quantification	Quantitative	Qualitative and quantitative
Relative Sensitivity	Low	High
Metabolite coverage	Low	High

2.1.3 Metabolomics in Liver Transplantation

Donor liver metabolic activity during organ procurement, cold preservation and transplantation has been studied extensively concerning IRI and post-transplant graft dysfunction(53, 54). These studies have identified intermediates in several critical metabolic pathways. These include metabolites of amino acid and nitrogen metabolism, anaerobic glycolysis, lipid catabolism, purine biosynthesis, energy metabolism and bile acid composition that may represent potential markers of graft dysfunction(53, 54). This systematic review examines the

published literature employing metabolomic analytical platforms in liver transplantation. The results reported from these studies are scrutinised against the metabolomic experiment workflow. All existing putatively identified molecular markers of graft dysfunction are examined, while discussing the associated principal metabolic pathways.

2.2 Methodology for Systematic Review of the Literature

2.2.1 Literature Search

The Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) protocol(64) was employed to conduct a literature search for peer-reviewed studies that reported on the metabolomic findings related to transplanted livers' clinical outcomes. MEDLINE and EMBASE OvidSp databases were consulted using the Medical Subject Heading (MeSH) terms *metabolomics*, *liver* and *transplant*. The titles and abstracts of studies were screened, and full texts of potentially relevant articles were retrieved. The reference lists of these articles were also scrutinised to identify other relevant studies.

Experimental studies investigating the metabolomic profiles of human biological samples from donor livers and transplant recipients were included in this review. The inclusion criteria were outlined: human donor liver studies investigating transplanted organs, applying metabolomic-based analytical platforms, reporting clinical outcomes of the investigated grafts, and published in English. Exclusion criteria included animal models, in vitro cell culture studies, other *omics* (proteomics, transcriptomics, and genomics), liver disease metabolomics and studies of non-transplanted donor livers. The information gathered from the full texts of all included articles were: first author, publication year, study design, metabolomic analytical platform, sample/population size, sample type, methodology, clinical outcome, and metabolite data. A

quantitative meta-analysis of the data was not undertaken due to the limited number of studies available and the significant variation in methodology, analytical platforms, multivariate analysis techniques and reporting of clinical outcomes.

2.2.2 Quality Assessment

QUADOMICS, an adaptation of QUADAS (Quality Assessment Tool for Diagnostic Accuracy Studies), was explicitly designed to appraise studies employing omics-based diagnostic research (65, 66) (see **Appendix 1** for tool template). Studies were listed according to the phase of biomarker discovery, which influences the number of items in the tool applied to each study(65). The percentage of applied items which scored positively in each study, as well as the percentage of each item that scored positively across all, were considered. Single case studies were not included in the quality assessment.

2.3 Results

2.3.1 Literature Search

The PRISMA flowchart for the literature search and selection process is outlined in **Figure 4**.

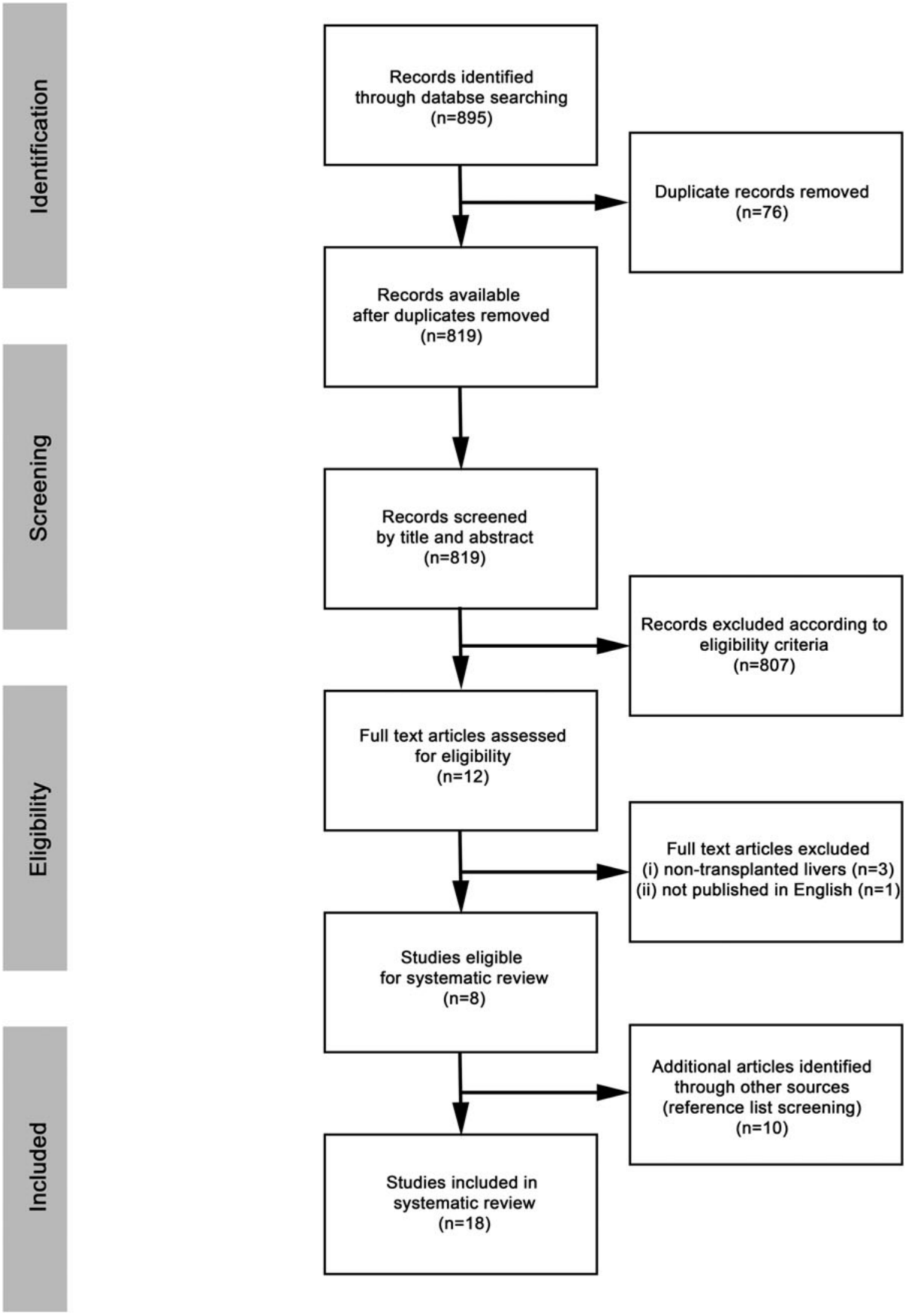

Figure 4. PRISMA flowchart outlining the search strategy for this systematic review.

2.3.2 Study Characteristics

The analytical platforms used in these studies were NMR, MS coupled to liquid chromatography (LC-MS), direct infusion MS, capillary electrophoresis fingerprinting and coulometric electrochemical array detection. Sample types consisted of blood, dialysate fluid from liver parenchyma, bile and liver tissue and most studies were cohort observational studies, followed by case series and single case reports. Notably, different criteria for the diagnosis of graft dysfunction were used. **Table 3** outlines the main characteristics of each study, including the main results and conclusions.

2.3.3 Quality Assessment of Included Studies

All studies were Phase 1 or exploratory studies of biomarker discovery. QUADOMICS items assessing patient spectrum representation (item 2) and clinical data availability (item 14) were therefore not applicable to these studies. In addition, none included a patient flow diagram detailing the patient selection process, and only one study scrutinised the observational data against an external validation set. All studies interpreted the index test results with knowledge of the reference standard outcome (see **Table 4**).

2.4 Discussion

2.4.1 The Effect of Sample Type

The liver's various functions can be broadly categorised into intermediary metabolism (carbohydrate, lipid, nitrogen and energy metabolism), protein synthesis, bile secretion, immunological activity, hormone degradation and xenobiotic detoxification(67). In addition, this organ is the primary driver of a wide array of crucial metabolic pathways, including glucose

balance, fatty acid metabolism, amino acid metabolism, ammonia detoxification and bile acid synthesis, among others. These occur across several compartments: the hepatocellular, cholangiocyte, immunological and vascular compartments, and involve different biological matrices; tissue, parenchymal fluid, bile, blood and serum(67). Consequently, the application of metabolomics to the assessment of liver function is a complex undertaking.

Interpretation of changes in metabolites must be made in the context of the biological sample analysed(55). Blood is a readily available bodily fluid which does not require chemical pre-treatment or an extractive step for metabolite quantification. Sampling is also the least invasive when compared to other sample types. However, during liver transplantation, the only sampling moments are during liver perfusion by the donor circulation, which may be too early for a decision to transplant, or recipient circulation, which would be too late. It is also the furthest sample matrix away from the liver metabolome. Bile secretion may be useful as a biofluid that is a direct product of liver metabolism, but its secretion following organ retrieval is variable and limited in quantity.

Parenchymal interstitial fluid is close to the liver metabolome and is more representative. Nevertheless, current extraction protocols involve mixing with a dialysate fluid, which may interfere with sample analysis. Tissue sampling is the most representative of the liver metabolome. Biopsies can be taken at any point during organ retrieval, preservation and transplantation and data obtained from samples can be correlated with histology from the same biopsy. However, it is an invasive procedure that may damage the organ if not done cautiously. It may also provide a heterogeneous view of the liver metabolome, particularly in poorly perfused or steatotic livers.

The growing use of NMP-L has introduced perfusate as another viable sample type; the perfusate injected into the perfusion device permeating the organ during NMP-L. Sample extraction is non-invasive and permits larger quantities due to the volume of perfusate required for the machine to function. Furthermore, in the case of NMP-L, samples can be obtained immediately before and while the liver is metabolically active at physiological temperatures. However, current protocols for perfusate composition vary according to operator preferences, and metabolomic data analysis must be conducted with full knowledge of the perfusate's constituents and their proportions to tease out metabolite perturbations that reflect changes in the liver metabolome.

2.4.2 Defining Graft Dysfunction

As illustrated in **Table 3**, most liver transplantation metabolomic studies in the medical literature use graft dysfunction as the primary clinical outcome. Primary graft dysfunction refers to poor functional recovery of a donor liver post-reperfusion. Generally detected by biochemical derangements in the blood, it occurs over varying degrees of severity. It is broadly categorised into early allograft dysfunction (EAD), the least severe, and primary non-function (PNF), the most. EAD refers to transient clinical and laboratory abnormalities that reflect the liver's poor functional status. PNF is a life-threatening, irreversible loss of graft function, manifested by high serum transaminases, coagulopathy, hyperlactataemia, hypoglycaemia, haemodynamic instability and multiorgan failure. While EAD is associated with lower graft and patient survival, it is a potentially recoverable condition. PNF, on the other hand, requires emergency re-transplantation. Despite the relevance of these conditions, there is currently no standardised definition of EAD or PNF. Many studies have attempted to establish the

parameters of these events, leading to varying diagnostic criteria and cut-offs(68-70), as can be appreciated in **Table 3**.

The most commonly employed set of criteria in this systematic review was that as defined by Clavien et al, followed by Strasberg et al and Olthoff et al. Three studies did not state the criteria used. One study did not have any livers that suffered graft dysfunction post-operatively, and the remaining reported other primary clinical outcomes. This inconsistency in clinical outcomes and lack of a standardised investigative approach poses a challenge to the global assimilation of metabolic data and their relevance to clinical outcomes. The following discussions will attempt to gather and rationalise the reported observations based on sample type to recognise patterns that may be significant for future research.

2.4.3 Liver Transplant Metabolomics

Blood

In a case report, Singh et al proposed the post-transplantation monitoring of glutamine and urea levels in the serum and urine as a predictor of graft dysfunction(71). The authors analysed the serum and urine from an 11-year-old male patient who underwent living-donated liver transplantation, in which a living person donates a portion of their liver, for decompensated Budd Chiari syndrome. In this case, the left lobe of the liver was donated by his 35-year-old mother. Recipient serum and urine samples were obtained after one and five hours post-operatively, followed daily until the fifth postoperative day. On the second postoperative day, the recipient developed portal vein thrombosis requiring operative intervention. On the third day, he developed portal vein and hepatic artery thrombosis and needed re-exploration. The authors commented that the liver appeared grossly normal during the procedure. However,

there was re-thrombosis of both vessels the same day postoperatively, and the patient's condition continued to deteriorate until he died on the sixth day. ^{1}H-NMR analysis revealed higher serum glutamine levels in both serum and urine as well as a decrease in urea in the urine on the third day following surgery. These observations corresponded with the decline in liver function, as measured by routine biochemical tests: serum alanine transferase (ALT), aspartate aminotransferase (AST), lactate and INR. Glutamine is hydrolysed in hepatocytes to provide ammonia and glutamate for urea synthesis and gluconeogenesis. The urea cycle is the principal mechanism for nitrogenous waste clearance in the liver, converting ammonia to urea that is expelled in the urine(71). Ammonia detoxification and gluconeogenesis are primary hepatic functions mutually connected through amino acid metabolism. The liver is rich in glutamate dehydrogenase, an enzyme catalysing the reversible oxidative deamination of glutamate to α-ketoglutarate (a key metabolite in the Krebs cycle) and ammonia, thereby bridging amino acid–to–glucose pathways(71). Therefore, the findings reported by Singh et al. suggest that impairment of glutamine metabolism and the urea cycle may be linked to graft dysfunction and affect clinical outcome(71).

Serkova et al analysed serum samples, using ^{1}H-NMR, from the same recipient who underwent two consecutive liver transplantations for hepatitis B complicated by hepatocellular carcinoma. The first graft, a right lobe from a living-related donor procedure, failed due to portal vein and hepatic artery thrombosis(72). A whole DCD graft was used for the second procedure. Blood samples were also taken from four healthy male subjects as baseline controls for comparison. Interestingly, PCA demonstrated separation of the blood metabolic profile of the first failed transplant as early as two hours post-operatively, when routine liver function tests were still unremarkable. The metabolic profile of the second transplant clustered with those of the healthy volunteers indicating similar metabolite compositions. The metabolites responsible for

this separation were lactate, uric acid, glutamine, methionine, citrate, and fatty acids. The first five metabolites had higher absolute concentrations following the first transplant, while fatty acid levels were lower(72). As previously described, lactate increases during ischaemia due to increased anaerobic glycolysis. Uric acid, an antioxidant which counters oxidative stress and is the end-product of the xanthine pathway, also accumulates under ischaemic conditions. The liver is the primary site of amino acid catabolism. An increase in circulating amino acids may therefore indicate reduced metabolism. Persistently high concentrations in these circulating metabolites may reflect hepatocyte dysfunction and the liver's inability to recover from ischaemia. Furthermore, as the central organ for fatty acid metabolism, the liver produces large amounts of fatty acids but stores only small quantities as triglycerides. Low circulating fatty acids may therefore indicate disturbances in lipid homeostasis due to graft dysfunction(72).

A ^{1}H-NMR study of nine patients, eight of whom underwent liver donor-related transplants and one cadaveric transplant, also revealed significant differences in amino acid levels(73). Moreover, these were significantly higher in patients who did not survive the post-operative period because of graft dysfunction. These included alanine, lysine, glutamine, methionine, asparagine, histidine, tyrosine and phenylalanine. Indications for transplantation were diverse; however, pre-transplant serum analysis demonstrated similar metabolic profiles between patients. Furthermore, amino acid concentrations globally exhibited an earlier and more consistent increase than routine liver function tests(73).

An investigation of 75 transplant recipients into the role of nitric oxide (NO) synthesis (NOS) inhibition in the assessment of graft recovery post-transplantation by Martin-Sanz et al. demonstrated a correlation between higher levels of L-N-monomethylarginine and asymmetric

dimethylarginine, both methylated arginine derivatives and NO inhibitors, in the blood of patients with post-operative graft dysfunction(74). Of note, shifts in these substrates were seen immediately following implantation of the organ when routine liver function tests were unremarkable. The relevance of these observations lies in NO's essential role in regulating liver physiology and blood flow. NO is produced by the nitric oxide synthases (NOS) from the substrate L-arginine and exerts protective effects in the liver by improving blood flow, antagonising neutrophil activation and adhesion, neutralising free radicals, and eliciting anti-apoptotic effects. The beneficial effects of the L-arginine-NO pathway have been reported in liver transplantation(74). Experiments using arginine supplementation and NO donors have demonstrated that NO improves IRI, cardiac output, and hepatic blood flow after liver transplantation. In addition, NOS inhibitor administration in the post-liver transplant setting has been shown to aggravate liver injury, indicating the beneficial role of L-arginine-NO synthase pathway in ameliorating IRI(74).

Parenchymal Interstitial Fluid

Silva et al also employed the microdialysis technique to investigate changes in metabolite concentrations attributed to IRI in 18 transplant patients. The perfusate samples' amino acid content was analysed using HPLC with fluorimetric detection of *o*-phthaldialdehyde derivatives(75). Post-operative graft function was assessed by monitoring serum transaminase levels and blood coagulation, which are standard biochemical methods of assessing graft recovery in the clinical setting. All patients included in this study had an uneventful recovery post-transplant, with no occurrences of PNF or Initial Poor Function (IPF) as dictated by biochemical results. The authors observed elevated levels of all carbohydrate metabolism intermediaries on reperfusion of these grafts, which subsequently normalised during the first

12 hours. The lactate/pyruvate ratio also showed a similar trend. Glycerol peaked on reperfusion, reaching baseline levels within 8 hours; however, this was not statistically significant. The levels of four amino acids (alanine, GABA, glutamate, and taurine) decreased at different rates over the monitoring period, while arginine concentrations increased. Alanine is involved in the glucose-alanine cycle, a nitrogen source for the urea cycle and pyruvate for glycolysis(75). GABA has been implicated in hepatic regeneration, disrupting GABA homeostatic mechanisms associated with extensive liver injury. Glutamate is involved in membrane excitability. Taurine is an osmolyte which helps maintain cell volume homeostasis. The reduction over time of all these metabolites in the microdialysate / interstitial fluid could reflect mechanisms of hepatic recovery post-reperfusion. Arginine is principally known for its role in the detoxification of ammonia in the urea cycle and the synthesis of nitric oxide (NO). Homeostatic mechanisms maintain a higher concentration of arginine outside rather than inside the cell. Therefore, this could be interpreted as an indicator of good hepatic recovery(75).

Another study by the same group analysed glucose metabolism in two different groups of patients – those that showed biochemical evidence of IR injury, with AST levels, as described by Strasberg et al. (1994), >2000 IU/L in the first 24 hours versus those that did not(76). Lactate levels were significantly higher during backbench preparation and took longer to normalise post-reperfusion in the IR injury group. No significant differences were observed in glucose, pyruvate, or glycerol concentrations at any point measured. Therefore, interstitial lactic acidosis was proposed to indicate functional impairment pre-implantation(76). In the same group of patients, Richards et al. analysed amino acid trends in microdialysate fluid during organ procurement, backbench preparation and up to 48 hours post-reperfusion(77). Although clinical outcomes for all grafts were favourable, differences were noted in the subset of grafts which displayed IPF. During procurement, extracellular aspartate was significantly higher in

grafts with subsequent IPF. Furthermore, on the backbench, IPF grafts had higher levels of β-alanine, GABA, glutamine and threonine. A marked progressive increase in extracellular arginine concentration was also noted in all grafts from donor procurement to post-implantation(77).

An exploration of IRI and graft dysfunction metabolomics was performed out by Perera et al. through microdialysis perfusate analysis(78). The metabolomic profiles and clinical outcomes of DBD and DCD liver grafts were compared. Microdialysate fluid samples obtained during cold storage and post-reperfusion were analysed through Colourimetric Electrochemical Array Detection (CEAD)(78). During the cold storage phase, homovanillic acid and methionine increased significantly with each 100-minute prolongation in cold ischemia time. Xanthine, uric acid, and kynurenine were in higher abundance in DCD grafts and 3-nitrotyrosine and 4-hydroxy-3-methoxymandelic acid in DBDs. Failed allografts (i.e. suffered PNF) demonstrated increased levels of reduced glutathione and kynurenine during cold storage as well as xanthine post-reperfusion(78). The findings relating to reduced glutathione and kynurenine during cold storage are of particular significance as they separate grafts according to outcome before transplantation. Furthermore, both metabolites have been demonstrated in hepatic IRI. Reduced glutathione is an antioxidant that acts as an intracellular ROS scavenger(79). Increased levels may be a response to increased ROS production in these livers. Kynurenine is generated from the breakdown of the amino acid tryptophan, a pathway that has been implicated in cellular stress mechanisms and inflammatory responses(80). Activation of the kynurenine pathway results in the production of kynurenine metabolites shown to contribute to oxidative stress, inflammation, and immune dysregulation, which may worsen liver injury(81). The authors postulated that increased kynurenine production may be related to DCD grafts' longer warm ischaemia time(78). Since a DCD liver is considered inferior, its metabolic

data could be extrapolated to grafts more likely to exhibit graft dysfunction. Those livers that suffered PNF in the same study exhibited a similar metabolic profile(78).

Bile

Bile acid synthesis is a complex but finely tuned physiological process. Bile acids are the end-products of cholesterol catabolism in the liver and are classified into a heterogeneous group of amphiphilic steroidal molecules(82). The major primary bile acids synthesised in the liver for secretion into bile are cholic acid (CA) and chenodeoxycholic acid (CDCA). They are conjugated with the amino acids taurine and glycine to form sodium salts in physiological pH to increase their solubility. Their synthesis stimulates bile flow and biliary secretion of bile acids, phospholipids, cholesterol, drugs and toxic metabolites into the duodenum(82). Most bile acids are reabsorbed in the terminal ileum and transported back to the liver via the portal blood in the enterohepatic circulation. This creates a negative feedback loop which regulates bile acid synthesis(82).

Conversion of cholesterol to bile acids is critical in maintaining cholesterol homeostasis and preventing accumulation of cholesterol, triglycerides, toxic metabolites, and injury to the liver and other organs. Bile acids are essential for intestinal absorption of dietary lipids, fat-soluble vitamins, and biliary secretion of lipids, toxic metabolites, and xenobiotics into the gut(82). They also act as signalling molecules and metabolic regulators that activate nuclear and G protein-coupled membrane receptors to regulate hepatic, lipid, glucose and energy homeostasis. In addition, their synthesis stimulates bile flow to prevent their accumulation in the liver, which may cause inflammation, apoptosis and cell death(82). Disorders in bile acid metabolism are associated with cholestatic liver disease, dyslipidaemia, fatty liver disease, and

have also been linked to diabetes(83). In liver transplantation, bile secretion is generally accepted as an indicator of graft recovery. Therefore, it has been hypothesised that alterations in bile acids before and during liver transplantation could reflect graft functional performance and predict post-operative complications(84).

Bile analysis studies were initially conducted by Vilca-Melendez et al. By ligating the cystic duct (preventing mixing of stored gallbladder bile and hepatic bile) and cannulating the common bile duct, hepatic bile composition could be analysed in the context of bile flow to determine what is known as *Apparent Choleretic Activity* (ACA)(84). This concept stems from the understanding that bile acid production drives biliary canicular flow. Therefore, an increase in bile acid production should lead to an increase in bile flow(84). In these studies, the majority of grafts considered to be suboptimal had varying degrees of steatosis (according to the gross macroscopic assessment of the procurement surgeon), and primary graft dysfunction (PGD) was defined according to the criteria set by Clavien et al. (68). A preliminary study, employing ^{1}H-NMR and GC analysis, revealed peak differences between suboptimal and ideal grafts. Phosphatidylcholine (PC) peaks were more prominent in steatotic livers(84). PC is a component of the lipids stored in steatotic livers, and this finding may indicate increased biliary lipid secretion stimulated by the steatotic environment. In addition, UW preservation solution clearance peaks took longer to clear in the suboptimal grafts that developed PGD, possibly indicating a prolonged recovery in bile flow. Lastly, bile acid secretion was slower in PGD grafts(84).

A subsequent randomised study by the same group enrolled a cohort of 35 patients. It used the same criteria to separate suboptimal from ideal grafts and postoperative outcomes(85).

Analysis was carried out using a more targeted approach, combining enzymatic assays, HPLC and GC to decipher bile acid composition. All grafts that had developed PGD had been deemed suboptimal pre-transplant(85). While there was no difference in bile flow to differentiate so-called suboptimal from ideal grafts, samples from the former had a higher concentration of bile acids. This indicated that bile flow did not increase appropriately with the higher bile acid concentrations. It was postulated that this could be related to water secretion impairment at a canalicular level or a reduction in bile-acid-independent promoters of bile flow, such as glutathione, bicarbonate, calcium, sodium, potassium, glucose, amino acids and organic acids. Furthermore, the donor bile from suboptimal grafts pre-reperfusion had a higher proportion of CA and a lower proportion of CDCA with a reversal of proportions in the ideal grafts. In the literature, higher proportions of "less hydrophobic" bile acids (such as CA) compared to "more hydrophobic" bile acids have been associated with reduced bile flow. Post-reperfusion, there was no statistically significant difference between sub-optimal and ideal grafts for bile acid composition and ACA(85).

Papaspyridonos et al employed capillary electrophoresis fingerprinting of pre- and post-transplant human bile samples in a prospective randomised observational study of six donor livers and five recipients(86). The sixth recipient suffered PGD, as per Clavien et al. 1994, and no bile was produced for sample collection post-transplant. Multivariate data analysis (using PCA and PLS-DA methods) showed that donor and recipient samples clustered separately. Interestingly, the recipient considered to have received the healthiest graft (non-steatotic appearance of liver, youngest age, shortest ITU stay and shortest cold ischaemia time (CIT) was situated closest to the donor cluster, indicating the least metabolic disruption or quickest metabolic recovery. A PLS-DA model for the pre-transplant donor bile samples of the PGD liver revealed higher levels of taurocholic acid (TCA) and taurodeoxycholic acid (TDCA)

when compared to non-PGD livers. The authors attributed this to the moderately steatotic condition, which translated into a lower ACA and reduced bile flow. While this study illustrated the high efficiency and fast analysis time of CE with minimal sample volume and preparation, the results of this analysis must be confirmed with a larger cohort of PGD livers(86).

Duarte et al collected human hepatic bile from one mildly steatotic donor liver (confirmed histologically) during procurement and subjected these samples to an analysis involving multiple analytical platforms (^{1}H-NMR spectroscopy, HPLC-NMR/MS and UPLC-MS) to identify and characterise as many metabolites as possible(87). Detection of several lipids and carbohydrate-based compounds, amino acids, and primary bile acids (CA, deoxycholic acid and CDCA) were possible with ^{1}H-NMR. However, confident spectral identification of glycine and taurine conjugated acids required the application of HPLC and MS. On the other hand, the bile acid isomers glycodeoxycholic acid and glycochenodeoxycholic acid could only be identified by ^{1}H-NMR. Interestingly, the authors observed a higher proportion of CA compared to CDCA(87). This finding matched what Vilca-Melendez et al(85) reported, leading the authors to conclude that this reflected the suboptimal status of the steatotic liver. This study also demonstrated the complementary potential of the various analytical platforms available for metabolomic analysis. However, it also illustrated the heterogeneity of results between them – a fact that limits the assimilation of results for interpretation between studies investigating the same outcome.

Looking at acute cellular rejection, Hedaya et al found no difference in bile acid composition in pre- and post-transplant bile from living-donor-related transplants with and without acute

cellular rejection(88). However, there was a statistically significant difference between the recipient and donor bile acid and TCBA ratios from the seventh day post-operatively (p=0.038). Furthermore, a recipient-to-donor ratio of 0.5 or greater was associated with better ACR-free survival(88). The most recent published work on bile analysis was by Legido-Quigley et al(89) using Vilca-Melendez et al(90) bile collection method. Serial donor and recipient samples were collected from 10 transplants (seven ideal, one mildly fatty and two moderately fatty – histologically confirmed), all of which had successful recipient outcomes, parameters in line with Clavien et al(68). UPLC-MS and multivariate analysis showed that metabolic profiles of the moderately steatotic livers did not have a different trajectory from the normal ones. Furthermore, bile acid metabolic profiles were time-dependent, with the post-reperfusion profiles found to be initially very different from their donor counterparts but moved closer towards the donor profile after transplantation. Tricarboxylic cycle metabolism was also significantly increased following transplantation(89).

Tissue

Duarte et al obtained tru-cut biopsies from the left lobe of 6 donor livers during procurement, at the end of cold storage and following reperfusion. Metabolomic analysis was performed using High-Resolution Magic-Angle-Spinning NMR spectroscopy (HR-MAS), ideal for profiling of intact tissues(91). Histological assessment revealed four normal (<5% fat infiltration), one mildly steatotic (20% fat infiltration) with focal parenchymal necrosis and one moderately steatotic (40% fat infiltration) biopsies. Five recipients survived. The remaining patient, who had required the graft as a retransplant for recurrence of hepatitis C, developed PGD (according to Clavien et al criteria(68)) which was complicated by a haemorrhagic episode, rendering the graft ischaemic(91). Despite another retransplantation, the patient died

of multi-organ failure. Metabolomic analysis distinguished the more steatotic grafts by revealing higher triglycerides and unsaturated lipid concentrations, together with lower phospholipids levels. This correlated with the degree of steatosis as determined by histological examination. Furthermore, on examining the metabolic trend over time for each liver, a consistent observation was the decrease in glycophosphocholine (GPC, a phospholipid degradation product), from retrieval to implantation, in all livers except one – that which developed PGD. In the latter, GPC remained constant throughout. The authors postulated that this could reflect an increase in cell turnover in a functioning liver as it recovers from the ischaemic insult and regenerates(91).

Hrydziuszko et al(92), using FTCIR-MS and CEAD, demonstrated metabolic changes on reperfusion involving upregulation of the urea cycle with increased urea and urea cycle intermediates and increased bile acid levels. More recently, Hyrdziuszko et al(93) to characterised the differences in metabolic activity between DCD vs DBD grafts by conducting a Direct Infusion Mass Spectrometry (DIMS) approach using Fourier transform ion cyclotron resonance mass spectrometry (FT-CIR MS) analysis on liver biopsies from both groups. The most significant finding related to amino acid metabolism, involving higher kynurenine levels in DCD grafts ($p<0.05$) in the cold phase and post-reperfusion.

Cortes et al examined 124 donor biopsies from transplanted livers to develop a predictive model of EAD based on metabolomic data(94). Ninety-six biopsies were used for model development and the remaining 28 for model validation. A lipidomic fingerprint consisting of significantly higher tissue concentrations of lysophosphatidylcholines, lysophosphatidylethanolamines, phosphatidylcholines, phosphatidylethonalmines and sphingomyelins was detected in EAD livers, using UPLC-MS. This was attributed to increased

cell membrane lysis and cell death, compromising tissue integrity. Interestingly, bile acids and products of histidine metabolism were also noted to be in significantly higher concentrations in these livers(94). Faitot et al also assessed the predictive value of metabolites for graft dysfunction in 42 transplant recipients(95). HR-MAS NMR detected higher levels of lactate, glutamate, glutamine, alanine, valine, isoleucine and choline derivatives in EAD livers. Lactate and phosphocholine gave high accuracy for the prediction of graft dysfunction. Receiver Operator Characteristic curve analysis showed a high predictive value for lactate, and multivariate analysis for graft dysfunction showed that graft lactate content was the only independent predictor ($p = 0.046$)(95).

Table 3. Main features and conclusions of included studies

Author	Analytical Platform	Study Design	Population No.	Investigated Outcomes	Measured Outcomes	Increased Metabolites	Decreased Metabolites	Proposed Affected Metabolic Pathways
Blood Metabolomics								
Singh et al (2003)(71)	^{1}H-NMR	Case study	1 recipient	Graft dysfunction	Graft dysfunction criteria not stated.	serum: glutamine, urea	urine: urea	Urea (Ornithine) Cycle impairment as a sign of graft failure. Monitoring of recipient serum glutamine levels as a predictor of graft dysfunction.
Martin-Sanz et al. (2003)(74)	HPLC-MS	Prospective cohort	75 recipients	Graft dysfunction	Grade I (n=40): AST<1000 U/L for the first 3 days post-OLT, with good bile production (>40 mL/day) and evidence of coagulation. Grade 2 (n=19): initial AST >1000 U/L, but decreased over next 48 hours with adequate bile flow and coagulation. Grade 3 (n=12): AST >2500 U/L during first 48 hours post-OLT, bile production <40 mL/day, with severe coagulopathy. Grade IV (n=4): rapidly increasing AST with no bile production and severe coagulopathy.	methylarginine, dimethylarginine	-	Increased inhibition of Nitric Oxide Synthesis through the action of arginine derivatives related to EAD. MMA and ADMA levels in graft preservation solution as a potential predictor of early liver function.

Serkova et al. (2007)(72)	^{1}H-NMR	Case study	1 recipient	Graft dysfunction	Graft dysfunction criteria not stated.	lactic acid, uric acid, citrate, glutamine, methionine	fatty acids	Lactate, end product of anaerobic glycolysis, increased in ischaemia. Uric acid, end product of xanthine pathway, accumulates during IRI. Lipid dysregulation and decreased circulation of fatty acids associated with graft failure. Amino acid levels as predictor of graft dysfunction. High levels attributed to decreased amino acid metabolism and liver nitrogen metabolism as a result of ischaemic injury.
Tripathi et al. (2009)(73)	^{1}H-NMR	Case series	9 recipients	Graft dysfunction	Mortality: survivors (n=6) versus non-survivors (n=3)	lactate, alanine, lysine, glutamine, methionine, asparagine, histidine, tyrosine and phenylalanine	-	Decreased amino acid metabolism and impairment of urea (ornithine) cycle related to graft dysfunction.
Interstitial Fluid / Dialysate Fluid Metabolomics								
Silva et al.(2005)(75)	CEAD / HPLC and fluorometric detection	Prospective cohort	17 recipients	Graft dysfunction	All patients were reported as having an uneventful post-operative course.	During SCS and post-reperfusion: lactate, pyruvate, glycerol, alanine, glutamate, GABA, taurine (all with reduction over time)	Arginine (initially decreased post-reperfusion but increased >19 hours increased post-transplant)	Increased lactate and pyruvate during backbench preparation - High lactate to pyruvate ratio post-reperfusion as indicator of cell ischaemia due to increased anaerobic glycolysis during SCS. Initially high glycerol may be indicative of cell lysis. Reduction in amino acids (GABA, glutamate, taurine, alanine) over time may reflect recovery of amino acid metabolism. Increased extracellular arginine may be related to Nitric oxide Synthase activity.
Silva et al. (2006)(76)	CEAD / HPLC and fluorometric detection	Prospective cohort	15 recipients	Graft dysfunction	Graft dysfunction as defined by Strasberg et al.: AST >2000 IU/L in the first 24 hours (n=6), versus	lactate	-	High lactate in donor graft pre-transplantation as a marker of IRI and development of IPF.

					those that did not (n=9).			
Richards et. al. (2007)(77)	CEAD / HPLC and fluorometric detection	Prospective cohort	15 recipients	IPF	Graft dysfunction as defined by Strasberg et al.	IPF grafts during procurement: aspartate IPF grafts during SCS: β-alanine, GABA, glutamine, threonine and arginine	-	Increased extracellular amino acid concentrations in procurement and SCS potentially due to release following hepatocyte death or active release as protective / restorative response.
Perera et al. (2014)(78)	CEAD	Prospective cohort	40 recipients	Graft dysfunction / IRI / DBD vs DCD	Graft dysfunction criteria not stated. DCD (n=13) DBD (n=27)	DCD: xanthine, uric acid, and kynurenine (SCS) DBD: 3-nitrotyrosine and 4-hydroxy-3-methoxymandelic acid (SCS) DBD and DCD: homovanillic acid and methionine (SCS) PNF graft: reduced gluthathione and kynurenine (SCS) and xanthine (post-reperfusion)	-	Xanthine and uric acid (products of the purine catabolism) are modulated by xanthine oxidoreductase, an enzyme implicated in reactive oxygen species generation, aggravated by tissue hypoperfusion. Upregulation of reduced glutathione (cellular antioxidant) as a marker of increased cellular oxidative stress. Kynurenine, as a product of tryptophan metabolism, implicated in mediation of cellular stress response.
Bile Metabolomics								
Vilca-Melendez	^{1}H-NMR	Case series	4 recipients	Graft dysfunction	Graft dysfunction as defined by Clavien et al.: first day AST	Steatotic livers: PC PGD grafts: persistence of	-	Increased PC in steatotic livers suggestive of higher abundance of biliary lipids from increased lipid secretion in bile.

et al. (2001)(84)					≥2000 IU/L or a transient increase in AST ≥1000 IU/L or a persistently high PT >20 (or the equivalent INR >1.4) for at least 3 days. EAD (n=1), PNF (n=1) and good graft function (n=2)	preservation solution component peaks (indicating decreased clearance of solution)		
Vilca-Melendez et al. (2004)(85)	Reverse ion pair HPLC and GC	Prospective Cohort	35 recipients	Graft dysfunction	Graft dysfunction as defined by Clavien et al. PGD (n=12), non-PGD (n=23).	Suboptimal graft: Total Bile Acid Output rate + CA	Normal liver: CDCA	Higher Total Bile Acid abundance in suboptimal grafts may be due to impaired bile flow. High amount of "less hydrophobic" CA relative to "more hydrophobic" CDCA may indicate impaired water secretion in bile for bile flow.
Papaspyridonos et al. (2008)(86)	CE finger-printing	Case series	6 recipients	Graft dysfunction	Graft dysfunction as defined by Clavien et al. PGD (n=1), non-PGD (n=5).	PGD and steatotic liver: TCA + TDCA	-	Upregulation of tauro-conjugated bile acids associated with liver injury, thereby reflecting metabolic dysfunction in moderately steatotic livers.
Hedaya et al. (2009)(88)	^{1}H-NMR	Prospective cohort	41 recipients	Acute cellular rejection	Biopsy-proven ACR according to clinical suspicion. ACR (n=12),non-ACR(n=29)	Recipient/Donor TCBA ratio ratio ≥0.5 on days 7 and 9, and ≥0.38 on day 11 post-transplantation	-	Recipient and Donor TCBA ratios as post-operative predictor of ACR.
Legido-Quigley et al. (2011)(89)	UPLC-MS	Prospective cohort	10 recipients	Graft Steatosis / Graft dysfunction	Graft steatosis by histopathological assessment. Graft dysfunction as defined by Clavien et al. No PGD livers. Mildly steatotic (n=2), moderately steatotic (n=1), normal (n=7).	Non-steatotic liver: glycodeoxycholic acid (GDCA), glycocholic acid (GCA) and glycochenodeoxycholic acid (GCDCA) (pre-transplantation) Normal and steatotic livers:	-	Different proportions of bile acids in grafts could be a sign of organ recovery or functional impairment.

						TCA and TCDCA (post-transplant), secondary bile acids.		
Liver Tissue Metabolomics								
Duarte et al. (2005)(91)	HR-MAS NMR	Case series	6 recipients	Graft dysfunction / Graft Steatosis	Graft dysfunction as defined by Clavien et al. PGD (n=1), non-PGD (n=5).	Steatotic grafts (mild and moderate): triglycerides and unsaturated lipids	Good functioning livers: GPC (post-reperfusion) Steatotic livers: amino acids, glucose and products of nucleotide metabolism	Decreased GPC (a cell membrane-derived phospholipid degradation product) in post-operative good functioning livers attributed to increased cell turnover reflecting and active cellular regeneration. GPC as a potential biomarker of liver function.
Hrydziuszko et al. (2010)(92)	FTCIR-MS CEAD	Case series	8 recipients	Graft dysfunction	Graft dysfunction as defined by Strasberg et al. IPF (n=1), non-IPF (n=7).	On reperfusion: Urea and urea cycle intermediates Bile acids Formate, orthophosphate, ADP, fumarate, succinate (intermediaries of energy metabolism)	-	Metabolite profile on reperfusion demonstrates recovery of liver metabolic function (increased urea synthesis, bile acid synthesis) and intermediaries of energy metabolism.
Cortes et al. (2014)(94)	UPLC-MS	Prospective cohort	124 recipients	Graft dysfunction / PNF	Graft dysfunction according to Olthoff classification - at least one of the following criteria for a diagnosis of graft dysfunction as EAD: bilirubin ≥10 mg/dl on day 7; INR ≥1.6 on day 7; AST or	Lysophosphatidylcholines lysophosphatidylethanolamines, phosphatidylcholines, phosphatidylethanolamines, sphingomyelins,	-	Disruption of phospholipid metabolism as a reflection of cell membrane breakdown. Lysophosphatidylcholines are toxic metabolites generated by phospholipase A2-catalyzed phospholipid hydrolysis, implicated hepatocyte apoptosis. Disruption of bile acid homeostasis has been shown to affect bile flow recovery.

					ALT >2000 IU/L within the first 7 days. EAD (n=60), IGF (n=64). Model development: EAD (n=)48 and IGF (n=48). Model validation: EAD (n=12), IGF (n=12), PNF (n= 4).	bile acids and products of histamine metabolism.		Increased histamine levels may reflect an adaptive response to ischaemic injury in which cytokine release is reduced through activation of the H4 histamine receptor.
Hrydziuszko et al. (2016)(93)	FTCIR MS	Prospective cohort	37 recipients	Graft dysfunction / PNF / DBD vs DCD	Graft dysfunction criteria not stated. DBD (n=27), DCD (n=10). PNF: 1 DBD and 1 DCD.	DCDs and PNF: kynurenine	-	Kynurenine is a product of tryptophan metabolism, implicated in immune modulation. Suboptimal quality of DCD livers due to warm ischaemia could be reflected in the higher kynurenine levels. Kynurenine postulated as a potential biomarker of graft dysfunction.
Faitot et al. (2018)(95)	HR-MAS NMR	Prospective cohort	42 recipients	Graft dysfunction	Graft dysfunction according to Olthoff classification. EAD (n=7), no EAD (n=35).	lactate and phosphocholine	-	High lactate is indicative of the anaerobic environment during SCS, potentially acting as a marker of graft tolerance to cold ischaemia. High phosphocholine can be linked to cell membrane disruption. It is converted to glycerophosphocholine by the endoplasmic reticulum, therefore sustained high levels could reflect endoplasmic reticulum stress.

Abbreviations: **ACR** acute cellular rejection **ADMA** asymmetric dimethylarginine **ADP** adenosine diphosphate **CA** cholic acid **CDCA** chenodeoxycholic acid **CE** Capillary Electrophoresis **CEAD** Coulorimetric Electrochemical Array Detection **DBD** donation after brainstem death **DCD** donation after circulatory death EAD early allograft dysfunction **FTCIR** Fourier Transform Infrared Spectroscopy **GC** Gas Chromatography **GCA** glycocholic acid **GCDCA** glycochenodeoxycholic acid **GDCA** glycodeoxycholic acid **GPC** glycophosphocholine H-NMR Proton Nuclear Magnetic Spectroscopy **HPLC** High Performance Liquid Chromatography **HR-MAS** High Resolution Magic Angle Spectroscopy **IPF** initial poor function **MMA** monomethylarginine **MS** Mass Spectrometry PC phosphocholine PGD primary graft dysfunction **PNF** primary non function **SCS** static cold storage **TCA** taurocholic acid **TCBA** taurine-conjugated bile acids **TCDCA** taurochenodeoxycholic acid **TDCA** taurodeoxycholic acid **UPLC** Ultra Performance Liquid Chromatography

Table 4. Quality assessment of included study methodology using the QUADOMICS tool for systematic review

Study	**Phase**	**QUADOMICS items**																**Applied Items (%)**		
		1	**2**	**3**	**4.1**	**4.2**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**YES**	**NO**	**UNCLEAR**
Martin-Sanz et al. (2003)	1	N	NA	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	N	Y	NA	N	N	66.7	33.3	0
Tripathi et. al. (2009)	1	N	NA	Y	N	N	Y	Y	Y	Y	Y	Y	Y	N	Y	NA	N	N	60	40	0
Silva et al. (2005)	1	N	NA	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	N	Y	NA	N	N	66.7	33.3	0
Silva et al. (2006)	1	N	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	NA	N	N	73.3	26.7	0
Richards et al. (2007)	1	N	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	NA	N	N	73.3	26.7	0
Perera et al. (2014)	1	N	NA	Y	Y	Y	Y	Y	?	Y	?	Y	N	N	Y	NA	Y	N	60	26.7	13.3
Vilca-Melendez et al. (2001)	1	N	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	NA	N	N	73.3	26.7	0
Vilca-Melendez et al. (2004)	1	N	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	NA	N	N	73.3	26.7	0
Papaspyridonos et al. (2008)	1	N	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	NA	N	N	73.3	26.7	0
Hedaya et al. (2009)	1	N	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	NA	N	N	73.3	26.7	0
Legido-Quigley et al. (2011)	1	N	NA	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	N	Y	NA	N	N	66.7	33.3	0
Duarte et al. (2005)	1	N	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	NA	N	N	73.3	26.7	0
Hrydziuszko et al. (2010)	1	N	NA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	NA	N	N	73.3	26.7	0
Cortes et al. (2014)	4	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	88.2	11.8	0
Hrydziuszko et al. (2016)	1	N	NA	Y	Y	Y	Y	?	?	N	?	Y	N	N	Y	NA	Y	N	46.7	33.3	20
Faitot et al. (2018)	4	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	N	82.4	17.6	0
YES (%)		0	12.5	100	93.7	93.7	81.2	93.7	87.5	93.7	87.5	100	87.5	0	100	12.5	25	6.3			
NO (%)		100	0	0	6.3	6.3	18.8	0	0	6.3	0	0	12.5	100	0	0	75	93.7			
UNCLEAR (%)		0	0	0	0	0	0	6.3	12.5	0	12.5	0	0	0	0	0	0	0			
NOT APPLIED (%)		0	87.5	0	0	0	0	0	0	0	0	0	0	0	0	87.5	0	0			

Phase of biomarker discovery is as proposed by Lumbreras et al. (2008). Non-applicable items are marked as 'NA'. A positive score is denoted by 'Y' and negative score by 'N'. If applied items cannot be scored, this is denoted by '?'. Percentage of applied criteria for each study as well as scores for each criterion across studies are listed. Questions for each item can be found in ***Appendix 1****.* ***Y****: yes;* ***N****: no;* ***?****: unclear;* ***NA****: Not Applied.*

2.5 Conclusions

2.5.1 Overview of Current Literature

Specific perturbations in metabolic pathways occurring before transplantation may be linked to the graft dysfunction risk following transplantation. This makes investment in further metabolomic research worthwhile. It will enable biomedical researchers to understand better the molecular mechanisms involved in graft dysfunction. It will also facilitate the identification of clinically applicable biomarkers of graft viability pre-transplantation. There are, however, significant limitations on the current body of research that have hindered its clinical application. No single metabolite or metabolite panel has been consistently observed to be correlated with graft outcome with the statistical confidence to translate it into clinical medicine. The metabolomic experiment consists of an extensive network of pre-analytical, analytical and data-processing steps influenced by the characteristics of the population under study, biological sample procurement and processing, the analytical platform available and the intended outcomes. These must be considered carefully in the study design, or data interpretation may become an issue.

The studies in this review sought to identify changes in metabolite compositions and their potential as possible biomarkers of liver function. External validation of their predictive capabilities for clinical applicability was rarely considered. In this regard they are therefore discovery phase studies involving comparatively small cohorts of livers. The application of different analytical platforms simultaneously may increase the yield of metabolites on account of their different sensitivities. However, the lack of a standardised investigative may lead to inconsistent results. Donor livers are singular organs, with many donor-related variables that influence graft quality. Small population data may therefore be plagued by confounding factors

thus warranting cautious interpretation and extrapolation of results. Of note, most studies in this review gathered data from convenience sampling rather than a randomised or consecutive patient protocol. Furthermore, the risk-of-bias assessment demonstrates the variable metabolomic data reporting quality of these studies. Another point worth considering is differences in graft dysfunction diagnostic criteria which also limits comparability between studies due to different diagnostic thresholds. This also lowers confidence in small population studies. Data must be reproducible against unbiased outcomes to ensure its clinical validity and utility. Collaboration between different centres, both clinical and scientific institutions, is key with standardised analytical and clinical protocols to maximise population numbers and increase the validity of relevant and viable data for biomarker discovery.

2.5.2 Recommendations for Standardisation of Metabolomics Results

Standardising the metabolomics workflow between clinical teams and scientific laboratories across various sites is crucial to ensure reproducibility and comparability of results. Below are some recommendations on how this could be achieved following appraisal of the current metabolomics literature in liver transplantation.

i. A well-defined research question: a clear research question or hypothesis which defines the patient population and the phenotype under study.
ii. Standardisation of clinical outcomes and diagnostic thresholds: this will increase confidence in the clinical validity and applicability of the data and increase acceptance of potential metabolic biomarkers.
iii. Appropriate study design: standardisation of donor liver inclusion and patient recruitment protocol. Consistent and standardised protocols for sample collection, storage, and handling will minimise variability and ensure reliability of metabolomics

measurements. Samples should ideally be obtained at a time point when results would influence clinical decision making. They should be representative of the population under study and should include sufficient numbers to provide statistical power. Clear documentation of the steps involved in sample preparation is also important, including extraction methods and derivatisation procedures.

iv. Establishing standardised analytical methods: agreement on analytical methods, including instrument settings, data acquisition, and data processing parameters. This ensures consistency in data generation and analysis.

v. Implementation of quality control measures: inclusion of appropriate quality control samples throughout the analysis to monitor instrument performance and data quality. This can involve the use of internal standards, blank samples, and replicate analysis.

vi. Robust data analysis: agreement on and application of appropriate statistical methods and bioinformatics tools to further ensure consistency in data output. This includes data normalisation, feature selection, multivariate analysis, and metabolic pathway analysis.

vii. Adoption of standardised reporting formats and metadata standards: This will facilitate data sharing and comparison between laboratories. It includes the use of controlled vocabularies and data repositories that comply with established metabolomics data standards, such as the Metabolomics Standards Initiative, to ensure adherence to best practices.

viii. Data validation and replication: validating initial findings using independent methods or platforms helps ensure reliability of metabolomics results. Replication in different patient cohorts can further strengthen a study's findings.

ix. Collaboration between clinical teams and laboratories: fostering an environment that promotes sharing of information between laboratories to exchange knowledge and best practices.

2.5.3 Avenues for Future Research

The metabolomic studies described in this systematic review describe metabolic changes in donor livers preserved in static cold storage before transplantation. Basic science perfusion research has been able to employ metabolomics to differentiate steatotic and DCD livers(58, 59, 80, 96). The next logical step is therefore metabolomic interrogation of livers that undergo NMP-L pre-transplantation in order to compare to clinical outcomes. This would be of great value, especially to establish the metabolic behaviour of ECD livers and determine those pathways that influence viability for transplantation. This could be explored in a controlled *ex vivo* environment. Therefore, metabolomics could potentially inform methods of liver preservation optimisation during perfusion, thus providing the ideal environment for the "less ideal" liver.

Chapter 3 Protocol for Metabolomic Analysis of Extended Criteria Donor Liver Function During End-Ischaemic Normothermic Machine Perfusion

The work described in this chapter represents a collaboration between the author as a clinical researcher with the VITTAL clinical trial team, the Centre for Liver and Gastrointestinal Research at the University of Birmingham and the Phenome Centre Birmingham. The clinical trial had been set up prior to the author joining the team. The author established links with Professor Warwick Dunn of the Phenome Centre Birmingham to design and incorporate the metabolomics study protocol into the clinical trial and was responsible for sample collection, quenching and storage as well as clinical data collection. The author was also one of the perfusionists who conducted the liver perfusions during the trial. The clinical trial protocol and result have been published previously.

Trial protocol: **BMJ Open. 2017 Nov 28;7(11):e017733.doi: 10.1136/bmjopen-2017-017733.**

Trial results: **Nat Commun. 2020 Jun 16;11(1):2939. doi: 10.1038/s41467-020-16251-3.**

3.1 Introduction

3.1.1 The VITTAL clinical trial

The Viability Testing and Transplantation of Marginal Livers (VITTAL) using Normothermic Machine Perfusion (NCT02740608) Trial is a registered open-label, non-randomised, prospective, single-arm clinical trial carried out at the Queen Elizabeth Hospital University Hospitals Birmingham NHS Foundations Trust, an institution with extensive experience in transplantation of high-risk livers and NMP-L. The trial was set up in collaboration with the Institute for Biomedical Sciences at the University of Birmingham. It investigated the feasibility and safety of employing organ viability testing to end-ischaemic NMP-L to assess procured ECD livers rejected by all transplant centres in the United Kingdom to salvage potentially transplantable livers from being discarded. As part of the research work (Work Package 2), a sampling protocol for metabolomic analysis was incorporated into the trial. This would enable direct correlation and comparison of metabolomic data with the trial's viability parameters for transplantation. Work Package 2 was designed to identify sensitive point of care liver quality tests and propose novel biomarkers or panels associated with viable livers.

One of the principal aims of this clinical trial was to establish objective transplant viability criteria during end-ischaemic NMP-L. The objectives of our metabolomics study were to understand the metabolic changes occurring during perfusion, how they relate to liver viability testing and to investigate whether post-transplant outcomes are reflected in pre-transplantation perfusate metabolic phenotypes. This would also facilitate an exploration of the metabolic pathways that influence ECD liver viability. Moreover, it may inform and direct future machine perfusion research towards potential molecular targets for therapeutic intervention that

influence donor liver function and viability pre-transplantation, thereby driving efforts to continue expanding the donor pool.

3.1.2 VITTAL Clinical Trial Protocol Overview

A detailed description of the protocol for the VITTAL clinical trial has been published previously(97). The following is an overview of the protocol with emphasis on the aspects of the trial most relevant to the metabolomics study.

Recipient Recruitment

Consented transplant recipients selected for inclusion were low-to-moderate-transplant-risk adult candidates, as deemed by the local liver transplant listing multidisciplinary meeting, listed for elective orthotopic liver transplantation, and over 18 years of age. Low-risk candidates were patients with a low UKELD score, no history of cardiovascular disease and no previous major upper abdominal surgery, good functional and nutritional status, and documented evidence of a patent portal vein. Patients requiring urgent liver transplantation were excluded.

Donor Liver Selection

Figure 5 provides a schematic of the donor liver pathway for the trial. Each donor liver offer had to pass a two-tier inclusion system. The first tier stipulated that the liver had to have been rejected by all UK liver transplant centres in the United Kingdom for transplantation. These livers were procured with the intention of transplantation and subsequently rejected via the normal or fast track pathway due to the graft's perceived marginality or for logistical reasons.

Cold ischaemia time could not exceed 16 hours for DBDs and 10 hours for DCDs. The second tier required that the donor organ satisfy one of seven criteria confirming its ECD status.

ECD criteria included: Donor Risk Index more than 2.0; graft macrovesicular steatosis greater than 30%; BAR score greater than 9; donor warm ischaemia time greater than 30 minutes; cold ischaemia time greater than 12 hours for DBD or 8 hours for DCD grafts; evidence of suboptimal graft perfusion and donor transaminases over 1000 IU/L.

At the retrieval hospital, each liver would be submerged in cold UW solution and packed in a sterile bag, then placed on top of slush ice solution in a suitable bowl. The bowl would be packed in a second sterile and vacuumed bag before being submerged in ice. The transport box would also contain a set of iliac vessels, spleen, lymph nodes and blood samples and be accompanied by the relevant documentation. As soon as our centre received the liver, it underwent bench surgery. This prepared the graft for connection to the perfusion device. Extraneous tissues were removed, and all vascular cuffs were cleaned and fashioned appropriately. The graft was kept in sterile slush ice and UW solution at 4 °C during bench surgery to avoid rewarming. A perfusion-giving set with cold UW was set up to perfuse the liver and check portal vein and arterial tree integrity.

Normothermic Machine Perfusion of the Liver

Following bench surgery, the graft was removed from the slush ice bath and connected to the perfusion device. The Organox Metra (Oxford, UK) machine perfusion system was selected for the VITTAL trial (**Figure 6**). The system consists of a sterile containment unit which would

accommodate the liver, a perfusate reservoir, oxygenators, pressure pumps, sensors to monitor and emulate physiological parameters and tubing to connect the inflow of the liver to the circuit. The liver's outflow via the remnant of the vena cava was left to drain freely into a reservoir below the liver that recycled the perfusate back into the circulation. Nutrient-rich and oxygenated perfusate bathed the liver while sensors monitored vascular flows and pressures in the circuit and maintained the organ's temperature between 36 to 37 ^{o}C. The perfusate's base mixture consisted of red cell concentrate for oxygen carriage, a nutrient source, acid-base agents, and antibiotics. In addition, throughout the perfusion procedure, constant infusions of vasodilators, antithrombotic agents, insulin and bile salt solution were maintained. During perfusion, biochemical analysis of the blood-based perfusate was performed using a Cobas biochemical point-of-care analyser (Roche Diagnostics), which analysed the perfusate's pH, pO2, pCO2, bicarbonate level, base excess as well as concentrations for calcium, chloride, sodium, potassium, haemoglobin, haematocrit, lactate and glucose. Sensors monitored arterial and portal venous flows, resistances and pressures (**Figures 6 and 7**).

The liver was perfused for a minimum of four hours, at which point the principal investigator and on-call transplant surgeon assessed its parameters to determine if they satisfied the viability criteria, as outlined below. If these were met, the liver was deemed transplantable, and the recipient was prepared for theatre. The organ remained on the machine until explantation of the recipient's native liver was complete and the operative field was ready to receive the donor liver. While the liver was connected to the perfusion device its parameters were monitored at regular intervals to determine if they still fell within the viability criteria. If there was concern in this regard, the principal investigator and transplant surgeon were informed. The liver was disconnected from the machine in tandem with the recipient hepatectomy. The organ was

flushed with three litres of histidine-tryptophan-ketoglutarate solution at 4 ºC and handed over to the transplant surgeon. At this point, the NMP-L procedure was complete.

Transplantation Viability Criteria

Viability Criteria used for the VITTAL trial are found below. In addition, each liver had to satisfy the major criterion and any two of the minor criteria within four hours of NMP-L. These criteria were selected for the trial protocol based on the transplant centre's previous experience with viability assessment during NMP-L.

Major criterion

- Maintenance of perfusate lactate levels ≤2.5 mmol/L

Minor criteria (any two)

- pH perfusate maintenance greater than 3.0
- Evidence of bile production
- Stable arterial flow greater than 150 ml/min and portal vein flow more than 500 ml/min
- Evidence of glucose metabolism
- Homogenous graft perfusion with soft consistency of parenchyma

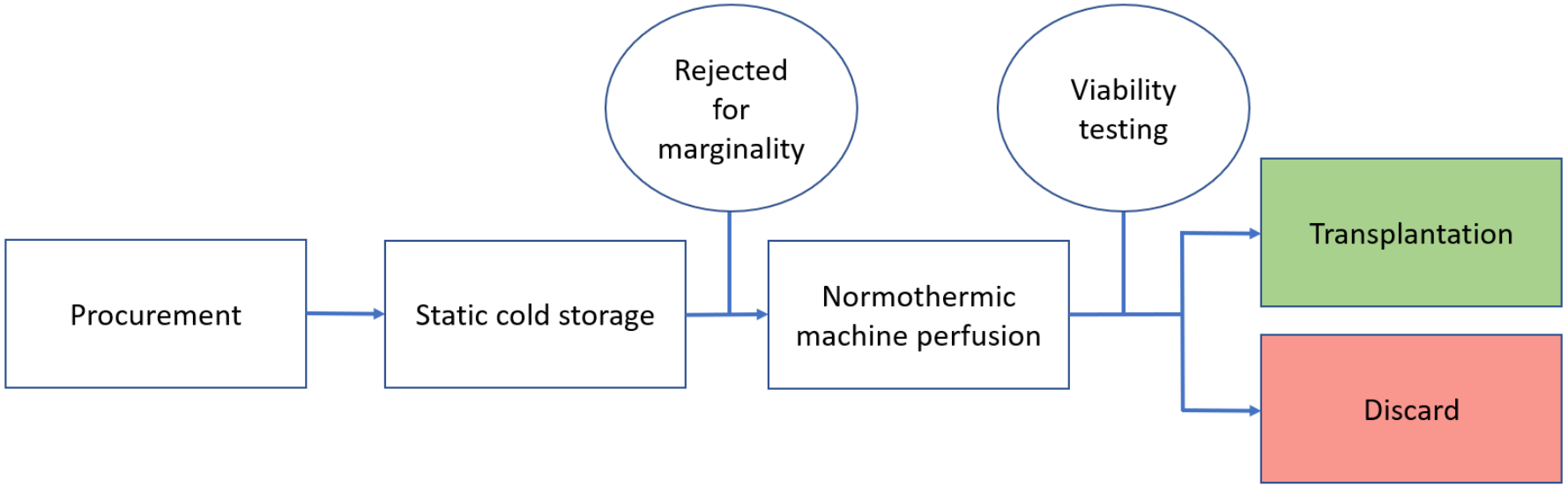

Figure 5. Schematic representation of NMP-L protocol for donor livers in VITTAL clinical trial. Samples for metabolomic analysis were acquired during the Normothermic Machine Perfusion phase.

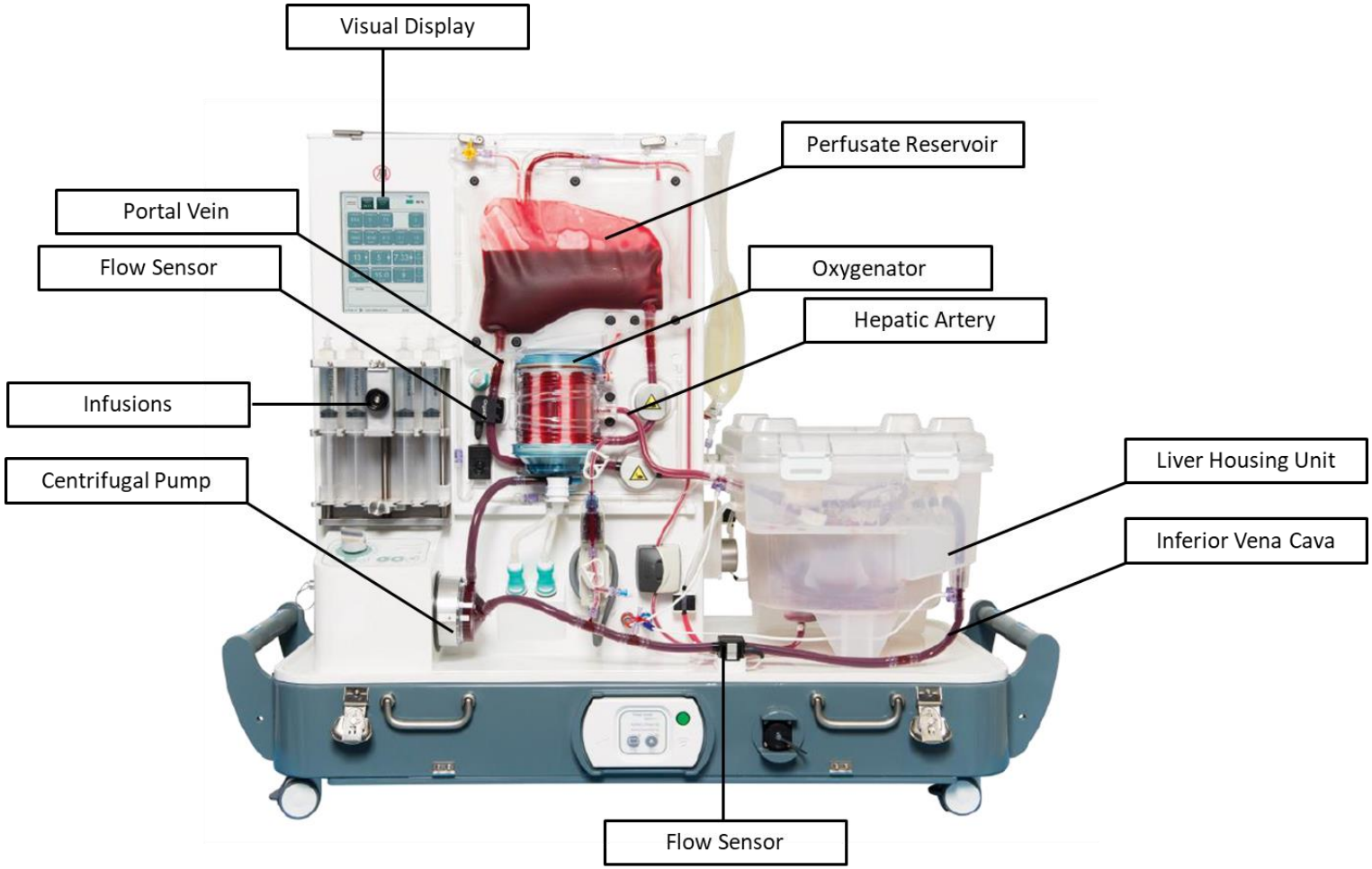

Figure 6. Organox Metra Liver Perfusion Device. A schematic of this device is provided in the figure overleaf.

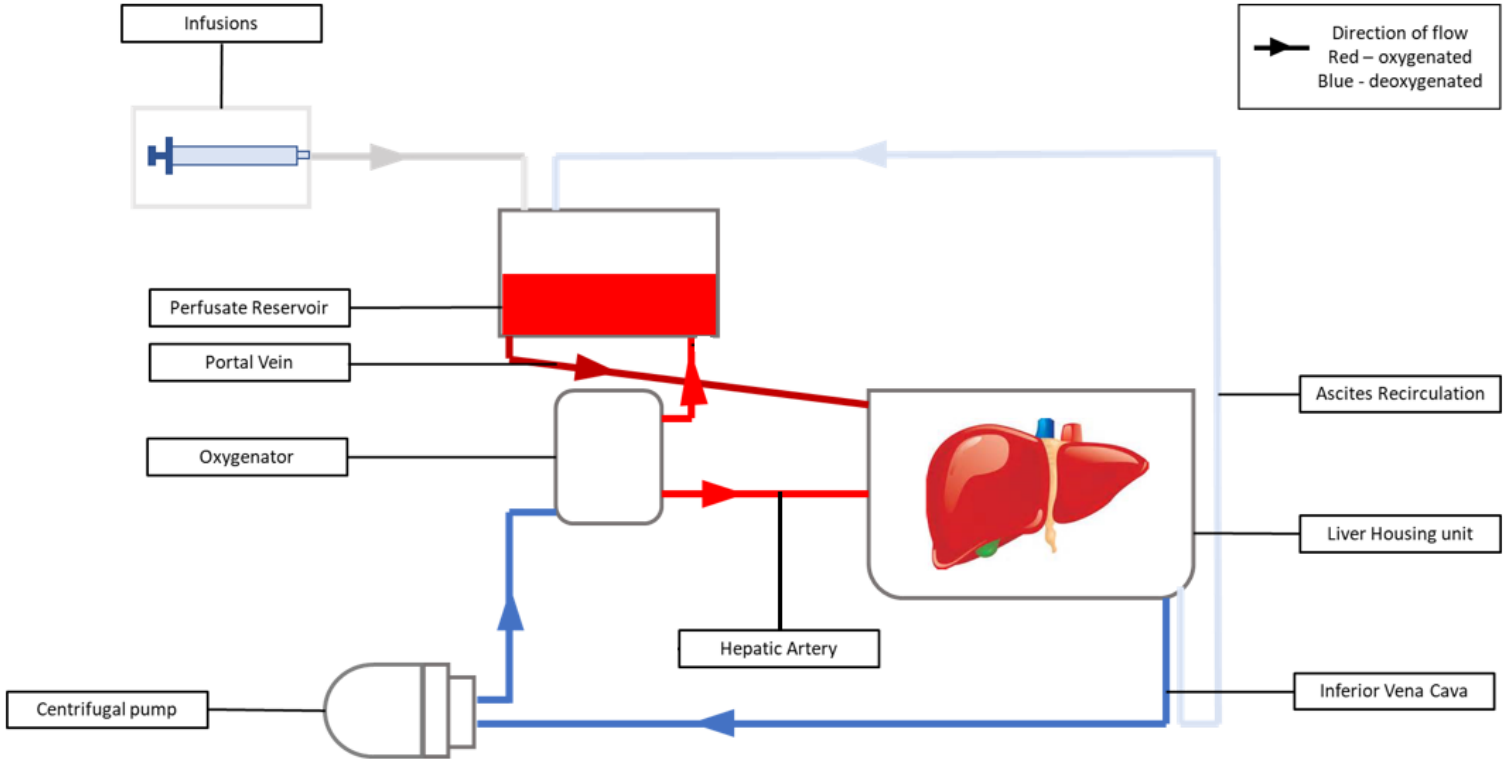

Figure 7. Schematic for NMP-L using Organox Metra Device

Figure 7. Schematic for NMP-L using Organox Metra Device

3.2 NMP-L Metabolomics Study Protocol

3.2.1 Study Design

The study was designed within the framework of the metabolomics experiment workflow, as outlined in **Chapter 2**. This was an exploratory study and therefore an untargeted analytical approach was chosen in order to assess the sample metabolome in its entirety. This maximises the number of metabolites detected and increases the likelihood of observing unexpected or previously undescribed changes while simultaneously reducing bias in the results.

3.2.2 Research Questions

The primary outcome was to perform a mechanistic study relating to the ECD NMP-L metabolome and understand the metabolic changes occurring during perfusion and their relation to viability testing. The parameters for viability testing have been outlined above. The secondary outcome was to determine whether VITTAL post-transplant outcomes are reflected in pre-transplantation perfusate metabolic phenotypes. The outcomes were selected for their clinical relevance and known association with post-operative graft dysfunction and an unfavourable recipient outcome: (i) EAD, and (ii) post-reperfusion syndrome (PRS). In the VITTAL clinical trial these outcomes were defined as follows.

EAD: presence of one or more of the following: bilirubin ≥10 mg/dL on post-operative day 7; INR ≥1.6 on postoperative day 7; ALT or AST >2000 IU/mL within the first seven post-operative days.

PRS: more than 30% decrease in mean arterial pressure from baseline lasting longer than one minute within the first 5 minutes of reperfusion.

3.2.3 Group Definition

With the research questions established, groups for metabolomic data interrogation were defined as follows. For the primary outcome: ECD livers that met transplantation viability criteria versus those that did not. This included the entire study population of 31 livers (22 transplanted and nine non-transplanted livers). For the secondary outcomes: Transplanted ECD livers that had (i) EAD versus no EAD, (ii) PRS versus no PRS. This included all 22 transplanted livers.

3.2.4 Biological Experiment

The VITTAL clinical trial, as already described.

3.2.5 Sample Choice

NMP-L perfusate was the sampling medium chosen for metabolomic analysis, and this was for several reasons. The perfusate metabolome is in direct contact with the liver metabolome and it can be assumed that changes in one would be reflected by changes in the other. From a clinical standpoint, perfusate sampling is preferable to tissue biopsies as sample extraction is non-invasive and does not risk direct trauma to the liver tissue. As a result, sampling could be carried out more safely than if tissue biopsies were taken. Metabolic perturbations in liver parenchyma may be influenced by the area of sampling due to local alterations in tissue

architecture, such as steatosis, and may therefore be heterogenous compared to perfusate. Moreover, the main determining factor for liver viability as per the Birmingham Liver Unit's criteria was perfusate lactate concentration after four hours of NMP-L perfusion. Perfusate metabolomic analysis would enable correlation of other metabolic perturbations in the same medium. Bile analysis was not included in the sampling protocol as bile secretion is highly variable between livers, may be limited in quantity and may be negligible if ECD liver resuscitation is unsuccessful during NMP-L. **Appendix 8** contains details of the starting perfusate composition.

3.2.6 Sample Collection and Quenching Method

Perfusate samples for metabolomic analysis were procured at three points during each perfusion procedure: (i) within the first 15 minutes of the start of liver perfusion, (ii) at the two-hour time point, and (iii) at the four-hour mark, when the principal investigator and transplanting surgeon assessed the organ's viability for transplantation. Samples were obtained from a sterile port in the device's perfusion circuit using a sterile syringe and aliquoted into 1.5 ml conical microtubes. These were placed into a centrifuge to displace the heavier cellular and particulate matter. Sample quenching was performed by extracting the supernatant, distributing it in vials and flash freezing in liquid nitrogen. Samples were stored at -80 ^{o}C before being transported for metabolomic analysis at Phenome Centre Birmingham.

3.2.7 Instrument Set-Up and Analysis

The study's explorative nature and objective to obtain an unbiased and comprehensive understanding of the ECD NMP-L metabolome warranted an untargeted metabolomics

analytical approach. For this reason, MS was selected as the analytic tool due to its inherent and well-documented advantages over NMR in metabolic profiling. These include the higher sensitivity and resolution of MS for metabolite coverage. Following sample quenching and storage, further sample processing as well as metabolomic data processing and statistical analysis were performed by Phenome Centre Birmingham.

3.3 Results

3.3.1 Donor demographics

31 donor livers underwent end-ischaemic NMP-L during the trial. **Table 5** below outlines the inclusion criteria met for each graft. Fourteen (45 %) grafts were DBDs and 17 (55 %) were DCDs, 13 (42 %) and 18 (58 %) were from male and female donors respectively. Median donor age was 57 years (range: 30 – 80 years). Median DRI and BAR scores were 2.1 (range: 1.2 – 3.8) and 3 (range: 1 – 11) respectively. Median graft weight was 1750 g (range: 1172 – 2604 g). Median cold ischaemia time was 453 minutes (range: 324 – 890 minutes). For DCDs, median functional warm ischaemia time was 21 minutes (range: 16 – 46 minutes).

3.3.2 Viability Criteria Assessment

23 livers ultimately satisfied the transplant viability criteria, eight did not. Twenty-five livers demonstrated satisfactory lactate clearance down to 2.5 mmol/L. Unfortunately, a suspicious donor colonic lesion biopsy during one procurement procedure confirmed malignancy, making one liver unsuitable for transplantation. Although three livers initially fulfilled the viability criteria, an increase in perfusate lactate beyond 2.5 mmol/L was observed with subsequent blood gas measurements. In two of these cases, the livers were discarded as the transplant had

not been started. However, in the third instance, the recipient hepatectomy had begun, so the transplant went ahead. For the purposes of our metabolomics study, groups were analysed according to whether viability status was achieved as opposed to whether or not the organ was transplanted. **Table 6** outlines the donor and organ characteristics when grouped according whether viability criteria were met. **Table 7** lists the criteria met by each individual graft.

Table 5. Inclusion criteria met and viability outcome for each graft that underwent end-ischaemic NMP-L in the VITTAL trial

Liver no.	Graft Inclusion Criteria							Viability Criteria Met
	DRI	BAR	Steatosis	CIT	fWIT	Perfusion	Transaminases	
1	Y	NA		Y				Y
2				Y	NA			Y
3	Y	Y			NA			Y
4		Y		Y	NA			Y
5	Y			Y	NA			Y
6	Y			Y	NA			Y
7	Y				NA		Y	Y
8	Y			Y				Y
9	Y	NA					Y	N
10	Y	NA						N
11					NA		Y	Y
12	Y	NA		Y				N
13	Y					Y		Y
14	Y				Y			Y
15	Y							Y
16			Y		NA			Y
17				Y	NA	Y		Y
18	Y				Y			Y
19	Y				Y			Y
20	Y					Y		Y
21	Y							Y
22	Y	NA			NA		Y	Y
23	Y			Y		Y		Y
24	Y	NA	Y	Y	NA			N
25	Y	NA		Y	NA			N
26		NA			NA	Y		N
27		NA			NA	Y		N
28	Y							Y
29			Y		NA			N
30	Y				NA			Y
31	Y				NA			Y

DRI: donor risk index; BAR: balance-of-risk score ; CIT: cold ischaemia time; fWIT: functional warm ischaemia time; Y: yes; N: no; NA: criterion not applicable

Table 6. Donor demographics and graft characteristics grouped according to achievement of transplant viability criteria.

	Met Viability Criteria (n= 23)	**Did Not Meet Viability Criteria** (n=8)	**p**
Donor Type (n),			
DBD	11	3	0.698
DCD	12	5	
Donor Age (years),			0.378
median (range)	60 (36 – 84)	53 (30 – 70)	
Donor Gender (n),			
Male	12	6	0.412
Female	11	2	
Donor DRI (score),			
median (range)	2.1 (1.2 – 3.8)	2.2 (1.6 – 3.0)	0.682
Donor BAR (score),			
median (range)	3 (1 – 8)	3 (3 – 11)	0.346
Graft CIT (minutes),			
median (range)	453 (324 – 890)	517 (360 – 804)	0.619
DCD fWIT (minutes),			
median (range)	21.0 (17.0 – 46.0)	19.5 (16.0 – 23.0)	0.409
Graft weight (g),			
median (range)	1660 (1172 – 2353)	1989 (1522 – 2604)	0.009
Steatosis on histology*			
small droplet macrosteatosis			
<30%	13	1	0.045
>30%	10	7	
large droplet macrosteatosis			
<30%	23	6	0.06
>30%	0	2	

***** Histological data was provided retrospectively by an experienced histopathologist

Table 7. Viability criteria outcomes for each individual liver

Liver ID	Perfusate Lactate	pH>7.30	bile production	glucose metabolism	vascular flows	homogenous graft perfusion
1	Y	N	Y	Y	Y	Y
2	Y	N	N	N	Y	Y
3	Y	Y	Y	N	Y	Y
4	Y	N	Y	Y	Y	Y
5	Y	Y	Y	N	Y	Y
6	Y	Y	Y	N	Y	Y
7	Y	N	Y	N	Y	Y
8	Y	Y	Y	N	Y	Y
9	N	N	Y	Y	Y	Y
10	N	Y	Y	N	Y	Y
11	Y	Y	Y	Y	Y	Y
12	N	Y	N	N	Y	Y
13	Y	Y	N	N	Y	Y
14	Y	Y	N	Y	Y	Y
15	Y	Y	Y	Y	Y	Y
16	Y	N	N	N	Y	Y
17	Y	Y	N	N	Y	Y
18	Y	Y	Y	Y	Y	Y
19	Y	Y	Y	Y	Y	Y
20	Y	N	Y	Y	Y	Y
21	Y	N	Y	Y	Y	Y
22	Y	N	Y	Y	Y	Y
23	Y	Y	Y	Y	Y	Y
24	N	N	N	Y	Y	Y
25	N	N	Y	N	Y	Y
26	N	N	Y	N	Y	Y
27	N	N	Y	N	Y	Y
28	Y	Y	Y	Y	Y	Y
29	N	N	Y	Y	Y	Y
30	Y	N	Y	Y	Y	Y
31	Y	Y	Y	N	Y	Y

3.3.3 Transplantation Outcomes

22 livers were transplanted and 9 were discarded. 10 transplants exhibited PRS (45.5 %), while 7 (31.8 %) demonstrated EAD. **Table 8** outlines the outcomes for each individual liver.

Table 8. Outcomes for all perfused livers

Liver no.	Graft Type	CIT (minutes)	NMP-L Time (minutes)	Total Preservation Time (minutes)	PRS	EAD
1	DCD	550	NA	NA	NA	NA
2	DBD	440	695	1135	No	Yes
3	DBD	360	665	1025	No	No
4	DBD	790	477	1267	No	No
5	DBD	625	580	1205	No	Yes
6	DBD	890	540	1430	No	No
7	DBD	390	1063	1453	No	No
8	DCD	573	450	1023	Yes	Yes
9	DCD	360	NA	NA	NA	NA
10	DCD	436	NA	NA	NA	NA
11	DBD	324	705	1029	No	No
12	DCD	599	NA	NA	NA	NA
13	DCD	389	1143	1532	Yes	Yes
14	DCD	429	620	1049	Yes	Yes
15	DCD	332	765	1097	Yes	No
16	DBD	715	593	1308	No	Yes
17	DBD	720	327	1047	No	No
18	DCD	464	414	878	Yes	No
19	DCD	420	557	977	No	No
20	DCD	354	327	681	Yes	No
21	DCD	412	427	839	Yes	No
22	DBD	516	NA	NA	NA	NA
23	DCD	600	840	1440	Yes	No
24	DBD	720	NA	NA	NA	NA
25	DBD	804	NA	NA	NA	NA
26	DBD	360	NA	NA	NA	NA
27	DBD	715	NA	NA	NA	NA
28	DCD	334	540	874	No	No
29	DBD	375	528	903	Yes	Yes
30	DBD	466	960	1426	Yes	No
31	DBD	453	680	1133	No	No

CIT: cold ischaemia time; NMP-L: normothermic machine perfusion of the liver; PRS: post-reperfusion syndrome; EAD: early allograft dysfunction; DBD: donation after brain death; DCD: donation after cardiac death; NA: not applicable

3.3.4 Samples for Metabolomics Analysis

A total of 93 perfusate samples (three samples per perfusion) were procured and stored for metabolomic analysis at a later date. These included samples at three specific time points: within the first 15 minutes of NMP-L after the liver was connected to the perfusion device (*t0.25*), at the two-hour time point (*t2*) and four-hour time-point (*t4*, when organ viability was determined to establish whether transplantation will proceed). The results of the metabolomic analysis will be outlined and discussed in the following chapter.

3.4 Discussion

Metabolomic studies have been conducted on transplanted livers to establish the molecular events that render some livers more susceptible to IRI and graft dysfunction. However, these studies have mainly involved heterogenous populations of cold-stored livers. Metabolomic studies investigating NMP-L have thus far shown great potential in increasing our understanding of donor liver metabolism. Nonetheless, the majority are basic science perfusion studies with a significant lack of data relating to clinical perfusions. Our study attempted to address this knowledge gap by scrutinising viability parameters and clinical outcomes against metabolomic data.

In this respect, we anticipated that our metabolomics study has a number of advantages. First and foremost, samples were procured from a well-defined cohort of ECD livers, as illustrated by the donor demographics and organ characteristics in the VITTAL trial, thus ensuring that the data obtained would be representative of this population. Secondly, it would enable a direct comparison of metabolomic data to functional parameters (namely acid-base balance, glucose

metabolism, bile production and vascular flow) rather than static donor variables and allow for a time series metabolomic analysis of dynamic organ function. Furthermore, an analysis of groups based to viability status achievement may generate metabolic data that could be used to further inform and develop transplant viability criteria. Finally, the incorporation of our study into a clinical trial would also allow for comparison with clinically relevant outcomes, that is PRS and EAD.

Chapter 4 Ultra-High Performance Liquid Chromatography – Mass Spectometry of Extended Criteria Donor Liver Perfusate Extracted During End-Ischaemic Normothermic Machine Perfusion

The work described in this chapter represents collaborative work. The author provided the samples and clinical data for analysis. Ultra-High Performance Liquid Chromatography and Mass Spectrometry (UHPLC-MS) sample analysis was performed by the Phenome Centre Birmingham. Professor Warwick Dunn carried out metabolomic data processing, quality assessment and biostatistical analysis. The author interpreted the biostatistical results, correlated the biological data with clinical outcomes and provided the intellectual contributions to the manuscript which is currently being prepared for peer review.

4.1 Introduction

The main purposes of a mass spectrometer are threefold. It is employed to identify unknown compounds, quantify known molecules, and establish their chemical structure and properties(57, 62). Identification of unknown compounds occurs via determination of their molecular mass once a sample has been broken down into its molecular components(57, 62). The basic principles behind how a mass spectrometer works are as follows. The platform imparts an electrical charge to the sample, converting it into a gas of positively charged ions which are molecular fragments of different masses. These ions are accelerated and deflected by magnetic field. The number of positive charges on the ion also affects the degree of deflection. This creates a beam of ions which generated an electric current that is detected and analysed by the data system(57, 62). The lighter the mass the greater the deflection. The flux generated from these electrically charged ions in turn is converted into an electric current which is read by the system to produce data. These processes are carried out in a vacuum to avoid inadvertent collisions with air molecules, which would affect the data output(57, 62). The Q Exactive Focus mass spectrometer described in this work uses orbitrap technology, so-called because it uses an outer electrode and inner spindle electrode to trap ions in orbital motion for detection and analysis(98).

Liquid chromatography is a separation technique which is used in the laboratory to separate a mixture into its different components. It consists of a mobile phase (which in this case is a liquid) that carries the mixture through a stationary phase. The different affinities of the mixture's components to the particles of the stationary phase cause them to separate(57, 62). In high performance liquid chromatography the sample is passed through the stationary phase at high pressure. It uses two types of columns of mobile and stationary phases, such as

Hydrophilic Action Liquid Chromatography (HILIC) and Reverse Phase Liquid Chromatography (RPLC). In RPLC, alkyl or aromatic ligands covalently bonded to a silica substrate creates a hydrophobic stationary phase through which through which compounds are separated passed on hydrophobicity(57, 62). In HILIC, the mobile phase consists of a weak organic solvent with water as a strong eluting solvent. This provides a polar stationary phase for compound separation. Coupled with MS, HPLC increases the molecule separating power of the platform(57, 62).

4.2 Untargeted Metabolomic Analysis – A Methodology

Untargeted metabolomic profiling of primary metabolites was performed using UHPLC-MS. Phenome Centre Birmingham carried out sample extraction, UHPLC-MS data acquisition and raw data processing, as described below, under the supervision of Professor Warwick Dunn. The author observed the process in order to gain an understanding of the experimental workflow and the factors that may influence biological interpretation of the results. The details of the methodological steps were provided by Professor Warwick Dunn.

4.2.1 Sample preparation

Perfusate samples from each liver perfusion were initially left to thaw on ice. Aliquots from each sample were then prepared for HILIC, and Reverse Phase C_{18} liquid Chromatography assays. 50 µL aliquots from each sample were mixed with 150 µL of ice-cold acetonitrile (LCMS grade, HiPerSolv, VWR) and vortexed for 20 seconds. All samples were centrifuged at 20,000g at 4°C for 20 minutes and 150 µL of supernatant were loaded into HPLC vials (VI-04-12-02RVG 300µl Plastic, Chromatography Direct, UK). For the reverse phase C_{18} liquid chromatography assay, the samples were prepared using the same protocol as for the HILIC

assay but propan-2-ol (IPA) (LCMS grade, LiChrosolv, Merck) was used instead of acetonitrile. For Quality Control (QC), 75 µL from each perfusate sample were pooled together and thoroughly vortexed for 5 minutes. These QC samples were then prepared following the same protocol as the rest of the samples. Extract blanks were prepared by the same methods using 50 µL of water (LCMS grade, LiChrosolv, Merck) instead of the perfusate sample.

4.2.2 Ultra-High Performance Liquid chromatography-Mass spectrometry

Sample analysis was carried out via two UHPLC-MS methods using a Dionex UltiMate 3000 Rapid Separation LC system (Thermo Fisher Scientific, MA, USA) coupled with an electrospray Q Exactive Focus mass spectrometer (Thermo Fisher Scientific, MA, USA). Samples were extracted with acetonitrile and IPA for polar and non-polar metabolites respectively.

Samples extracted with acetonitrile were analysed on the Accucore-150-Amide-HILIC column (100 x 2.1 mm, 2.6 µm, Thermo Fisher Scientific, MA, USA). Using this instrument set-up, mobile phase A consisted of 10 mM ammonium formate and 0.1% formic acid in 95% acetonitrile/water and mobile phase B consisted of 10 mM ammonium formate and 0.1% formic acid in 50% acetonitrile/water. Injection volume was set at 2µL and column temperature at 35 ^{o}C. The flow rate was set at 0.50 mLmin^{-1} with the gradient: *t*=0.0, 1% B; *t*=1.0, 1% B; *t*=3.0, 15% B; *t*=6.0, 50% B; *t*=9.0, 95% B; *t*=10.0, 95% B; *t*=10.5, 1% B; *t*=14.0, 1% B. All changes were noted to be linear with a curve equal to 5. Data were acquired in positive and negative ionisation modes separately within the mass range 70 – 1050 *m/z* at resolution 70,000 (FWHM at *m/z* 200). The ion source parameters were set as sheath gas 53 arbitrary units, auxiliary gas 14 arbitrary units, sweep gas 3 arbitrary units, spray voltage = 3.5kV, capillary

temperature 269 °C and auxiliary gas heater temperature = 438 °C. Data-dependent MS^2 in 'Discovery mode' was used for the MS/MS spectra acquisition, applying a resolution of 17,500 (FWHM at m/z 200), isolation width 3.0 *m/z* and stepped normalised collision energies (stepped NCE) of 25, 60, 100%. Finally, spectra were acquired in mass ranges: 50 – 200 *m/z*; 200 – 400 *m/z* and 400 – 1000 *m/z*. These assays are defined as HILIC positive ion mode and HILIC negative ion mode.

Samples extracted with IPA were analysed on Hypersil GOLD C_{18} column (100 x 2.1mm, 1.9 µm; Thermo Fisher Scientific, MA, USA). Mobile phase A consisted of 10 mM ammonium formate and 0.1% formic acid in 60% acetonitrile and water and mobile phase B consisted of 10 mM ammonium formate and 0.1% formic acid in 90% propan-2-ol and water. In this case, injection volume was set at 2 µL and column temperature 55 ^{o}C the flow rate was set at 0.40 $mL.min^{-1}$ with the gradient: *t*=0.0, 20% B; *t*=0.5, 20% B, *t*=8.5, 100% B; *t*=9.5, 100% B; *t*=11.5, 20% B; *t*=14.0, 20% B with all changes noted to be linear at a curve equal to 5. The ion source parameters were set at sheath gas 50 arbitrary units, auxiliary gas 13 arbitrary units, sweep gas 3 arbitrary units, spray voltage 3.5kV, capillary temperature 263 °Ca and auxiliary gas heater temperature 425 °C. Data dependent MS^2 in 'Discovery mode' was used for the MS/MS spectra acquisition. This was done using a resolution of 17,500 (FWHM at m/z 200), isolation width 3.0 *m/z*; and stepped normalised collision energies (stepped NCE) 20, 50, 80%. Spectra with mass ranges 200 – 400 *m/z*; 400 – 700 *m/z* and 700 – 1500 *m/z* were subsequently acquired.

A Thermo Exactive Tune 2.8 SP1 build 2806 was used as instrument control software in both cases described above and data were acquired in profile mode. QC samples were analysed as

the first ten injections in the system followed by two QC samples at the end of the analytical batch. Samples from individual time points from the same liver perfusions were measured together, each time followed by a QC sample. Four blank samples were analysed in each assay, with the first sample analysed as the sixth injection in the system, second and third samples analysed at fixed points in the injection sequence and the fourth sample analysed at the end of each batch.

4.2.3 Metabolomics Data Analysis

Raw Data Processing

Raw data acquired in each analytical batch were converted from the instrument-specific format to the mzML file format through ProteoWizard open access software. Deconvolution was performed with XCMS software using minimal peak width (4 for HILIC and 6 for lipids); maximum peak width (30), ppm (12 for HILIC and 14 for lipids), mzdiff (0.001), gapInit (0.5 for HILIC and 0.4 for lipids), gapExtend (2.4), bw (0.25) and mzwid (0.01). The resulting data matrix consisted of metabolite features (*m/z*-retention time pairs) versus samples with peak areas where the metabolite feature was detected for each sample. PUTMEDID-LCMS workflows operating in the Taverna workflow environment were used to putatively annotate metabolites or metabolite groups. 5 ppm mass error was applied and a retention time range of 2 seconds in feature grouping and molecular formula and metabolite matching was used. Different metabolites can be detected with the same accurate *m/z* due to different molecular structures, such as isomers having the same molecular formula, and therefore multiple annotations were observed for a single detected metabolite feature. Conversely, a single metabolite may be detected as multiple molecules as a consequence of different fragmentation and ionisation patterns leading to, for example, different types of ions (e.g. protonated and

sodiated ions derived from the same metabolite). Throughout this report, the term *metabolite* refers to either single metabolites or groups of molecules with the same retention time and the same accurate *m/z*. All molecules detected in the data matrix were annotated in line with Metabolomics Standards Initiative level 2, guidelines for reporting of chemical analysis results.

Data analysis comprised metabolite feature extraction, quantitation, statistical analysis, metabolite identification and pathway enrichment analysis. Data and statistical analysis were carried out using MetaboAnalyst, a free online platform for processing NMR and MS-derived metabolomic data. Raw data processing and conversion to a data matrix (as described above) produced a .xls spreadsheet data file. This was converted to .csv format to enable upload to and processing by MetaboAnalyst.

Data Pre-Treatment

Data pre-treatment involved a number of steps for data reduction to minimise the influence of missing values and outliers on the biological interpretation of the metabolite dataset. The initial step was QC to evaluate overall data quality and assess the analytical variance in the data. Using the data from pooled QC samples, the relative standard deviation (RSD) of each metabolite feature across all QC samples was calculated to assess data reproducibility. Features with RSD greater than 30% and found to be missing more than 50% of QC samples failed to pass QC and were removed from the dataset. This was followed by a missing values imputation by applying a K-Nearest Neighbours algorithm under the assumption that a feature's value can be estimated based on the values of the features closest to it. For multivariate analysis, data were normalised to the sum of peak intensities and underwent generalised log transformation to bring the multivariate data closer to normality. Pareto scaling, using the square root of the

standard deviation as a scaling factor, was applied to the peak intensity data to adjust for the differences in fold changes between different metabolites. This adjustment is more pronounced in metabolites with larger fold changes, thus maintaining the dimensions and original structure of the data. Visual clustering of QC samples constructed PCA. For pre-treatment of data for univariate analysis, data were normalised to the sum of peak intensities only.

Data analysis

Following the QC check, the QC samples were removed from the dataset and multivariate analyses were carried out for samples obtained at *t0* and at *t4* separately. These were carried out using the same data pre-treatment procedures as described above except for QC sample processing. A PCA algorithm was initially constructed, using the first two principal components explaining the maximal amount of variation in the samples, to visually assess the data for natural separation of the sample groups. The dataset was then subjected to supervised learning methods to construct a partial least squares discriminant analysis algorithm demonstrating components of variation between a labelled training dataset. The algorithm was subsequently cross-validated to assess the percentage of variation (R^2) and predictive ability of the model (Q^2) to determine a goodness-of-fit.

Univariate analysis using non-parametric methods (Wilcoxon Rank Sum test) was finally conducted using T4 data to identify metabolites with significantly different abundances between groups following four hours of NMP-L. Significance was determined with a threshold <0.05 after correction for multiple testing, false discovery rate (FDR) correction. A fold change analysis was performed to measure the degree of change of significant metabolites between groups. Pathway analysis was applied through submission of a list of all metabolite annotations

reported as statistically significant with each annotation as a separate entry (input type = compound name). Manual correction of metabolite annotations not automatically matched was performed with the correct match or no match being submitted. The visualization method was scatter plot, the Enrichment method was Hypergeometric test, the topology analysis was Relative-betweeness Centrality, the Reference metabolome was Use all compounds in the selected pathway library. The pathway library used was Homo sapiens (KEGG). Enriched pathways were reported with a q-value of <0.05 and a minimum of 5 metabolites from the pathway present in the submitted list of metabolite annotations.

Fold changes in metabolites over perfusion time were expressed as t0.25 / t2.0 and t0.25 / t4.0. Therefore, a fold change of less than one denotes an increase in abundance whereas a fold change of more than one signifies a decrease. Fold changes for the viability assessment analysis were expressed as non-viable livers / viable livers.

4.3 Results

4.3.1 Metabomics Data Overview

Untargeted metabolomics data for 93 perfusate samples (31 livers, three time points per liver [0.25 hours, 2 hours and 4 hours]) were collected applying four complementary UHPLC-MS assays (HILIC positive ion mode, HILIC negative ion mode, C_{18} lipids positive ion mode and C_{18} lipids negative ion mode). The quality of the data collected was visually assessed applying unsupervised multivariate PCA analysis (*Supplementary File*). Pooled QC samples demonstrated a high level of clustering compared to the biological samples and therefore a high-quality dataset where further data analysis could be performed.

4.3.2 ECD Liver Perfusate Metabolic Phenotype during NMP-L

ECD Liver Metabolite Profile

Unsupervised multivariate PCA for data acquired applying the two HILIC assays demonstrated that the metabolic phenotype of perfusate collected within the first 15 minutes of perfusion (t0.25) of perfusion was different to perfusate collected at two and four hours (*t*2 and *t*4 respectively) from all 31 donor livers. As exhibited by the PCA plots in **Figure 8** there was clustering of all three timepoints with complete separation of *t*0.25 from *t*2 and *t*4. There was some separation of samples collected at two and four hours, but the overlap suggested that there was less of a metabolic difference between these later time points. This may indicate that the most significant metabolic changes are observed at up to two hours of NMP-L, though with a less significant change in metabolic phenotype also being observed between two and four hours. This trend was not observed for the data acquired applying the two lipidomic assays, with no visual separation of the data at each of the time points.

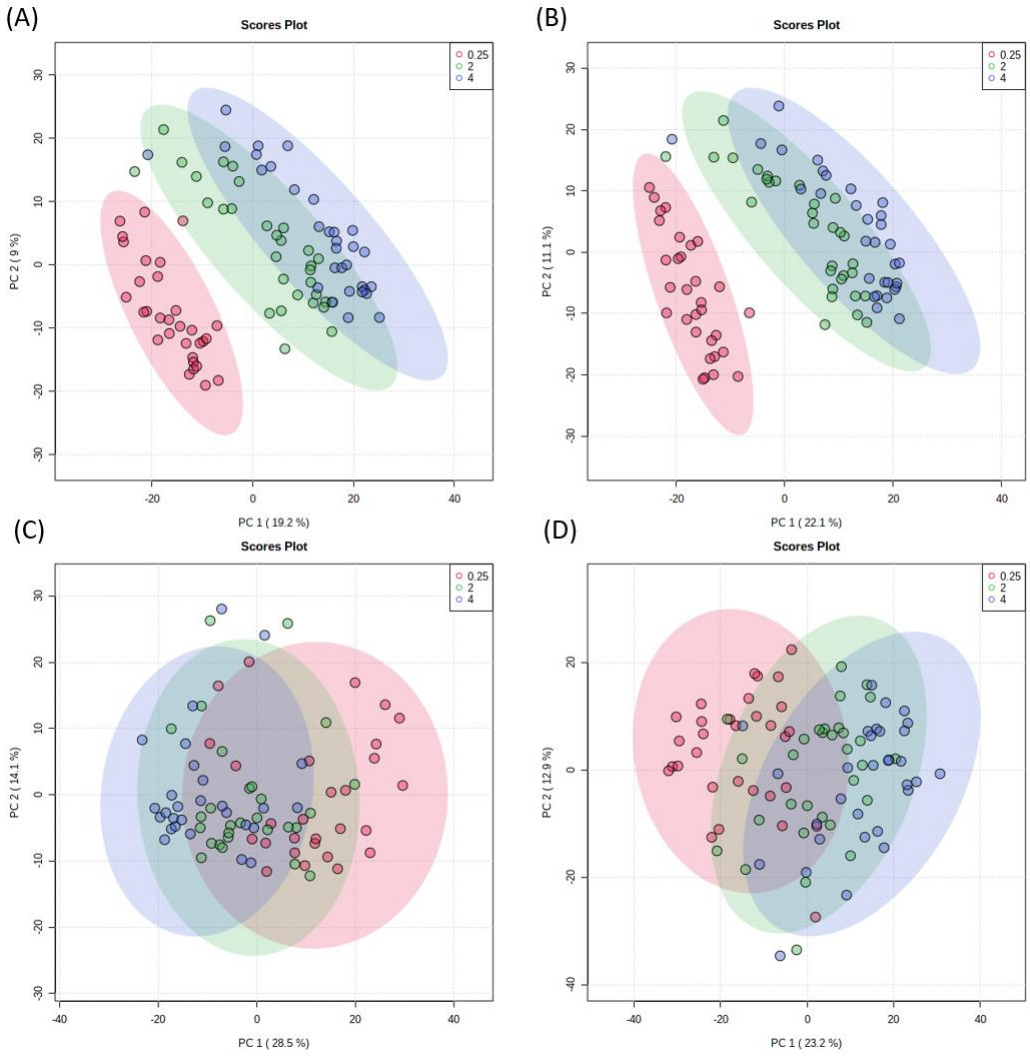

Figure 8. Principal Components Analysis describing the distribution of data for each of three perfusate sampling time points after the start of liver perfusion (t0.25, t2 and t4 hours) for all 31 livers for data collected applying HILIC negative ion mode (A), HILIC positive ion mode (B), C_{18} lipids negative ion mode (C) and C_{18} lipids positive ion mode (D). Scores plots are shown for PC1 vs. PC2.

One-way repeated measures ANOVA was performed ($q<1x10^{-10}$ after correction for multiple testing). 392 unique metabolite features were statistically significant. 260 metabolite features were present at higher abundances at t2 and t4 compared to t0.25 hours and represent metabolites being released from the liver tissue (*Supplementary Files*). These included 9 acyl carnitines (median fold change 0.65, range 0. 31 – 9.96, n=16, $q<0.05$), 29 ceramides (median

fold change 0.53, range 0.25 – 2.20, n=30, q<0.05), 76 glycerophospholipids (median fold change 0.64, range 0.26 – 2.68, n=97, q<0.05), 12 lysoglycerophospholipids (median fold change 0.34, range 0.19 – 0.70, n=12, q<0.05) and 19 triacylglycerides (median fold change 1.44, range 0.39 – 2.73, n=44, q<0.05) (**Figure 9**). 127 metabolites were present at higher abundances at t0.25 compared to t2 and t4 and represent metabolites being consumed from the perfusion solution. These included 7 acyl carnitines, 1 ceramide, 18 glycerophospholipids and 25 triacylglycerides. Ceramides, glycerophospholipids and lysoglycerophospholipids were predominantly released from the liver. The raw data concerning the specific lipid related metabolite features can be found in Appendix 4. Amino acid abundances also decreased as the perfusion time increased. This indicates significant liver uptake of these metabolites during perfusion and assumes that these metabolites are depleted in the liver prior to perfusion.

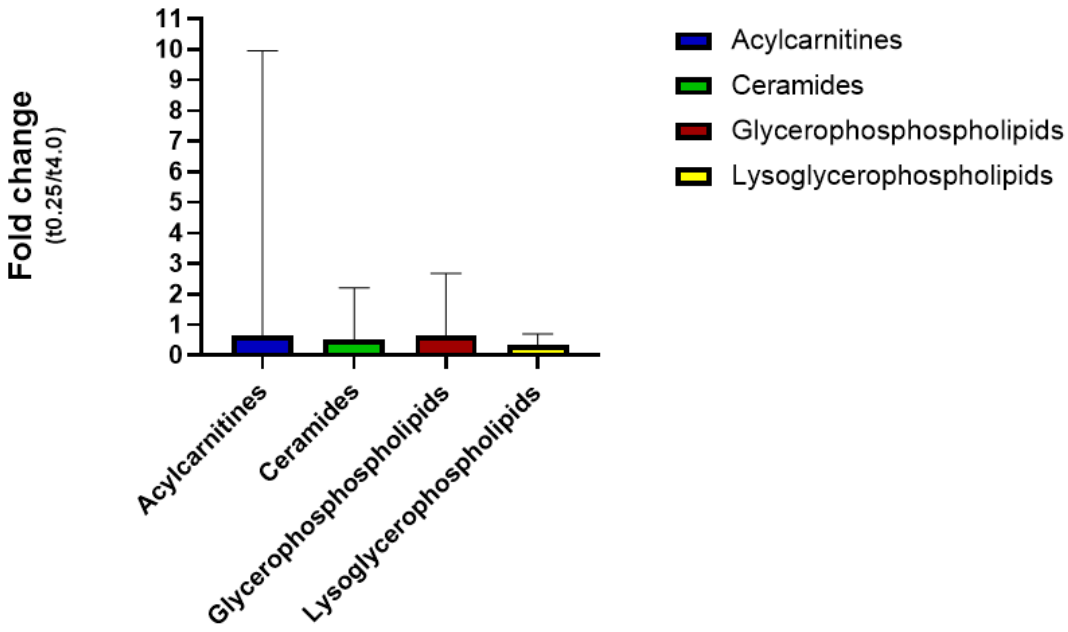

Figure 9: Fold changes in acylcarnitines and lipid-based metabolites in ECD liver perfusate for all ECD livers from t0.25 to t4.0.

Pathway Enrichment Analysis

Pathway Enrichment Analysis of all non-lipid and mixed class defined metabolite annotations derived from the one-way repeated measures ANOVA results demonstrated enrichment of five metabolic pathways. These were primarily related to amino acid metabolism: the glycine /serine /threonine metabolic axis, lysine degradation, histidine metabolism and valine /leucine biosynthesis (**Table 9**).

Table 9. *Pathway Enrichment Analysis applying all non-lipid and mixed class defined metabolite annotations derived from the one-way repeated measures ANOVA results assessing metabolic changes over time independent of outcome. The match status (X/Y) defines the number of metabolites in the pathway (Y) and the number of statistically significant metabolites from the pathway that were reported (X).*

Pathway Name	**Match Status (X/Y)**	**p-value (FDR)**	**Statistically Significant Pathway Metabolites (q<0.05)**
Glycine, serine and threonine metabolism	9/33	0.0071194	Betaine; Guanidinoacetate; Glycine; 5,10-Methylenetetrahydrofolate; L-Threonine; Tetrahydrofolate; 3-Phosphonooxypyruvate; L-2-Amino-3-oxobutanoic acid; Methylglyoxal
Nicotinate and nicotinamide metabolism	6/15	0.0071194	Nicotinamide D-ribonucleotide; Nicotinamide; Iminoaspartate; Nicotinate; N1-Methyl-4-pyridone-5-carboxamide; N1-Methyl-2-pyridone-5-carboxamide
Histidine metabolism	6/16	0.0080767	Urocanate; L-Histidine; Imidazole-4-acetaldehyde;; N(pi)-Methyl-L-histidine; N-Formimino-L-glutamate; Imidazole-4-acetate
Lysine degradation	7/25	0.01567	L-Lysine; 4-Trimethylammoniobutanoate; N6,N6,N6-Trimethyl-L-lysine; Protein N6,N6-dimethyl-L-lysine; L-Pipecolate; L-Hydroxylysine; (3S)-3-Hydroxy-N6,N6,N6-trimethyl-L-lysine
Valine, leucine and isoleucine biosynthesis	4/8	0.015785	L-Threonine; (S)-3-Methyl-2-oxopentanoic acid; 3-Methyl-2-oxobutanoic acid; 4-Methyl-2-oxopentanoate

Table 10: Heat map showing fold changes of all non-lipid and mixed class defined metabolite annotations derived from the one-way repeated ANOVA results assessing metabolic changes over time independent of outcome and matched in the pathway enrichment analysis. Fold change less than 1.0 signifies an increase in concentration over time.

Adjusted P-value	Fold change			Metabolite annotation	Metabolic Pathway
	0.25hours/2 hours mean	2 hours/4 hours mean	0.25 hours/4 hours mean		
1.14E-22	0.4	0.59	0.24	N6,N6,N6-Trimethyl-L-lysine	Lysine Degradation
1.06E-15	6.25	1.2	7.97	Lysine; Pipecolic acid	
3.00E-16	6.46	1.21	8.3	Lysine	
1.58E-11	2.51	1.51	3.73	5-Hydroxylysine	
1.09E-19	2.58	1.95	5.11	4-Trimethylammoniobutanoic acid	
7.42E-12	0.77	0.72	0.56	3-Hydroxy-N6,N6,N6-trimethyl-L-lysine	
7.96E-17	0.45	0.71	0.32	Ne,Ne dimethyllysine	
2.44E-26	0.33	0.56	0.19	Formiminoglutamic acid	Histidine metabolism
1.57E-13	1.39	1.06	1.47	Imidazole-4-acetaldehyde	
1.72E-20	7.49	3.54	31.89	Formiminoglutamic acid	
3.76E-24	0.35	0.68	0.23	Imidazoleacetic acid	
1.02E-30	0.35	0.8	0.28	3-Phosphonooxypyruvate	Glycine, serine and threonine metabolism
2.07E-27	0.42	0.68	0.29	2-amino-3-oxo-butanoic acid	
8.93E-24	0.16	0.93	0.15	Tetrahydrofolic acid	
2.76E-19	0.47	0.77	0.35	Methylglyoxal	
9.25E-23	3.45	2.34	6.93	Threonine	
3.24E-30	0.3	0.55	0.17	5,10-Methylenetetrahydrofolate	
1.80E-19	0.55	0.63	0.35	Betaine	
7.85E-21	4.49	1.51	6.42	Glycine	
1.09E-13	0.62	0.78	0.5	3-Methyl-2-oxobutanoic acid; 3-methyl-2-oxo-butanoic acid	Valine, leucine and isoleucine biosynthesis
1.44E-10	2.41	1.19	2.79	3-Methyl-2-oxopentanoic acid; 4-Methyl-2-oxopentanoate	
9.25E-23	3.45	2.34	6.93	Threonine	
4.10E-26	0.26	0.54	0.15	Nicotinic acid	Nicotinate and nicotinamide metabolism
3.76E-24	0.35	0.68	0.23	Imidazoleacetic acid	
3.12E-12	0.67	0.72	0.5	Nicotinamide ribotide	
2.07E-27	123.65	1.34	158.56	Niacinamide; Nicotinamide	
1.76E-22	24.01	5.19	60.85	2-Methylnicotinamide; N-Methylnicotinamide	

4.3.3 ECD Liver Perfusate Metabolic Phenotype According to Viability Criteria

ECD Liver Metabolite Profile

Univariate t-test analyses ($q<0.05$ after correction for multiple testing) were applied separately at each of the three sampling time points (*t*0.25, *t*2 and *t*4) to identify metabolic differences between the ECD livers which did and did not meet the transplantation viability criteria. 247, 643 and 299 metabolites were statistically significant at *t*0.25, *t*2 and *t*4 respectively.

Acylcarnitines were found to be present in significantly higher proportions in the non-viable liver group at t0.25 (median fold change 5.96, range 3.14 – 13.22, n= 13, $q<0.05$) and t2 (median fold change 3.10, range 0.05 – 21.96, n=21, $q<0.05$) (**Figure 10**). This observation was still present but less pronounced at t4 with a lower number of higher fold-change acylcarnitines in the non-viable liver perfusate (median fold change 3.17, range 2.09 – 8.26, n=3; $q<0.05$). Ceramides and glycerophospholipids were in significantly higher proportions in non-viable versus viable livers throughout NMP-L. Lysoglycerophospholipids were in significantly higher abundances in the non-viable liver perfusate solely at the start of perfusion (median fold change 4.21, range 0.02 – 7.43, n=9, $q<0.05$). Furthermore, glycosphingolipids were also found to be significantly higher in the non-viable group at all three time points. The raw data regarding lipid-related metabolite features can be found in Appendix 5, 6 and 7.

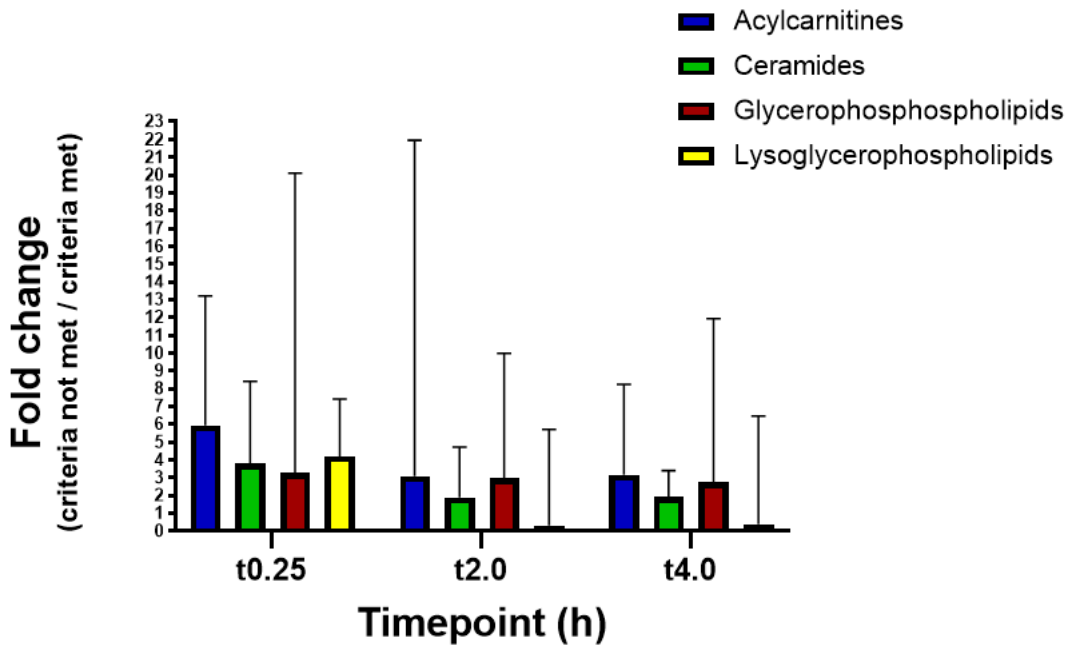

Figure 10: Fold changes in acylcarnitines and lipid-based metabolites in ECD liver perfusate for viable versus non-viable livers.

Pathway Enrichment Analysis

Pathway enrichment analysis of all non-lipid and mixed class defined metabolite annotations was performed for each time point separately. No pathways were enriched for data collected at *t*0.25 and *t*4. 12 pathways were enriched for data collected at *t*2 (**Table 11**). These again were primarily amino acid-related metabolic pathways: arginine and proline metabolism, arginine biosynthesis, alanine / aspartate / glutamate metabolism, tryptophan metabolism, histidine metabolism and glycine / serine / threonine metabolism. The related metabolic pathways of purine metabolism, pyrimidine metabolism and caffeine metabolism were also enriched.

Table 11. Pathway Enrichment Analysis results applying all non-lipid and mixed class defined metabolite annotations derived from the analysis of livers which did not meet transplantation viability criteria compared to livers that did at t2. The match status (X/Y) defines the number of metabolites in the pathway (Y) and the number of statistically significant metabolites from the pathway that were reported (X).

Pathway Name	**Match Status**	**p-value (FDR)**	**Statistically significant pathway metabolites (q<0.05)**
Caffeine metabolism	10/10	4.28E-09	1,7-Dimethylxanthine; 1-Methylxanthine; Theobromine; 7-Methylxanthine; Caffeine; 1-Methyluric acid; 3,7-Dimethyluric acid; 1,7-Dimethyluric acid; 5-Acetylamino-6-formylamino-3-methyluracil; 7-Methyluric acid
Arginine and proline metabolism	17/38	4.38E-07	L-Arginine; Guanidinoacetate; Creatine; 4-Aminobutanoate; Putrescine; Spermidine; N4-Acetylaminobutanal; L-4-Hydroxyglutamate semialdehyde; L-1-Pyrroline-3-hydroxy-5-carboxylate; L-Proline; (S)-1-Pyrroline-5-carboxylate; L-Glutamate; Phosphocreatine; 4-Guanidinobutanoate; 4-Acetamidobutanoate; 1-Pyrroline-2-carboxylate; 1-Pyrroline-4-hydroxy-2-carboxylate
Alanine, aspartate and glutamate metabolism	12/28	8.90E-05	L-Asparagine; N-(L-Arginino)succinate; L-Alanine; Succinate semialdehyde; L-Glutamate; 4-Aminobutanoate; (S)-1-Pyrroline-5-carboxylate; Citrate; Oxaloacetate; Fumarate; Succinate; 2-Oxoglutarate
Arginine biosynthesis	7/14	0.0026729	L-Glutamate; L-Arginine; N-Acetylornithine; N-(L-Arginino)succinate; L-Citrulline; 2-Oxoglutarate; Fumarate
Pantothenate and CoA biosynthesis	8/19	0.0030737	Pantetheine 4'-phosphate; Pantothenate; 3-Ureidopropionate; 5,6-Dihydrouracil; L-Valine; L-Cysteine; beta-Alanine; Uracil
Butanoate metabolism	7/15	0.0030738	(R)-3-Hydroxybutanoate; Acetoacetate; 4-Aminobutanoate; L-Glutamate; Succinate semialdehyde; 2-Oxoglutarate; Succinate
Tryptophan metabolism	12/41	0.0032466	L-Tryptophan; N-Acetylserotonin; 5-Hydroxyindoleacetate; 5-Hydroxykynurenamine; 2-Aminomuconate semialdehyde; L-Kynurenine; Formylanthranilate; Formyl-N-acetyl-5-methoxykynurenamine; Formyl-5-hydroxykynurenamine; 5-Methoxyindoleacetate; Anthranilate
Histidine metabolism	7/16	0.0037708	L-Glutamate; L-Histidine; Imidazole-4-acetaldehyde; Methylimidazole acetaldehyde; Histamine; N(pi)-Methyl-L-histidine; Imidazole-4-acetate
Glycine, serine and threonine metabolism	10/33	0.0061765	Betaine; Guanidinoacetate; Glycine; Sarcosine; D-Glycerate; Tetrahydrofolate; Creatine; 3-Phosphonooxypyruvate; L-Cysteine; 2-Oxobutanoate

Citrate cycle	7/20	0.013329	2-Oxoglutarate; Succinate; Isocitrate; Oxaloacetate; cis-Aconitate; Citrate; Fumarate; [
Pyrimidine metabolism	10/39	0.019117	Orotidine 5'-phosphate; UMP; UTP; Uridine; 5,6-Dihydrouracil; 3-Ureidopropionate; Deoxycytidine; Thymidine; Thymine; Uracil; beta-Alanine
Purine metabolism	13/65	0.044996	Xanthine; 2-(Formamido)-N1-(5'-phosphoribosyl)acetamidine; dADP; Adenosine; dAMP; Deoxyinosine; Xanthosine; Hypoxanthine; Inosine; Guanine; Deoxyguanosine; dIDP; 5-Amino-4-imidazolecarboxyamide

Table 12: Heat map showing fold changes of all non-lipid and mixed class defined metabolite annotations derived from the one-way repeated ANOVA results assessing metabolic changes at t2 and matched in the pathway enrichment analysis. Fold change greater than 1.0 denotes a higher abundance in non-viable liver perfusate.

Adjusted P-value	Fold Change (Criteria not met/criteria met)	Metabolite annotation	Metabolic Pathway
0.017585	3.49	Histidine	Histidine metabolism
0.0078257	0.19	Imidazoleacetic acid	
0.0055343	0.45	Histamine	
0.0038084	0.62	Imidazole-4-acetaldehyde	
0.040177	1.7	Glutamic acid	
0.011226	2.24	1-Methylhistidine||3-Methylhistidine||N(pi)-Methyl-L-histidine	
0.015973	0.52	Imidazole-4-acetaldehyde	
0.0032603	0.57	Imidazole-4-acetaldehyde	
0.0090588	0.1	Indolelactic acid; 5-Hydroxyindoleacetate	Tryptophan metabolism
0.043435	0.37	2-Aminomuconic acid semialdehyde	
0.0060163	0.37	Formylanthranilic acid	
0.044438	0.43	Tryptophan; N-Acetylserotonin; Formyl-N-acetyl-5-methoxykynurenamine	
0.010875	3.3	2-Aminomuconate semialdehyde	
0.020627	3.49	Tryptophan	
0.030697	0.17	Formyl-5-hydroxykynurenamine; Kynurenine; 5-Hydroxyindoleacetate	
0.00096118	0.48	3-Hydroxykynurenamine; 5-Hydroxykynurenamine	
0.0013035	0.49	Formyl-5-hydroxykynurenamine; Kynurenine	
0.0058845	0.52	Anthranilate	
0.0027218	0.54	Acetyl-N-formyl-5-methoxykynurenamine; Formyl-N-acetyl-5-methoxykynurenamine	
0.035043	2.18	Creatine	Arginine and Proline metabolism
0.0072307	4.62	Spermidine	
0.0032603	3.39	Arginine	
0.043435	0.37	(S)-1-Pyrroline-5-carboxylate; 1-Pyrroline-2-carboxylate; 1-Pyrroline-2-carboxylic acid; 1-Pyrroline-5-carboxylic acid; Proline	
0.0070553	0.37	4-Acetamidobutanoate	

0.0038084	0.62	1-pyrroline-3-hydroxy-5-carboxylic Acid; 1-Pyrroline-4-hydroxy-2-carboxylate	
0.040177	1.7	Glutamic acid; 4-Hydroxyglutamate semialdehyde	
0.042906	2.46	4-Guanidinobutanoic acid	
0.013983	3.57	Aminobutanoate	
0.0072307	3.9	4-Aminobutanoate	
0.022696	7.62	N4-Acetylaminobutanal	
0.023823	0.65	Creatine	
0.034734	1.81	Putrescine	
0.046302	1.86	4-Guanidinobutanoate	
0.012968	2.39	Creatine	
0.001856	30.36	Guanidoacetic acid	
0.021382	2.67	Asparagine	Alanine, aspartate and glutamate metabolism
0.0025292	0.25	Dihydroxy-dioxohexanoate||2-hydroxy-3-oxoadipic acid||Ascorbic acid||Citraconic acid||Itaconic acid||Fumaric acid||Maleic acid||Diaminobutanoate	
0.0039027	0.34	Argininosuccinic acid	
0.043435	0.37	(S)-1-Pyrroline-5-carboxylate||1-Pyrroline-2-carboxylate||1-Pyrroline-2-carboxylic acid||1-Pyrroline-5-carboxylic acid||2-Aminomuconic acid semialdehyde||Proline	
0.018996	0.5	Citric acid||Isocitric acid||Dehydroascorbic acid||cis-Aconitic acid||trans-Aconitic acid	
0.02361	0.76	3-methoxy-3-oxopropanoic acid||4-Hydroxy-2-oxobutanoic acid||Methylmalonic acid||Succinic acid	
0.040177	1.7	2-Oxo-4-hydroxy-5-aminovalerate||Glutamic acid||4-Hydroxyglutamate semialdehyde||N-Acetylserine||N-Methyl-D-aspartic acid||N-lactoyl-Glycine||O-Acetyl-L-serine	
0.034303	2.22	Methylmalonic acid semialdehyde||2-Methyl-3-oxopropanoate||2-Oxobutanoate||Acetoacetic acid||Succinate semialdehyde	
0.039952	2.26	2-Oxoglutarate||3-Oxoglutaric acid||Oxoglutaric acid	
0.036514	3.2	Methylmalonic acid||Succinic acid||3-Hydroxy-2-oxobutanoic acid	
0.013983	3.57	Aminobutanoate||O-Acetylethanolamine||Alanine||Sarcosine||beta-Alanine||Amino-methylpropanoate	
0.0072307	3.9	3-Amino-2-methylpropanoate||beta-Aminoisobutyric acid||2-Aminobutanoate||2-Aminoisobutyric acid||3-Aminobutanoic acid||3-Aminoisobutanoic acid||4-Aminobutanoate||gamma-Aminobutyric acid||Valine||Threitol||Erythritol	
0.036886	4.78	2-Oxoglutarate||3-Oxoglutaric acid||Oxoglutaric acid||Oxalacetic acid||Isocitric acid||Citric acid	
0.0050942	5.12	Alanine||Sarcosine||beta-Alanine	
0.0080916	2.85	N-Acetylornithine	Arginine biosynthesis
0.0032603	3.39	Arginine	
0.031145	3.02	Citrulline	

0.0025292	0.25	Fumaric acid	
0.0039027	0.34	Argininosuccinic acid	
0.042164	3.17	2-Oxoglutarate	
0.036886	4.78	2-Oxoglutarate	
0.045999	5.01	N-Acetylornithine	
0.035043	2.18	Glycerylphosphorylethanolamine||Beta-Guanidinopropionic acid||Creatine	Glycine, serine and threonine metabolism
0.0040966	0.5	Tetrahydrofolic acid	
0.040308	0.7	Glyceric acid	
0.034303	2.22	Methylmalonic acid semialdehyde||2-Methyl-3-oxopropanoate||2-Oxobutanoate||Acetoacetic acid||Succinate semialdehyde	
0.032002	3.38	3-Phosphonooxypyruvate	
0.013983	3.57	Aminobutanoate||O-Acetylethanolamine||Alanine||Sarcosine||beta-Alanine||Amino-methylpropanoate	
0.023823	0.65	Beta-Guanidinopropionic acid||Creatine||Creatinine	
0.012968	2.39	Beta-Guanidinopropionic acid||Creatine	
0.0059855	2.56	2-Amino-3-methylbutanoic acid||2S-amino-pentanoic acid||4-Methylaminobutyrate||4-amino-pentanoic acid||4R-aminopentanoic acid||4S-aminopentanoic acid||5-Aminopentanoate||5-Aminopentanoic acid||5-amino-pentanoic acid||Amyl Nitrite||Betaine||Valine	
0.014766	2.82	Cysteine	
0.018129	3.58	Glycine	
0.0050942	5.12	Alanine||Sarcosine||beta-Alanine	
0.0032603	3.39	Pantothenic acid||Hydroxydecanedioic acid||Arginine	Pantothenate and CoA Biosynthesis
0.0078257	0.19	5,6-Dihydrothymine||Dihydrothymine||Imidazole||Hydantoin-5-propionate||Uracil||Imidazoleacetic acid||Thymine||2-Oxosuccinamate||Iminoaspartate	
0.0066087	0.32	Hydantoin-5-propionate||5,6-Dihydrothymine||Uracil||2-Oxosuccinamate||Thymine||Imidazole||Iminoaspartic acid	
0.013983	3.57	Aminobutanoate||O-Acetylethanolamine||Alanine||Sarcosine||beta-Alanine||Amino-methylpropanoate	
0.0072307	3.9	3-Amino-2-methylpropanoate||beta-Aminoisobutyric acid||2-Aminobutanoate||2-Aminoisobutyric acid||3-Aminobutanoic acid||3-Aminoisobutanoic acid||4-Aminobutanoate||gamma-Aminobutyric acid||Valine||Threitol||Erythritol	
0.006788	5.27	Pantothenic acid	
0.019607	0.23	Uracil	
0.015778	0.5	Pantetheine 4'-phosphate	
0.0059855	2.56	2-Amino-3-methylbutanoic acid||2S-amino-pentanoic acid||4-Methylaminobutyrate||4-amino-pentanoic acid||4R-aminopentanoic acid||4S-aminopentanoic acid||5-Aminopentanoate||5-Aminopentanoic acid||5-amino-pentanoic acid||Amyl Nitrite||Betaine||Valine	
0.014766	2.82	Cysteine	

0.0050942	5.12	Alanine||Sarcosine||beta-Alanine	
0.02361	0.76	3-methoxy-3-oxopropanoic acid||4-Hydroxy-2-oxobutanoic acid||Methylmalonic acid||Succinic acid	Butanoate metabolism
0.048877	1.52	2-hydroxybutyric acid||3-Hydroxybutanoate||3-Hydroxyisobutyric acid||4-Hydroxybutanoic acid	
0.040177	1.7	2-Oxo-4-hydroxy-5-aminovalerate||Glutamic acid||4-Hydroxyglutamate semialdehyde||N-Acetylserine||N-Methyl-D-aspartic acid||N-lactoyl-Glycine||O-Acetyl-L-serine	
0.034303	2.22	Methylmalonic acid semialdehyde||2-Methyl-3-oxopropanoate||2-Oxobutanoate||Acetoacetic acid||Succinate semialdehyde	
0.039952	2.26	2-Oxoglutarate||3-Oxoglutaric acid||Oxoglutaric acid	
0.042164	3.17	2-Oxoglutarate||3-Oxoglutaric acid	
0.036514	3.2	Methylmalonic acid||Succinic acid||3-Hydroxy-2-oxobutanoic acid	
0.013983	3.57	Aminobutanoate||O-Acetylethanolamine||Alanine||Sarcosine||beta-Alanine||Amino-methylpropanoate	
0.0072307	3.9	3-Amino-2-methylpropanoate||beta-Aminoisobutyric acid||2-Aminobutanoate||2-Aminoisobutyric acid||3-Aminobutanoic acid||3-Aminoisobutanoic acid||4-Aminobutanoate||gamma-Aminobutyric acid||Valine||Threitol||Erythritol	
0.036886	4.78	2-Oxoglutarate||3-Oxoglutaric acid||Oxoglutaric acid||Oxalacetic acid||Isocitric acid||Citric acid	
0.011807	3.41	Deoxycytidine||hydroxyoctanoic Acid	Pyrimidine metabolism
0.0078257	0.19	5,6-Dihydrothymine||Dihydrothymine||Imidazole||Hydantoin-5-propionate||Uracil||Imidazoleacetic acid||Thymine||2-Oxosuccinamate||Iminoaspartate	
0.0066087	0.32	Hydantoin-5-propionate||5,6-Dihydrothymine||Uracil||2-Oxosuccinamate||Thymine||Imidazole||Iminoaspartic acid	
0.013391	0.5	3'-UMP||Pseudouridine 5'-phosphate||UMP||Uridine 2'-phosphate||Uridine 3'-monophosphate||Uridine 5'-monophosphate	
0.0079818	0.56	3'-UMP||Pseudouridine 5'-phosphate||Uridine 2'-phosphate||Uridine 3'-monophosphate||Uridine 5'-monophosphate	
0.031145	2.01	Thymidine	
0.0302	3.56	Deoxycytidine	
0.013983	3.57	Aminobutanoate||O-Acetylethanolamine||Alanine||Sarcosine||beta-Alanine||Amino-methylpropanoate	
0.019607	0.23	Uracil	
0.0050942	5.12	Alanine||Sarcosine||beta-Alanine	
0.023061	3.31	Adenosine	Purine metabolism
0.013391	4.22	Hypoxanthine	
0.018495	2.57	Deoxyadenosine monophosphate||dAMP	
0.013636	2.73	dADP	
0.0083896	0.51	Xanthosine	
0.019014	2.5	N-Acetylneuraminic acid||S-Acetyldihydrolipoamide||Allopurinol riboside||Arabinosylhypoxanthine||Inosine	
0.0072307	5.01	1,3,7-Trimethyl-5-hydroxyisourate||5-Acetylamino-6-formylamino-3-methyluracil||Allopurinol riboside||Arabinosylhypoxanthine||Inosine	

0.0032603	0.04	Allopurinol riboside||Arabinosylhypoxanthine||Inosine	
0.02856	0.11	2,4-Diamino-6-hydroxypyrimidine||5-Amino-4-imidazolecarboxyamide||5-Methylthioribose 1-phosphate	
0.031704	0.46	6,8-Dihydroxypurine||Oxypurinol||Xanthine	
0.00096118	6.04	2-Hydroxyadenine||8-Hydroxyadenine||Guanine	
0.0025292	0.25	Dihydroxy-dioxohexanoate||2-hydroxy-3-oxoadipic acid||Ascorbic acid||Citraconic acid||Itaconic acid||Fumaric acid||Maleic acid||Diaminobutanoate	Tricarboxylic Acid cycle
0.018996	0.5	Citric acid||Isocitric acid||Dehydroascorbic acid||cis-Aconitic acid||trans-Aconitic acid	
0.02361	0.76	3-methoxy-3-oxopropanoic acid||4-Hydroxy-2-oxobutanoic acid||Methylmalonic acid||Succinic acid	
0.039952	2.26	2-Oxoglutarate||3-Oxoglutaric acid||Oxoglutaric acid	
0.042164	3.17	2-Oxoglutarate||3-Oxoglutaric acid	
0.036514	3.2	Methylmalonic acid||Succinic acid||3-Hydroxy-2-oxobutanoic acid	
0.036886	4.78	2-Oxoglutarate||3-Oxoglutaric acid||Oxoglutaric acid||Oxalacetic acid||Isocitric acid||Citric acid	
0.045005	8.36	Dehydroascorbic acid||cis-Aconitic acid||trans-Aconitic acid	
0.035574	17.27	Dehydroascorbic acid||4-Hydroxyglutamine||cis-Aconitic acid||trans-Aconitic acid	

4.3.4 ECD Liver Perfusate Analysis Based on Clinical Outcome

Statistically significant metabolites were observed prior to correction for multiple testing when the data was analysed in the context of PRS and EAD. However, this was not the case when correction for multiple testing ($q<0.05$) was applied to all p values. For this reason and to ensure robust reporting and consistency in the statistical analysis, data that displayed $q>0.05$ was not reported. Therefore, according to our analysis, metabolomics does not distinguish ECD liver perfusate based on the selected clinical outcomes.

4.4 Discussion

While several studies have demonstrated metabolic markers of IRI and graft dysfunction in cold-stored livers, few have investigated the dynamics of ECD liver metabolism during NMP-L. This has become a critical area of clinical research in protocol development for NMP-L as it may provide the groundwork for the development of prognostic biomarkers of graft function as well as liver-specific adjunct therapies prior to transplantation.

4.4.1 Analytical Approach

Untargeted, or discovery-based, metabolomics provides an unbiased global analysis and relative quantification of metabolites in a biological sample. It therefore offers the ability to identify altered and previously undescribed physiologically relevant molecules and metabolic pathways and link them to outcomes of interest. The simultaneous analysis of hundreds of metabolites would have generated hundreds of hypotheses in relation to the study outcome. This necessitated the adoption of the FDR method for correction for multiple testing to increase confidence in the statistically significant results of this high throughput data analysis, by

reducing the number of false positives. Correction for multiple testing attempts to assign an adjusted p-value to each test or reduce the p-value threshold from 5% to a more reasonable value(99). Another way to look at the difference is that a p-value of 0.05 implies that 5% of all tests will result in false positives. An FDR adjusted p-value (or q-value) of 0.05 implies that only 5% of significant tests (i.e. tests with $p<0.05$) will result in false positives(99). Additionally, the adoption of a pathway enrichment analysis aims to establish a link between statistically significant metabolites and provides mechanistic insight for biological interpretation, thus lending more weight to the results.

Our study offered a bioinformatics approach to the analysis of the ECD liver perfusate metabolome and revealed that perfusate metabolites show distinct profiles during reperfusion and NMP-L. Moreover, significant differences were also established when livers were classified according to achievement of transplant viability criteria. Notable changes included the accumulation of lipid-based and amino acid metabolites as well as the upregulation of identifiable metabolic pathways in the pathway enrichment analysis, several of which have been linked to IRI, impaired mitochondrial function and tricarboxylic acid cycle regulation. Our analysis, however, did not identify distinct metabolic signatures related to early allograft dysfunction or post-reperfusion syndrome.

Time series analysis of all 31 ECD livers demonstrated that the most significant metabolic changes occurred during the first two hours of perfusion (**Figure 8**). This corresponded with the re-warming phase of the metabolically suppressed organ to physiological temperatures as it is transferred from the ice bath to the perfusion circuit. This would indicate that this initial resuscitation period is a vital therapeutic window for the liver as it attempts to re-establish

homeostasis and may have a significant bearing on its subsequent metabolic behaviour. When scrutinising livers according to viability, the number of statistically significant metabolite features points to the transplant viable and non-viable livers being more metabolically similar early in perfusion (though with many areas of metabolism perturbed) with metabolic divergence over the first two hours, as demonstrated by the pathway enrichment analysis in **Table 11**. Beyond this time point, the reduction in the number of statistically significant metabolites at the four-hour mark suggests that these groups became more metabolically similar as NMP-L progressed with lipidomic perfusate profiles being primarily responsible for discriminating ECD livers on the basis of transplant viability (**Figure 10**). **Figure 11** below provides a simplified schematic linking several of the metabolic pathways revealed in the pathway enrichment analysis.

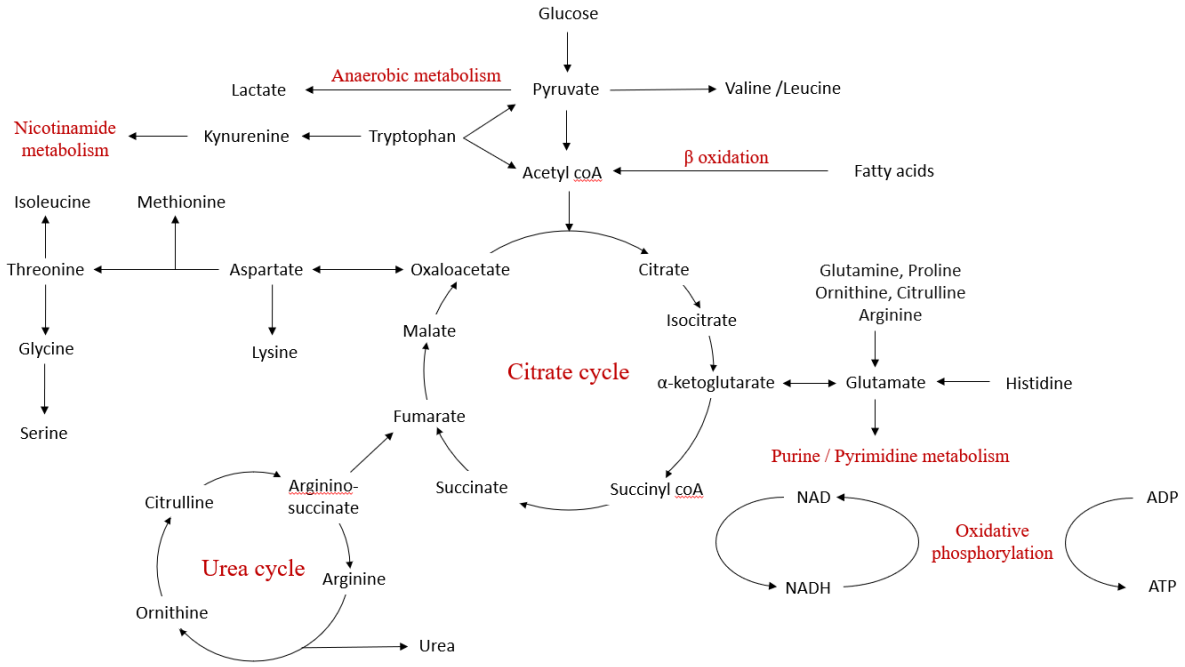

Figure 11: Simplified schematic of metabolic pathways in pathway enrichment analysis and demonstrating their relation to basic cellular pathways, using the KEGG pathway database as reference. NAD: Nicotinamide adenine dinucleotide, NADH: reduced nicotinamide adenine dinucleotide, ADP: Adenosine diphosphate, ATP: adenosine triphosphate

4.4.2 Lipidomic profile of ECD livers

The role of lipids in hepatic steatosis and their impact on hepatocyte and global liver function is well-established(100). Lipids are organic compounds insoluble in water with metabolic and non-metabolic functions. Not only do they represent a condensed energy substrate but have been implicated in key inflammatory processes as part of a network of soluble mediators at the interface of cellular metabolism and the immune system. The role of endogenous bioactive lipid mediators has been demonstrated in several acute and chronic inflammatory diseases(100).

The relative abundances of metabolites in ECD liver perfusate indicates that ceramides, glycerophospholipids and lysoglycerophospholipids were predominantly released from the livers during NMP-L (**Figure 9**). Interestingly, these metabolites were found in significantly higher proportions in non-viable versus viable livers throughout NMP-L, except for lysoglycerophospholipids which were in significantly higher abundances in the non-viable liver perfusate solely at the start of perfusion (**Figure 10**). Furthermore, glycosphingolipids were also found to be significantly higher in the non-viable group at all three time points. These molecules represent lipid classes which are both major components of cell membranes as well as being bioactive metabolites. This lipidomic pattern may therefore suggest a disturbance in phospholipid homeostasis indicative both of structural and functional disruption. Of note, lipidomic signatures have previously been linked to early allograft dysfunction in cold-stored livers.

Ceramides have been implicated in cellular death mechanisms(101). These are a class of sphingolipids which consist of *N*-acylated sphingoid bases linked to a fatty acid by an amide

bond. They are bioactive molecules involved in cell membrane signal transduction, influencing cell differentiation, senescence, migration, adhesion, growth arrest and apoptosis(101). Central to this is their interaction with mitochondria. Intracellular ceramide concentrations are known to increase in response to external insults, triggering a downstream array of signalling cascades which negatively impact the bioenergetic status of the mitochondrion(102). The mechanism of this interaction is still poorly understood but has the net effect of suppressing the ETC, generating ROS and releasing proapoptotic proteins which result in mitochondrial damage, and induce apoptosis. Increased de novo synthesis of ceramides in hepatocytes has also been linked to hepatic insulin resistance, gluconeogenic pathway dysregulation and therefore disruption of energy production in non-alcoholic fatty liver disease and type 2 diabetes(103). This is because of increased fatty acid release from adipose tissue and the activation of Toll Like Receptor 4, involved in inflammatory cytokine release, as well as via the Akt/PKB signalling inhibition, which influences cellular growth(103).

In vitro experiments have demonstrated that treatment of hepatocytes with TNFα or IL-1β, recognised inflammatory mediators, resulted in increased ceramide accumulation. Increased intracellular ceramide resulted in hepatocellular death in TNFα-treated hepatocytes, by activation of mitochondrial membrane permeability transition(101). Furthermore, ceramide synthesis inhibition has been shown to ameliorate hepatic inflammation and apoptosis in animal models(101). In our own study, the observation that perfusate ceramides were generally higher in the non-viable liver group may indicate that ceramide production and accumulation could be a therapeutic target of interest in future NMP-L studies.

Lysoglycerophospholipids are another class of bioactive lipids. They consist of a hydrophobic carbon chain and a hydrophilic head attached to a glycerol or sphingosine backbone and act as

intermediate precursors for the biosynthesis of other lipids. For this reason and due to their lipotoxic effect, they found in higher concentrations extracellularly(104). This may explain the general trend of an increase in perfusate lysoglycerophospholipids during NMP-L as the ECD-L attempts to re-establish lipid homeostasis. Glycerophospholipids are the main structural component of cell membranes. Increasing concentrations of these metabolites may therefore be explained by hepatocyte loss because of hypoxia and IRI. Similar lipidomic findings have been demonstrated in liver transplantation metabolomics, linking them to EAD(94).

4.4.3 Acylcarnitine metabolism

Acylcarnitines were found to be present in significantly higher proportions in the non-viable liver group during the first two hours of NMP-L (**Figure 10**). This observation was still present but less pronounced at four hours with a lower number of higher fold-change acylcarnitines in the non-viable liver perfusate. The liver is the body's main source of endogenous carnitine synthesis and metabolism, which is an essential cofactor in beta-fatty acid oxidation for cellular energy production(105). This is facilitated by way of acylcarnitines, a large class of metabolites that comprises esters of L-carnitine and fatty acids that act as transporters of activated long-chain fatty acids into mitochondria, where beta-oxidation takes place. These pathways are regulated by a number of enzymes including long-chain acyl-coenzyme(105) A synthetase, carnitine / acylcarnitine translocase and carnitine palmitoyl-transferases. Beta-fatty oxidation produces acetyl co-A, an essential precursor for the tricarboxylic acid cycle and ketone body production. It may also be converted to acetyl-carnitine by carnitine O-acetyltransferase and exported back into the cytoplasm where it is converted to malonyl-CoA which exerts a negative

feedback loop on beta-fatty acid oxidation to prevent the toxic build-up of acyl-CoA metabolic intermediaries in the mitochondria(105).

Carnitine is intimately involved in cellular energy metabolism. It is implicated in the transfer of the products of peroxisomal beta-oxidation to mitochondria for oxidation in the tricarboxylic acid cycle(106). It also regulates acyl-coA / CoA ratios, stores energy as acylcarnitine and is involved in the excretion of poorly metabolised acyl groups from amino acid metabolism as carnitine esters(106). They drive oxidative metabolism of long-chain fatty acids by shuttling them across the mitochondrial membrane from the cytosol into the mitochondrial matrix for beta-oxidation and cellular energy production. Carnitine and its derivatives have antioxidant, anti-inflammatory, and anti-apoptotic properties through several mechanisms. It is a direct scavenger of free radicals, a chelator of metals that promote ROS generation, inhibitor of ROS-generating enzymes (such as xanthine oxidase) and is integral in maintaining the integrity of the mitochondrial membrane. Carnitine derivatives also influence redox signalling and have shown to exert protective effects on IRI(107).

Alterations in acylcarnitine metabolism as a consequence of enzyme deficiencies have been implicated in several metabolic disorders. Changes in blood acylcarnitines has been significantly correlated with diabetes, both type I and II, and have also been observed in coronary artery disease, heart failure, dementia and certain types of cancer such as hepatocellular carcinoma and prostate cancer(105). In patients with non-alcoholic fatty liver disease and non-alcoholic steatohepatitis, absolute acylcarnitine levels have been shown to increase with disease progression(108). This may indicate an attempt at mobilising lipids for oxidation due to high lipid loads, the resulting mitochondrial lipotoxicity and altered lipid

metabolism. Interestingly, a study of pre-liver transplant carnitine supplementation demonstrated a lower incidence of primary graft dysfunction and primary non-function than the placebo group, although this difference was not statistically significant(109). Moreover, one-month survival was significantly higher in patients who were administered carnitine pre-operatively.

In our study, the higher proportion of acylcarnitines in the non-viable liver perfusate indicates lipid metabolism is an influential driver for energy production in this group during NMP-L via fatty acid oxidation. Enhancing fatty acid mobilisation and oxidation in these livers, such as through perfusate carnitine supplementation, may therefore improve energy production and utilisation and increase the probability of achieving a clinically viable transplant status. This has been demonstrated in pre-clinical ex situ NMP-L perfusion studies of steatotic livers, in which addition of a defatting cocktail to the perfusate resulted in significantly better metabolic parameters and a greater proportion of livers achieving viability, using the same criteria outlined in this clinical trial(52).

4.4.4 Pathway Enrichment Analysis of ECD Livers with Perfusion Time

A notable trend throughout the first fours of NMP-L was the gradual decrease in amino acid abundances as indicated by the metabolite annotations relating to the pathway enrichment analysis (**Table 10**). This trend in abundances may be representative of ongoing amino acid metabolism as these metabolites are processed by the liver during perfusion and one would therefore assume that these metabolites are depleted in the liver prior to perfusion. This is supported further by the pathway enrichment analysis which demonstrated that the significant non-lipid metabolic differences were predominantly driven by amino acid metabolic pathways

(**Table 9**). A time series analysis of all 31 ECD livers showed metabolic perturbations in the following pathways: metabolism of glycine, serine, threonine and histidine, lysine degradation, valine, leucine and isoleucine biosynthesis and nicotinate and nicotinamide metabolism.

Amino Acid Metabolism

As a major site of nitrogen and protein metabolism, the liver plays a central role in amino acid homeostasis. In addition to protein synthesis, amino acid metabolism is intimately linked to carbohydrate and energy metabolism, hormone and stress responses as well as anti-inflammatory and immune reactions(110). Amino acids are processed in different ways to execute these functions: deamination and transamination followed by conversion of non-nitrogenous part of these metabolites to glucose or lipids; conversion of ammonia to urea to prevent toxic build-up and hepatic encephalopathy; and synthesis of non-essential amino acids as well as plasma protein such as albumin. The liver therefore acts as 'gatekeeper' of amino acids absorbed in the portal circulation before passing into the systemic circulation(110).

The pivotal role of the liver in amino acid metabolism and its influence on plasma amino acid alterations in liver disease has generated a body of research relating to the study of circulating levels of amino acids as indicators of liver function(111). Therefore, the transplanted liver's ability to process plasma amino acids may potentially be linked to a functioning allograft. Temporal changes in amino acid concentration within the extracellular fluid may reflect efflux from, or uptake into, the intracellular compartment, both passively and actively. In addition, during times when circulatory supply to the graft is intact, blood may also influence levels of amino acids in the extracellular compartment. Interruption of the activity of amino acid transporter activity across the hepatocellular membrane will impact extracellular amino acid

levels. As demonstrated by the systematic review in Chapter 2, the succession of insults to the donor liver during procurement, preservation and transplantation has been shown to both affect intracellular and extracellular amino acid concentrations of cold-stored livers. These changes could be a sign of passive release following hepatocellular death, or they could represent active processes designed to signal a protective or restorative response. In our study, the individual variation in the kinetics of these amino acids during NMP-L and the abundance of metabolites linked to their metabolic pathways suggests that the observed changes are actively regulated and are most likely involved in the regulation of hepatic regeneration and function. The following accounts explore the most notable enriched metabolic pathways, their impact on liver function and potential therapeutic roles in ECD NMP-L.

Histidine metabolism

Perfusate metabolite annotations potentially indicate two main pathways (**Table 10**). As a gluconeogenic amino acid, histidine is processed for gluconeogenesis. Histidine is degraded by conversion to glutamate, and then oxidised to alpha-ketoglutarate by glutamate dehydrogenase. Elevated fold changes of glutamate, as well as other pathway intermediaries, with perfusion time may be point towards upregulation of this pathway, as the liver prioritises energy production in the energy-depleted environment following ischaemia.

Histidine is also a precursor for histamine, a mediator of local inflammatory response. Imidazole-4-acetic acid is a downstream product of histamine oxidation(112), suggesting that this pathway may also be upregulated during perfusion, acting as an adaptive response to ischaemic liver injury and cytokine release through H4 histamine receptor activation and mast cell degranulation(112). Upon release, histamine exerts several physiological and pathophysiological regulatory functions, with the net effect of inducing systemic vasodilation

and increasing capillary permeability. The liver itself plays a major part in clearing histamine from the circulation through the action of diamine oxidase and raised plasma histamine levels been associated with IRI, biliary injury and hepatic fibrosis. In fact, increased histamine plasma concentrations have been demonstrated in patients with cirrhosis and end-stage liver disease(113), most likely due to the reduced capacity of the liver to process the metabolite. In experimental models, rat hepatocytes subjected to IRI, histamine reduced cell growth, enhanced oxidative stress, and promoted apoptosis(114). In human donor livers, higher concentrations of the metabolic products of this histidine pathway have been demonstrated in cold-stored grafts that developed EAD(94). An experimental perfusion study also demonstrated upregulation of this pathway in NMP-L when compared to subnormothermic perfusion (20 ^{o}C), possibly due to the stronger IRI response at higher temperatures(80).

Livers undergoing NMP-L may subsequently benefit from histamine antagonism to prevent histamine release through mast cell degranulation and reduce the load of oxidative stress. This effect has been demonstrated in rat hepatocytes, in which the histamine H2 receptor antagonist cimetidine reduced the oxidative stress, apoptosis, and poor cell growth(114). Cimetidine also inhibits P450 activity, attenuating ROS production(115). Doxantrazole and sodium cromoglycate are radical scavenging mast cell stabilizers radical scavenging abilities, but have yet to be investigated in IRI(116).

Lysine Degradation

As a gluconeogenic and ketogenic amino acid, lysine catabolism occurs via several metabolic pathways, the most common of which is the saccharopine pathway(117). Lysine is catabolised to saccharopine and then 2-aminoadipic acid in mitochondria. 2-aminoadipic acid catabolism

undergoes decarboxylation reactions to produce a series of CoA esters which stops at acetyl-CoA. Lysine is also required for carnitine biosynthesis(118), which, as previously described, plays a crucial role in the mitochondrial carnitine shuttle, the transport of activated fatty acids across the inner mitochondrial matrix for beta-oxidation. Interestingly, lysine supplementation in mice has been reported to prevent hepatic steatosis by stimulating beta-oxidation(119). Stimulation of lysine metabolism in addition to carnitine supplementation may therefore be beneficial to support the ECD liver dependent on fatty acid oxidation for energy production.

A look at the downstream metabolites highlighted in the pathway enrichment analysis (**Table 10**) reveals that lysine may predominantly be funnelled into the metabolic pathway leading to carnitine biosynthesis. N^6,N^6,N^6-trimethyl-L-lysine is generated by the hydrolysis of proteins containing lysine that is trimethylated at their ε-amino group by a protein dependent methyltransferase in a *S*-adenosyl-L-methionine dependent reaction which occurs in lysosomes. The rate of carnitine biosynthesis depends upon the availability of N^6,N^6,N^6-trimethyl-L-lysine in the mitochondrial matrix, the site of trimethyllysine dioxygenase activity. The hydroxylation of N^6,N^6,N^6-trimethyl-L-lysine by trimethyllysine dioxygenase yields 3-hydroxy-N^6,N^6,N^6-trimethyl-L-lysine, which enters the cytosol from the mitochondria for the next step of the pathway, a pyridoxal phosphate-dependent aldolytic cleavage of 3-hydroxy-N^6,N^6,N^6-trimethyl-L-lysine to 4-trimethylammoniobutanal and glycine performed by a cytosolic aldolase. After dehydrogenation to γ-butyrobetaine by 4-trimethylaminobutyraldehyde dehydrogenase in the cytosol, this compound enters the circulation and is actively transported primarily into the kidney and liver. In these organs cytosolic γ-butyrobetaine dioxygenase hydroxylates this compound to produce L-carnitine(120).

Glycine, serine and threonine metabolism

The interplay between these amino acids constituents a metabolic axis linked to several important biological pathway including the tricarboxylic cycle and cellular antioxidant capacity via glutathione synthesis. It drives one-carbon metabolism, a cyclic metabolic network involving folate compounds, which is central to proteins, lipids, nucleic acid and cofactor synthesis(121). This may be reflected by the detection of folate-related compounds (5,10-Methylenetetrahydrofolate and tetrahydrofolate) in the perfusate which can be related to purine synthesis. A link to fatty acid beta-oxidation may also be evident through an increase in the abundance of betaine, a methyl group donor that is involved in carnitine biosynthesis.

Branched chain ammino acid biosynthesis

In addition to providing substrates for the tricarboxylic acid cycle, branched chain amino acids have been shown to have a protective effect on hepatic IRI and counteract the stressors of antioxidant activity(122). They have also been linked to hepatocyte apoptosis and regeneration and reducing insulin resistance(123). At present, most of the evidence available is related to experimental animal models(122, 124). However, there are a number of clinical trials that have demonstrated that branched chain amino acid supplementation improved patient outcomes in liver diseases including improving markers of hepatic metabolism and function in cirrhotic patients undergoing hepatectomy or liver transplantation(123). Whether these metabolites may play a role in enhancing liver function in NMP-L has yet to be established.

Nicotinate and nicotinamide metabolism

Nicotinate (niacin) and nicotinamide are precursors of the coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide-adenine dinucleotide phosphate. NAD synthesis takes place via two routes: de nuovo synthesis from amino acids or salvage from nicotinamide(125). The latter recycles nicotinamide as well as nicotinamide-containing molecules such as nicotinamide riboside. NAD's role in energy metabolism is well-established as a vital coenzyme regulating several metabolic pathways, including glycolysis, oxidative phosphorylation, and fatty acid beta-oxidation(126). NAD plays a central role for ATP synthesis in mitochondria via oxidative phosphorylation, as an electron donor. Its biologically active form, NAD^+ can be reduced to NADH by dehydrogenases and can also be phosphorylated to $NADP^+$ by NAD^+ kinases(126). The NAD^+/NADH redox couple regulates cellular energy metabolism, through glycolysis and mitochondrial oxidative phosphorylation, and therefore has a direct link to IRI(126).

Oxidative phosphorylation is the process by which ATP is synthesised using energy released from the ETC, a series of protein complexes located in the inner mitochondrial membrane(126). The ETC consists of four main protein complexes: Complex I (NADH dehydrogenase), Complex II (succinate dehydrogenase), Complex III (cytochrome bc1 complex) and Complex IV (cytochrome c oxidase). The process begins with the transfer of electrons from NADH to Complex I. As the electrons move through the protein complexes, their energy is used to pump protons across the mitochondrial membrane, creating an electrochemical gradient. The protons that accumulate in the intermembrane space flow back into the mitochondrial matrix through ATP synthase, which drives the synthesis of ATP from adenosine diphosphate and inorganic phosphate. Complex IV is the final component in the chain which accepts electrons from

cytochrome c and transfers them to molecular oxygen, which serves as the final electron acceptor(126). This reaction combines electrons, protons and oxygen to produce water, thereby completing the ETC. During ischaemia, the lack of oxygen leads to a deficiency of electrons. This leads to undirected flow and build-up of electrons. Upon reperfusion, the sudden influx of oxygen reacts with the accumulated electrons to produce ROS(126).

$NADP^+$ and its reduced form, NADPH, are involved in maintaining redox balance and the biosynthesis of fatty acids and nucleic acids. NAD and NADP therefore play vital roles in regulating the cellular redox state, energy metabolism, mitochondrial function, gene expression, and signalling pathways, making them indispensable for the maintenance of numerous essential biological processes(125). Metabolite shifts relating to nicotinate and nicotinamide metabolism during liver perfusion is therefore most likely related to the multitude of metabolic pathways responsible for maintaining cellular homeostasis and redox potential.

4.4.5 Pathway Enrichment Analysis of Donor Liver Transplant Viability

As previously stated, an analysis directed towards ECD liver transplant viability demonstrated that the persistent discriminating metabolic changes in the perfusate at fours of NMP-L were related to lipid-based and acylcarnitine-related metabolites (**Figure 10**). Interestingly, a pathway enrichment analysis of polar and mixed class metabolites revealed enriched pathways only at the two-hour perfusion time point, halfway through the designated window for viability testing. The majority of these enriched pathways were associated with amino acid metabolism: arginine and proline metabolism; arginine biosynthesis; alanine, aspartate and glutamate metabolism, tryptophan metabolism, histidine metabolism and glycine, serine and threonine metabolism. Other enriched pathways included fatty acid and nucleic acid metabolic pathways:

pantothenate and coA biosynthesis, butanoate metabolism, the tricarboxylic acid cycle, and pyrimidine and purine metabolism. Beyond the two-hour perfusion time point the number of statistically significant metabolites decreased such that only abundances in lipid-based molecules distinguished these groups.

This may indicate that as perfusion progressed the livers in both groups became more metabolically similar and there are a number of reasons that may explain the lack of enriched pathways in this analysis. Definitions for an ECD liver are broad and the criteria for ECD liver status in the clinical trial were multiple. Moreover, a graft needed only to satisfy one criterion to be included. For this reason, the donor liver cohort was highly heterogenous from an ECD standpoint. Furthermore, the major criterion for achieving transplant viability, perfusate lactate less than 2.5 mmol/L, resulted in a group of non-viable livers that did not reach this threshold to varying degrees. It may be reasonable to assume that non-viable livers that were closer to the threshold were potentially more metabolically similar to the viable livers than those livers that were further off the mark. The observations in the one-way ANOVA analysis may therefore be a manifestation of the different rates of metabolic recovery that these livers exhibit during NMP-L. Based on this interpretation of the data it is possible that the viability criteria employed in VITTAL, as well as the fixed time window in which these had to be satisfied, discarded a proportion of livers that could, from a metabolic perspective, have been considered viable for transplantation should the thresholds have been raised. These observations would therefore support a consideration for revision of the viability criteria employed in future clinical trials.

A closer look at the pathway enrichment analysis following two hours of perfusion (**Table 11**) reveals that in several metabolic pathways different metabolites were upregulated in either the viable or non-viable livers. Interestingly, there were certain metabolic pathways in which the metabolites were predominantly upregulated in one particular group. Of note, the majority of histidine and tryptophan metabolic intermediaries were more abundant in the perfusate of the viable livers (**Table 12**). Metabolites relating to butanoate metabolism and coA biosynthesis were predominantly elevated in the non-viable liver perfusate. This may indicate that, at that time point, non-viable liver metabolism was mainly directed towards energy production via fatty acid metabolism while pro and anti-inflammatory pathways, were active to a greater extent in viable livers.

Tryptophan metabolism via the kynurenine pathway is the main route of tryptophan catabolism in the liver and leads to NAD synthesis. This pathway has also been implicated in immune regulatory response. It is recently gathering attention in liver transplantation as it has been associated with graft dysfunction in metabolomic studies(78, 93), has been implicated in NMP-L related liver metabolic response(80) and may be linked to allogeneic liver graft tolerance(127). Regulation of tryptophan and histidine metabolism during NMP-L may enable control of inflammatory and anti-inflammatory response.

4.5 Conclusion

Our application of metabolomics to ECD NMP-L has revealed several metabolic differences affecting crucial metabolic pathways other than lactate metabolism. The data indicates that further investigation into these metabolic pathways and their role during NMP-L is warranted Implementation of current viability criteria is supported by significant metabolic differences

affecting crucial liver metabolic pathways other than lactate metabolism. The data indicates that further investigation into these metabolic pathways is warranted to further develop transplant viability criteria for machine perfusion. Furthermore, we have identified may perturbations in metabolic pathways during perfusion that can be targets for therapeutic interventions in ECD livers in the future.

Chapter 5 Ex Situ Normothermic Split Liver Machine Perfusion: Developing a Perfusion Protocol for Robust Comparative Controls in Liver Function and Metabolomic Assessment Suitable for Evaluation of Novel Therapeutic in the Preclinical Setting

This chapter describes the researcher's own work which has been peer-reviewed and published:

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Figures and tables have been adapted from the original manuscript.

5.1 Introduction

5.1.1 NMP-L in Basic Science Research

NMP-L has emerged as safe and feasible method of organ preservation at physiological temperatures, simultaneously enabling the assessment of metabolic parameters to determine the liver's viability for transplantation as it perfuses the liver with oxygen and substrate to kickstart cellular metabolism *ex vivo(37, 49)*. This has been shown to be advantageous for ECD livers, as dynamic organ function rather than static donor and graft variables can be appraised to increase a transplant surgeon's confidence in the liver's viability for transplantation and, therefore, the likelihood of a successful recipient outcome(97). NMP-L is also becoming an attractive alternative to SCS as it allows for the delivery of pharmacological and cytoprotective agents while monitoring organ viability. Subsequently, as our understanding of ECD liver metabolism during NMP-L and IRI grows, we are unlocking the potential not only to monitor liver function, but also manipulate it to improve graft quality and potentially turn a discarded liver into a transplantable organ through the administration of therapeutics(51).

Several basic science perfusion studies in human donor livers have yielded promising results to this effect, with novel therapeutic approaches put forward to ameliorate IRI and enhance organ quality(51). Unfortunately, there are limitations to these studies that impede their translation into clinical medicine. Firstly, donor livers that are accessible for research are in short supply and an even smaller proportion are available for machine perfusion studies. Consequently, these studies suffer from low population numbers, which reduces their statistical power and, therefore, confidence in their results. Secondly, there is a wide variation in donor characteristics and organ quality among different livers, limiting their capacity as suitable

comparative controls. The singularity of donor livers restricts interpretability of data from small series of whole organ perfusions(128).

A well-known and well-developed surgical approach that is utilised in transplantation to increase the number of available grafts is splitting of the donor liver(129). In this technique, the donor organ is split into two separate units that function independently, thus allowing for two recipients to "share" the same organ(129). This technique and its variations serve the fundamental principle of dividing the whole liver into portions, each with a suitable vascular pedicle, bile duct and venous outflow, along with sufficient functional hepatic mass(129).

5.1.2 Split Liver Transplantation

In the 1984, limited availability of donor livers for paediatric patients led Henri Bismuth to develop a graft size reduction method(130). This produced a graft of smaller volume than the parent graft that could be utilised for paediatric liver transplantation. In 1988, Rudolf Pichlmayr developed a liver splitting technique that produced two independently functioning grafts that could be transplanted into one paediatric patient and one adult patient(131). Later that same year, Henri Bismuth performed the first full right and full left split liver transplantation in two adult recipients(132).

Surgical Technique

The aim of split liver transplantation is to achieve two grafts with an intact blood supply and biliary drainage(133). When discussing surgical technique, two main types of split liver transplantation exist. The classical split produces a right extended graft (segments I, IV – VIII)

and a left lateral graft (segments II and III), for one adult and one paediatric recipient respectively. In contrast, the second technique splits the liver along Cantlie's line, a vertical plane that joins the gallbladder fossa to the inferior vena cava. This produces a right (segment V – VIII) and a left (segments I – IV) hemi-liver, both of which can be transplanted into two adult recipients(133).

In the classical split, dissection begins along the left side of the hepatoduodenal ligament to identify the left hepatic artery. The left portal vein is subsequently dissected, which generally sacrifices the portal branch to segment IV. Parenchymal division is carried out to the right of the falciform ligament and ends between the left and middle hepatic veins. The hepatic ducts are identified and preserved. The left hepatic artery, left hepatic vein and left portal vein are then divided to produce a left lateral segment with its own inflow and outflow(133).

With regards to the hemi-liver split, the plane for parenchymal division is wider without a clear anatomical landmark to guide dissection and may not be feasible if presented with an anatomical variant. A normal left hemi-liver a single portal vein, hepatic duct, venous outflow from middle and left hepatic veins but multiple arterial branches. A normal right hemi-liver consists of a single right hepatic artery, but generally has multiple venous branches, hepatic ducts and may also have several portal vein branches. For this reason it is generally recommended that the left hemi-liver retains the coeliac trunk and the right hemi-liver retains the common hepatic duct, main portal vein and the cava(133).

Split Liver Perfusion

Split livers have been shown to demonstrate comparable perfusion and functional characteristics during *ex-situ* subnormothermic machine perfusion(128). The study provided a controlled comparison between split lobes enabling each liver to act as its own internal control. However, the characteristics of split livers during perfusion remain underexplored or limited to case reports(134-136), in particular in relation to normothermic machine perfusion.

The work described in this chapter focuses on developing a normothermic machine perfusion protocol for the application of the split liver model to preclinical research. The hypothesis was that, when subjected to ex-situ end-ischaemic NMLP, individual lobes derived from the same liver would recover and function in a similar manner. The protocol not only provides a means of affording more liver units for perfusion experiments, but also allows each liver to act as its own internal control, which eliminates the inherent heterogeneity of whole organ perfusions.

5.2 Materials and Methods

5.2.1 Study Design

The principal objective of this proof-of-concept study was to examine the functionality of left and right lobes from the same human donor liver separately, compare their performance and investigate their suitability and feasibility as comparative controls during NMP-L in the pre-clinical setting. Each lobe was connected to a different perfusion device, with each lobe being supplied by inflow and outflow vessels having their biliary drainage independent of each other. (i) Assessment of liver function and (ii) Evaluation of perfusion parameters were the primary

outcomes investigated in this study. The perfusion machines employed were Liver Assist devices (Organ Assist, Groningen, The Netherlands).

5.2.2 Donor Liver Source and Selection

The right and left lobes of four different donor livers were included in this study, making up a total of eight perfusions. As per NHSBT policy, the livers used in this study were organs that were originally donated for potential transplantation. However, for various reasons, these livers were rejected by all UK liver transplant centres for transplantation. The decision to decline them was done solely by the transplant surgeons at each individual transplant centre, and none of the authors involved in this study had any influence on the decision to reject the donor livers for transplantation. NHSBT subsequently offered the donor livers for research purposes. Specialist nurses would have obtained informed consent from the donor's next of kin for using the donor organs for research purposes and overseen transport of the liver to the centre of the research group that would have accepted the liver. Authorisation for research in organ donation is overseen by each transplant centre's specialist nurse. All of the described methods were conducted in keeping with NHSBT guidelines and regulations. The London-Surrey Borders National Research Ethics Service (Reference Number 13/LO/1926) and the NHSBT ethics committee (Reference Number 06/Q702/61) granted study ethical approval. None of the donor livers utilised in this work were obtained from executed prisoners. All offers for research were considered while the study was active. The exclusion criteria for donor livers in this work were as follows: (i) presence of hepatic malignancy (ii) livers subjected to machine perfusion before being discarded (iii) asymmetric or poor perfusion shown while retrieving the donor livers (iv) gross macroscopic appearance that indicates moderate or severe steatosis.

5.2.3 Liver Splitting Protocol

During transportation, donor livers were preserved in the University of Wisconsin solution. As is standard practice in the UK, this was done at hypothermic temperatures (0 – 4 ^{o}C) in ice. Splitting of the donor livers into right and left lobes (**Figure 12**) was carried out with the liver in an ice bath to maintain it in cold storage (**Figure 13**). This was important as it ensured that both of the lobes had similar cold ischaemic times and were simultaneously assessed when attached to the perfusion devices.

Standard "bench surgery" was then performed to remove extraneous tissue and ascertain the quality of the organ. This involved assessing the liver tissue for macroscopic abnormalities as well as the quality of the vessels and bile duct to determine its suitability for study inclusion (**Figure 13**). Once this was complete the post-hepatic inferior vena cava was incised as the perfusion device enables open drainage of the perfusate from the liver's outflow via this vein. As shown in **Figure 13**, the incision created two separate patches leading to direct visualisation of the left, middle and right hepatic veins, and this was the initial step in order to determine the line of demarcation for parenchymal division. The right and left halves of the liver were divided along Cantlie's line for the scope of this study. Posteriorly, the line starts from the inferior vena cava at the level of the middle hepatic vein and ends antero-inferiorly in the centre of the gallbladder fossa. It therefore marks a watershed area between the right and left sided blood supply of the liver. Using Couinaud's classification system, this procedure resulted in a left lobe consisting of segments I to IV and a right lobe comprising segments V – VIII (**Figure 13**). Couinaud's system divides the liver into eight functionally independent segments, each with their own blood supply and biliary drainage.

Upon opening of the vena cava, excess tissue in the liver hilum was cleared to determine the left and right branches of the portal vein and hepatic artery respectively, as shown in **Figure 1**3. Special care was taken to clearly identify the arterial branch that supplied segment 4 of the liver, as during splitting this would have the greatest influence on the level of division of the branches. It was also important to clearly identify the left and right hepatic ducts as this would ensure that there was adequate biliary drainage for both halves (**Figure 1**3). The gallbladder was removed, and the cystic duct was dissected and ligated. Once all of the appropriate anatomy was successfully identified, division of the vessels and bile duct was carried out as follows: (i) division of the hepatic arterial tree was done in such a way that the left lobe of the liver retained the main trunk, while the right lobe kept the right hepatic branch, unless anatomical anomalies were present. (ii) The main trunks of the portal vein and bile duct were assigned to the right lobe, while the left lobe utlised its respective branches. Following this, parenchymal division was successfully carried out along Cantlie's line, as shown in **Figure 13**.

When the vessel length and diameter were found to be insufficient for safe cannulation of either of the two lobes, vessel reconstruction was conducted so as to allow for the proper insertion and siting of the cannulas for perfusion. This was particularly the case with respect to the isolated branches. Vessel reconstruction was conducted by using the native vessels to fashion a conduit for extra length. This was done in one of two ways: (i) The distal portion of the main arterial or venous trunk retained by either lobe was resected and subsequently anastomosed onto the branch of the other. (ii) Using a length of the donor iliac vessels for reconstruction (this was provided along with the donor liver should the need arise for arterial or venous reconstruction during transplantation (**Figure 13**). The method of vessel reconstruction ((i) or (ii)) was chosen depending on the conduit length that was needed as well as the size of the vessels had to be anastomosed together. Cannulation of the hepatic arteries was done using 10-

14 F cannulas, whilst the portal vein was cannulated using a 24 F cannula. The hepatic veins drained freely by gravity into the reservoir that housed the liver.

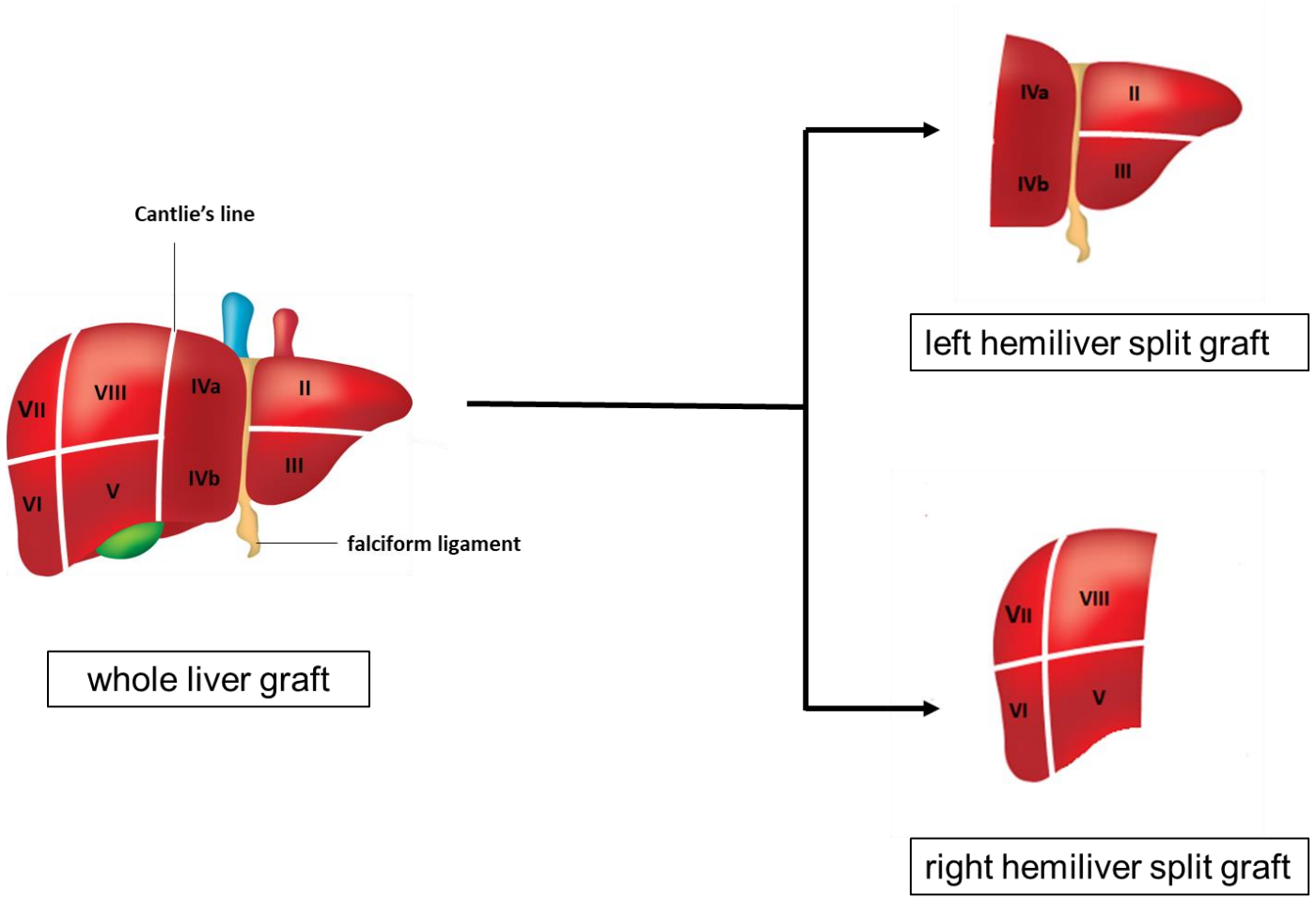

Figure 12. Schematic demonstrating the resulting hemilivers and their corresponding segments, as per Couinaud's classification system, following division of a whole graft at Cantlie's line.

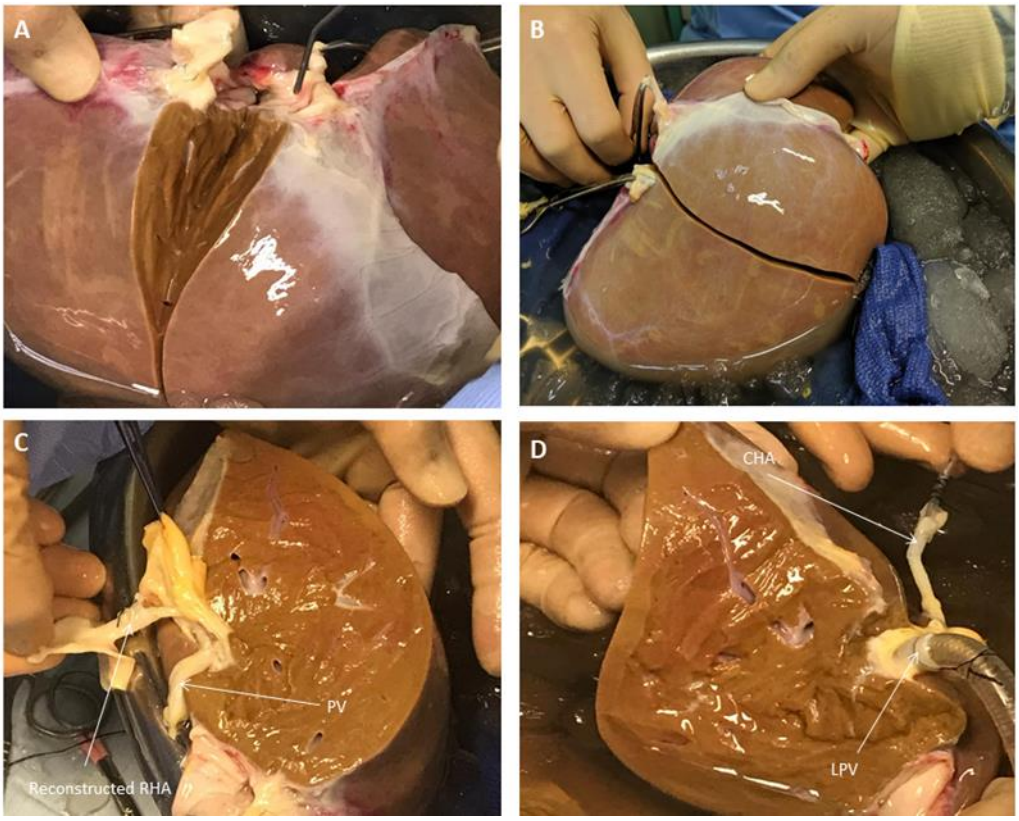

Figure 13. Liver Splitting Procedure: A Parenchymal transection across gallbladder bed B Parenchymal transection complete C Reconstruction of hepatic artery using donor celiac artery trunk D Cannulation of hepatic artery and portal vein branches. CHA: common hepatic artery; LPV: left portal vein; PV: portal vein; RHA: right hepatic artery.

5.2.4 Ex-situ Perfusion protocol

The two perfusion devices were primed using a perfusion protocol that can be found in the Appendix Chapter of this thesis, using Hemopure [HBOC-201, hemoglobin glutamer-250 (bovine); HBOC-201, Hemoglobin Oxygen Therapeutics LLC, Cambridge, MA] in place of packed red blood cells as the oxygen carrier (Appendix 9 for details on perfusate composition). Hemopure is a polymerised bovine hemoglobin-based acellular oxygen carrier, displaying low immunogenicity and has an oxygen-carrying capacity that is comparable to that of human haemoglobin at normothermic temperatures. Pre-clinical and clinical studies have demonstrated its efficacy as an alternative to blood-based machine perfusion fluid.

After splitting, each lobe was weighed before perfusion. Cannulae were inserted into the arterial and portal venous tracts and each lobe was positioned inside the organ reservoir in such a way so as to directly visualise the open drainage from the hepatic veins. Perfusion was initiated with oxygenated pulsatile flow and non-pulsatile flow in the hepatic artery and PV, respectively, at a temperature of 36 – 37 ◦C. It was ensured that perfusion of each lobe was commenced within 5 – 15 mins of each other (**Figure 16**). Key parameters including perfusion pressures and flow parameters, were constantly monitored. Each lobe was also perfused with epoprostenol, a prostaglandin and potent vasodilator, via a pump connected to each perfusion circuit at an initial rate of 4 ml/h. In order to maintain physiological parameters, the rate of prostaglandin infusion was adjusted in accordance with flow readings. The hepatic artery pressures were kept constant at 60 – 70 mm hg while the portal vein pressures were maintained at 10 mm Hg. Supply of oxygen was adjusted so as to maintain a perfusate oxygen partial pressure >10 kPa in the arterial circuit. Real-time analysis of serial perfusate samples was conducted using the Cobas b 221 point of care system (Roche Diagnostics, USA) Blood Gas

Analyser, whereby metabolic parameters, including perfusate oxygen partial pressures, glucose, and lactate levels, were monitored. These parameters were selected for monitoring as they have been described in previous research as being key parameters for monitoring liver function and viability in machine perfusion. Each lobe had an NMLP-L time of six hours. Core needle biopsies from each lobe were obtained at the start and end of perfusion for histological analysis. The biopsies were fixed in formalin, embedded in paraffin and were subsequently stained using haematoxylin and eosin for conventional examination. The biopsies were also stained with periodic acid schiff (PAS) for glycogen content and distribution.

5.2.5 Statistical Analysis

Prism 7 (GraphPad Inc., CA) was used to conduct data analysis. The Wilcoxon signed-rank test was used to compare continuous data at each timepoint. Statistical significance was set $p < 0.05$. The data is shown as per lobar mass as well as following normalization of pre-perfusion lobar weight per gram of tissue.

Figure 14. Liver Assist Perfusion Device

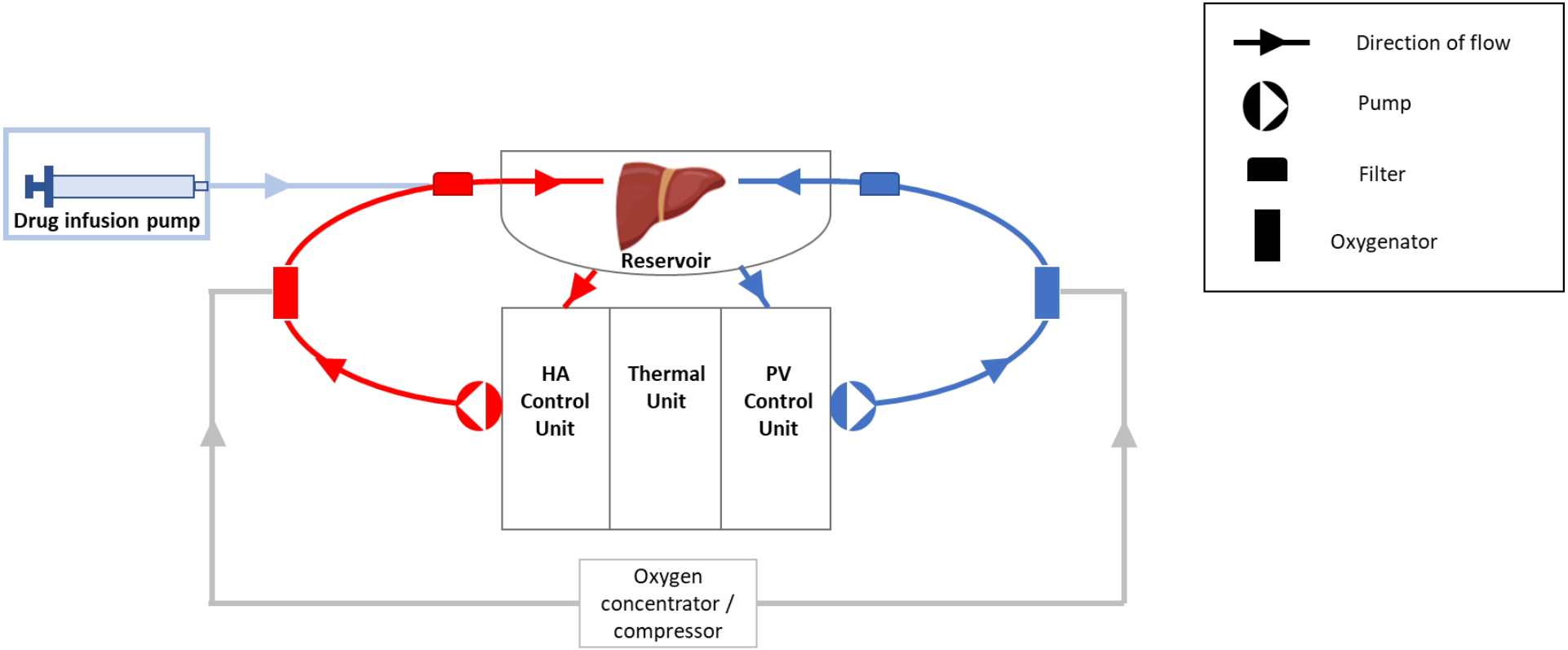

Figure 15. Schematic of split liver machine perfusion set-up, which was identical for right and left hemi-livers.

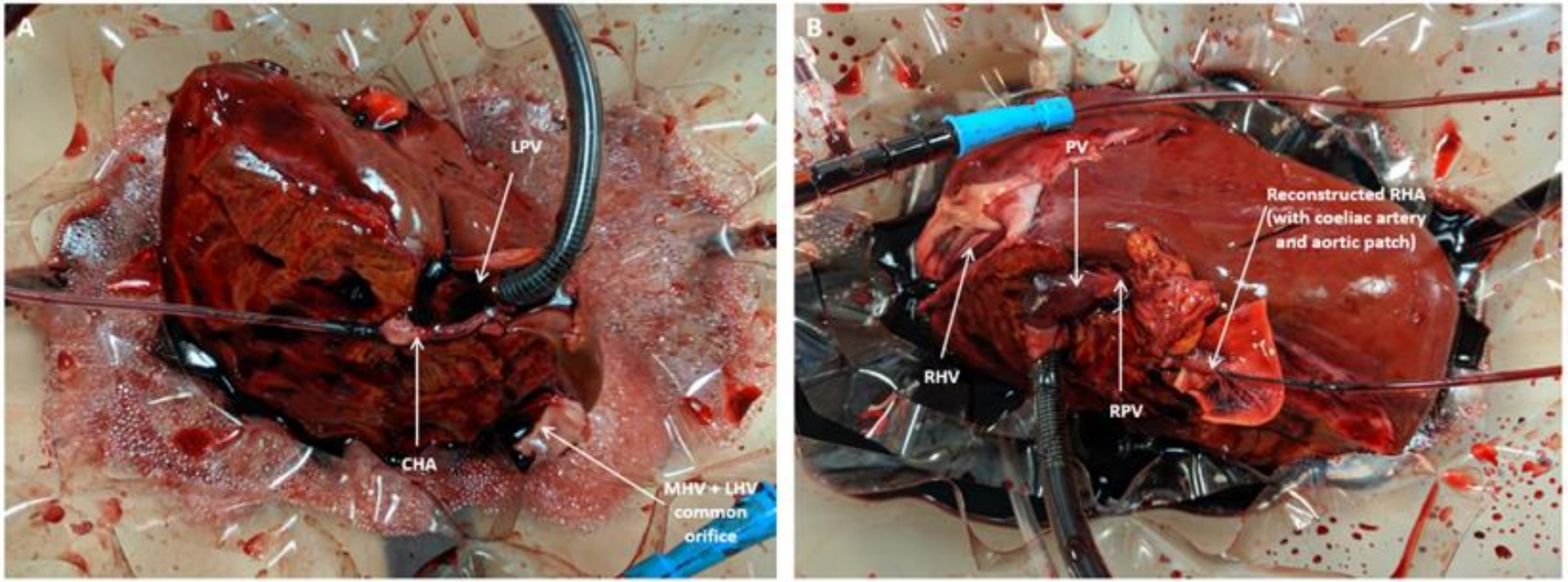

Figure 16. Split lobe perfusion: (A) Left lobe (B) Right lobe. CHA, common hepatic artery; LPV, left portal vein; MHV, middle hepatic vein; PV, portal vein; RHA, right hepatic artery; RPV, right portal vein.

5.3 Results

5.3.1 Donor Liver Characteristics

In all perfusion experiments, left lobar mass was significantly lower than their right lobe counterparts. Details regarding liver characteristics and donor characteristics may be seen in **Table 13** below.

Table 13: Donor liver demographics and characteristics.

Liver ID	**1**	**2**	**3**	**4**
Donor age (years)	31	75	57	73
Gender	male	male	female	female
DBD / DCD	DCD	DBD	DCD	DBD
Cold ischaemia time (min)	902	799	1108	1026
Weight (g)				
Right lobe	715	1120	957	739
Left lobe	488	851	851	363

DBD: donor after brainstem death; DCD: donor after cardiac death

5.3.2 Assessment of Liver Function

Figure 17 illustrates the perfusate lactate (**7A**) and glucose levels (**7B**) for every single split lobe over a period of six hours of end-ischaemic NMP. At T0 (*i.e.* the start of perfusion) the lactate levels were comparable, and significantly dropped until T6, the end of the perfusion experiment. In both lobes, there were similar trends in perfusate lactate clearance. The perfusate lactate levels in the left lobe tended to be higher than in the right lobe at any time

point when normalising for pre-perfusion tissue weight, however, the rate of reduction in the lactate levels was similar for both lobes in all the perfusion experiments. It is interesting to note that, during the perfusion experiment, there was a slight increase in perfusate lactate levels in two of the four livers, which occurred in both lobes at the same timepoint. At T0, the perfusate glucose levels were high and continuously decreased throughout the duration of the experiment. At all timepoints, lactate and glucose levels were comparable (**Figure 17**).

5.3.3 Perfusion Hemodynamics

Figure 18 shows the hepatic artery and portal vein flows for each of the split lobes over a period of six hours of end-ischaemic NMP-L. In both right and left lobes, these were found to be relatively consistent and as the perfusion experiment proceeded, there was a trend toward an increase in flow rate. Across all time points, the variation in flow rates for hepatic artery and portal vein were found to be statistically insignificant.

5.3.4 Liver Histology

Histological analysis was conducted in order to observe the behaviour of the left and right liver lobes throughout the perfusion experiments. PAS staining was scored using a descriptive assessment by an experienced histopathologist, as weak to strong positive, and the distribution described as patchy to uniform/even. No numerical data was acquired. It was found that both lobes acted in a similar way throughout the experiments. In both pre- and post-perfusion biopsies there was good preservation of lobar architecture. Although there was some variable centrilobular necrosis at both time points, this was present to the same extent before and during NMLP. All of the biopsies were shown to be PAS positive, with the intensity of staining

ranging from mild to strong. The distribution of staining ranged from even distribution throughout the parenchyma to patchy distribution. Although there were variations between livers, there were strong similarities between each of the paired lobes, with no changes observed throughout the duration of the perfusion experiments. Furthermore, there was no correlation between patterns of histology with any parameters of functional assessment. Evidence of significant macrovesicular steatosis was only found in one liver, and this did not change between lobes or throughout the perfusion experiments (**Figure 19**).

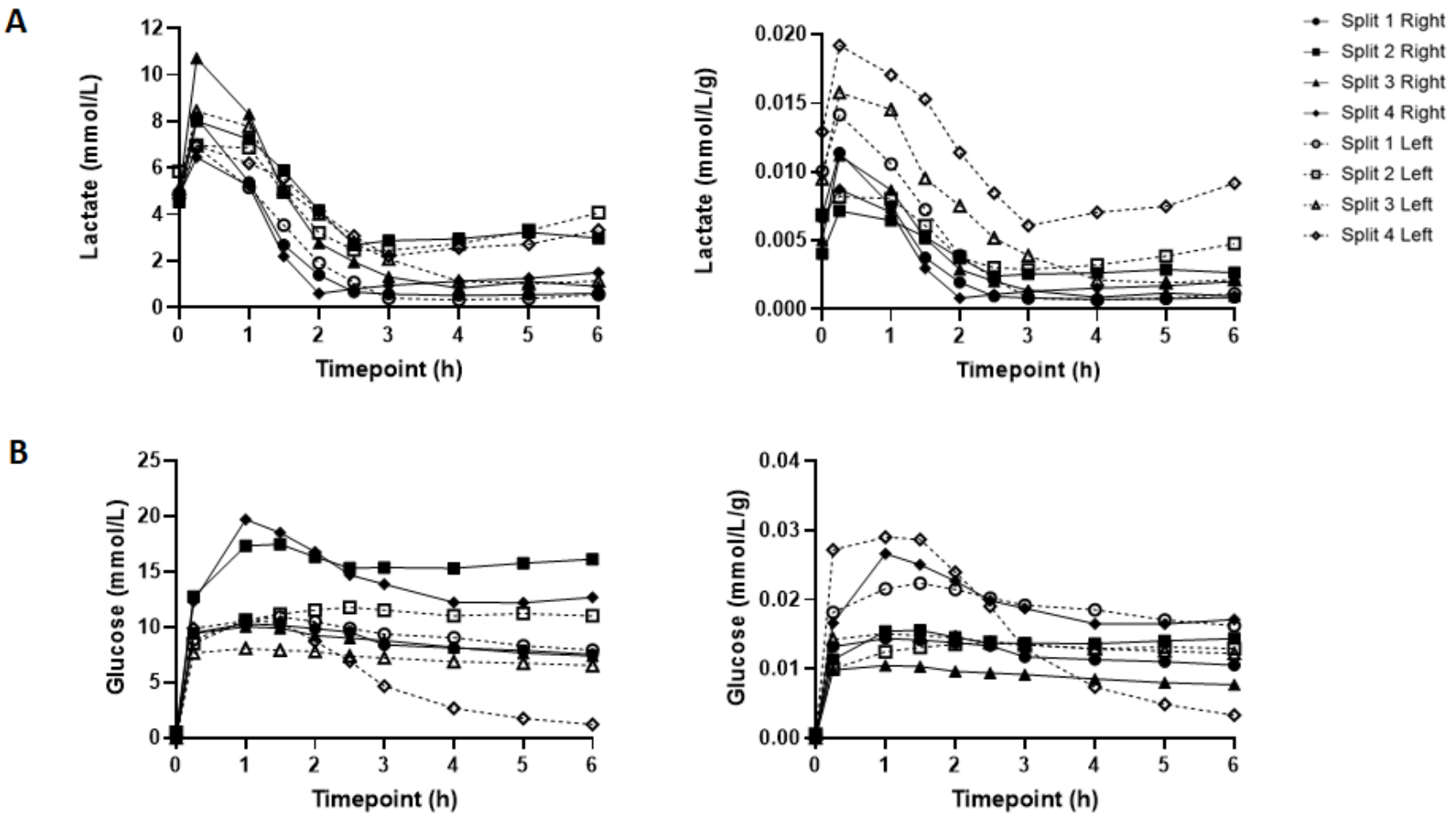

Figure 17.Perfusate lactate (A) and glucose (B) levels for each individual split lobe over 6 h of end-ischaemic normothermic machine perfusion.

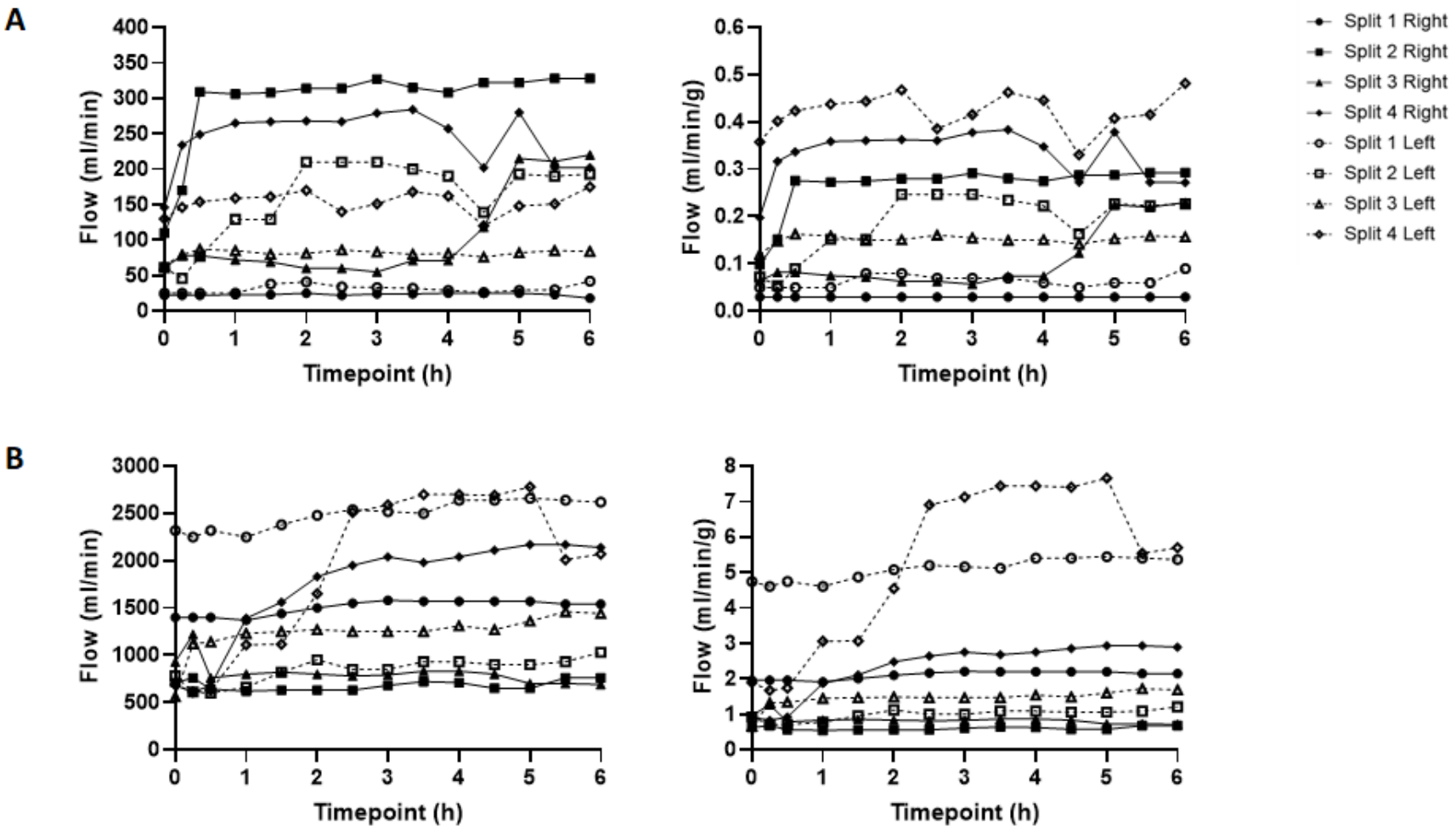

Figure 18. Hepatic artery (A) and portal vein (B) flows for each individual split lobe over 6 h of end-ischaemic normothermic machine perfusion.

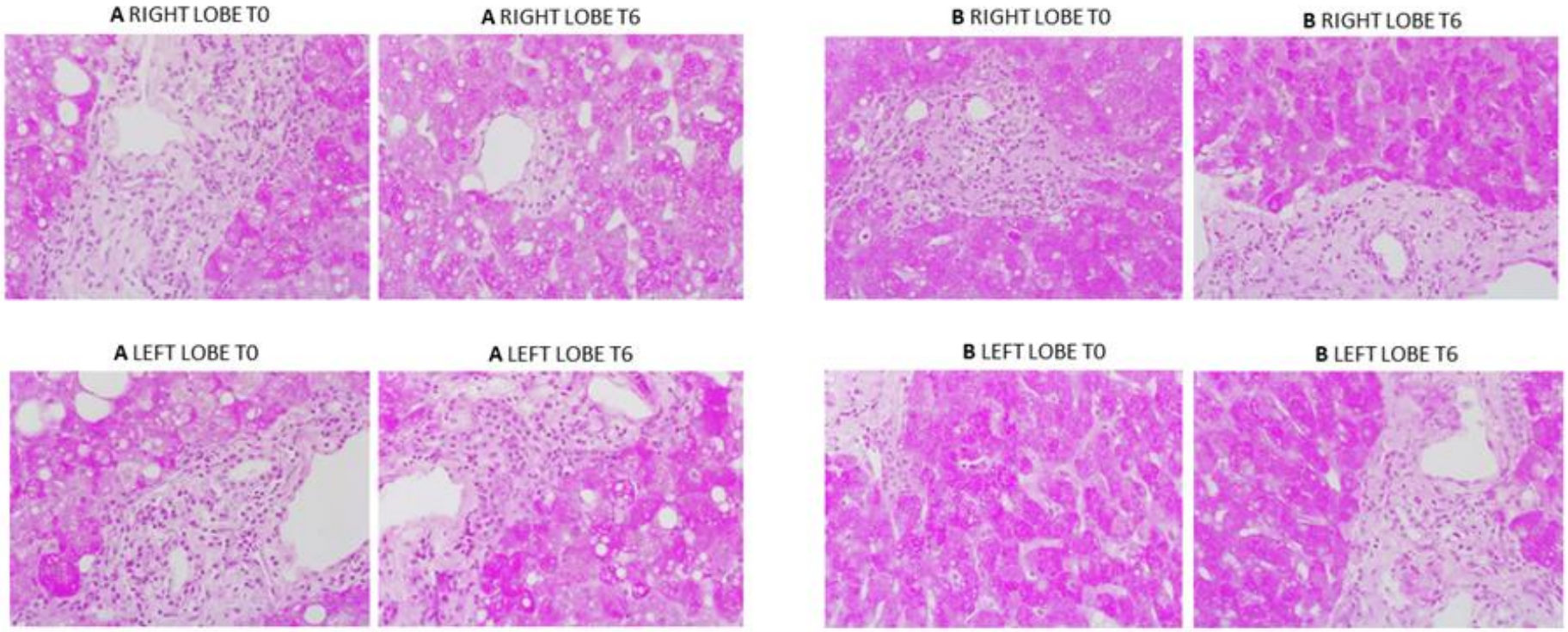

Figure 19. PAS staining from two representative perfusion experiments. (A) (from liver case number 2) shows mild to moderate macrovesicular steatosis, portal inflammatory cell infiltration and patchy PAS staining which was the same at commencement and end of perfusion. (B) (from liver case number 4) shows a liver with evenly distributed PAS staining throughout. Again, a degree of portal inflammation was seen. T0: pre-perfusion, T6: end of perfusion.

5.4 Discussion

Data on the behaviour of split livers during NMP-L is severely lacking. This area of research has recently garnered attention for its potential to provide a NMP-L platform in which livers can act as their own internal controls when testing pharmacological agents and perfusion protocols(128). We aimed to address this gap in the literature, whereby the split liver technique was adapted to the NMP-L model with the principal objective of developing a perfusion protocol that is more reliable by enabling each liver to provide its own internal control. The results of this study demonstrated that liver functionality is recovered post-splitting, with each lobe performing similarly during NMP-L. Therefore, there is great potential for this split liver model to ex situ end-ischaemic NMP-L to act as a suitable comparative control for investigating and assessing the effect of pharmacological agents and therapeutic interventions before subjection to rigorous clinical trials.

In previous work carried out by our research group, viability assessment of perfused livers was conducted after the organs were subjected to four hours of NMP-L. There are two main reasons as to why this four-hour window is vital in the setting of split liver machine perfusion. Primarily, following a variable cold ischaemic period, it provides sufficient time for each lobe to restore its metabolic function. Secondly, any variations in performance and parameters for each lobe can be sufficiently monitored and assessed prior to therapeutic intervention so as to be taken into account in the final analysis.

It is important to point out that the model developed in this study was done for experimental purposes only and did not factor in clinical transplantation, as this was outside the scope of our objectives. It must also be noted that the development of a standardised approach, utilised in

all livers, was made possible as a result of there being no variations in the inflow portal venous anatomy. The site of caval division bared no negative impact on lobe function since liver outflow was on free drainage to the reservoir. Since an open circuit perfusion system was incorporated, the blood was freely drained and collected in the reservoir of the perfusion device, meaning that no reconstruction of liver outflow veins was required. This included the segmental veins that drained into the middle hepatic vein. As such, the surgical technique and the perfusion parameters were not influenced by either the hepatic veins anatomy or inferior vena cava accessories. This would not have been the case if the set-up was a closed circuit or if we considered the clinical use of these segmental grafts.

As stated previously, one of the benefits of incorporating NMP-L to the split liver model, is the ability to assess liver function under conditions that are near-physiological. Furthermore, there are advantages to using an artificial acellular haemoglobin-based perfusion fluid in place of blood products. First of all, acellular haemoglobin-based perfusion fluid (Hemopure) has low immunogenicity, in contrast to blood products that are derived from a number of donors(137). This may prove advantageous when investigating liver-specific immune cell populations as well as mechanic studies. Secondly, it is possible to deploy Hemopure as a perfusion fluid under hypothermic conditions, which cannot be done when utilising blood products (limited to physiological temperatures)(137). Consequently, this enables our split liver model to be performed under hypothermic, sub-normothermic as well as normothermic conditions.

However, it is important to note that there are some limitations to this perfusion model design. Namely:

i. Extensive surgical skill and technique is required for liver splitting.

ii. Splitting of the liver increases cold ischaemia time more than what is currently considered normal when preparing a whole liver for machine perfusion.

iii. The right and left lobes cannot be split into units of equal mass without compromising blood supply, since the liver's anatomy dictates that the right lobe is invariably larger than the left. Any division beyond Cantlie's line in this scenario will jeopardise inflow and outflow to one or more segments.

iv. It is considerably more difficult to cannulate the hepatic artery and portal vein branches as they are shorter and smaller than the common hepatic artery, coeliac trunk or aorta. This issue was addressed by attempting to provide additional length for safe cannulation by retaining the main trunk of the vessel with one lobe while carrying out vascular reconstructions on the other. Vascular reconstruction was done using either the donor iliac vessels or segments from the coeliac or splenic artery if available. This does, however, increase the cold ischaemic time of the liver further and vascular reconstruction requires considerable surgical expertise to ensure that it is secure and does not threaten lobar perfusion.

v. Lastly, variations in arterial anatomy may preclude splitting of the liver since this could cause disruptions in branching patterns which would compromise the inflow of a segment of the lobe.

Although the data shown in this work is from a small number of human donor livers, it is a proof-concept-study that shows that there are very similar trends in metabolic parameters and functional recovery between the left and right lobes. This model could therefore be a very useful tool in assessing responses to therapeutic interventions in the pre-clinical NMP-L setting. Moreover, this model allows the same donor liver to be placed in treatment and control groups, which solves the issue that arises when using individual donor livers (as a result of

variations that exist between different livers). Also, since one donor liver generates two perfusion experiments for pre-clinical studies, this maximises use of resources which is vital due to the low availability of donor livers for research. This study has shown that liver splitting can be an effective tool to provide comparative controls for pre-clinical research in NMP-L. Future developments that would enhance its feasibility include optimising the method to minimise the need for considerable surgical expertise when splitting the liver for machine perfusion.

Chapter 6 Discussion and Concluding Remarks

Machine perfusion of the donor liver is an area of research which has been and still is rife with activity, especially over the last decade. This is in no small part due to the versatility in its clinical application, with multiple perfusion platforms and protocols being subjected to the rigor of clinical trials. The advent of machine perfusion has seen the increased use of extended criteria donor livers. It offers an organ preservation strategy which serves not only to reduce (or even eliminate) cold ischaemia time but also enable transplant viability testing based on *ex vivo* metabolic function, thus predicting clinical outcome and potentially provide a unique opportunity to recondition organs that would have previously been deemed unsuitable for transplantation. In so doing, it has demonstrated its ability to add organs to the donor pool. There is also another facet to the clinical utility of machine perfusion. This is its applicability to basic science research in assessing donor liver function and targeting pathophysiological pathways implication in liver IRI and graft dysfunction.

The research outlined in this thesis began with a systematic review to explore the peer-reviewed literature that has identified metabolic markers and molecular mechanisms of graft dysfunction and IRI using metabolomics high-throughput analytical technologies that may predict clinical outcome pre-transplantation. It was found that the main body of research is related to cold-stored livers. However, *omics* technologies are increasingly being used in basic science perfusion studies to compare different perfusion modalities as well as to determine the molecular signatures of steatotic livers that may provide further insight into their susceptibility to IRI. It is also interesting to note that of all the *omics*, metabolomics is the field that appears to be gaining most traction in machine perfusion research. This is most likely a result of the fact that metabolite flux may occur over a matter of minutes, as opposed to events at higher levels of cellular function. Assessing perturbations in metabolite concentrations therefore

offers an ideal opportunity to assess donor liver function during machine perfusion, which may only be of a few hours' duration.

The next part of this thesis sought to apply metabolomic analytical tools to investigate the metabolic behaviour of ECD livers included in a clinical trial. The opportunities in this regard were manifold. Firstly, it allowed us to analyse high throughput ECD liver metabolic data and determine any links clinical outcomes. Secondly, current perfusion viability parameters could be scrutinised against metabolic phenotypes. Thirdly, using high-throughput bioinformatics, we could establish potentially new and previously unobserved metabolic pathways that influence ECD liver metabolic behaviour.

The results did not establish a definitive link between clinical outcome and ECD liver phenotypes. This may have been due to the cohort of livers, small for a metabolomics study that had to operate within the parameters of the clinical trial. Interestingly, however, they did offer valuable insight into the viability parameters employed during the trial. Sustained differences in the perfusate metabolic phenotypes between viable and non-viable livers during the four-hour perfusion window were predominantly related to fatty acid and lipid metabolism, which were significantly different at all three time points. An analysis of polar metabolites revealed that, while there were significant differences during the first two hours of perfusion, these differences disappeared by the four-hour mark, indicating that, at least in this regard, these groups of livers were metabolically similar. Nevertheless, our lipidomic findings as well as the metabolic pathways highlighted at the two-hour perfusion time point with respect to transplant viability may warrant further lines of inquiry for future investigation. Together with the pathway enrichment analysis of the entire ECD liver cohort over NMP-L, several metabolic

pathways have been pinpointed that may prove useful in two respects. These are optimisation of perfusion solution to provide a favourable environment for the ECD liver to enhance its metabolic function and provide targets for therapeutic intervention to manipulate donor liver metabolism.

The second part of this thesis sought to investigate a pre-clinical NMP-L protocol that would address the persistent challenge of perfusion studies: low numbers of heterogenous donor livers. This proof-of-concept study demonstrated that hemi-livers derived from the same graft can be employed in perfusion studies as their own internal controls due similar trends in their behaviour as demonstrated by assessment of liver function, perfusion haemodynamics and liver histology. This offers an advantage over current methods of conducting perfusion research as it may increase confidence in the data relating to the effects of perfusate additives and pharmacological agents. Due to the small number of donor livers, a metabolomics protocol was not included in the study design, as its application fell outside the scope of this particular study. However, interestingly, the concept of split liver machine perfusion may also prove useful for future metabolomic studies. This is because reducing the effect of liver heterogeneity may hypothetically reduce the amount of "metabolic noise" that is associated with the high sensitivity of high-throughput analytical technologies. Further study is warranted.

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Appendix 1

Quadomics Assessment tool for metabolomics systematic reviews (as employed in Chapter 2)

Adapted from: Clin Biochem. 2008 Nov;41(16-17):1316-25. doi: 10.1016/j.clinbiochem.2008.06.018. Epub 2008 Jul 9.

Item	Yes	No	Unclear	Not applied
1. Were selection criteria clearly described?				
2. Was the spectrum of patients representative of patients who will receive the test in practice?				
3. Was the type of sample fully described?				
4. Were the procedures and timing of biological sample collection with respect to clinical factors described with enough detail?				
4.1. Clinical and physiological factors				
4.2. Diagnostic and treatment procedures.				
5. Were handling and pre-analytical procedures reported in sufficient detail and similar for the whole sample? And, if differences in procedures were reported, was their effect on the results assessed?				
6. Is the time period between the reference standard and the index test short enough to reasonably guarantee that the target condition did not change between the two tests?				
7. Is the reference standard likely to correctly classify the target condition?				
8. Did the whole sample or a random selection of the sample receive verification using a reference standard of diagnosis?				
9. Did patients receive the same reference standard regardless of the result of the index test?				
10. Was the execution of the index test described in sufficient detail to permit replication of the test?				
11. Was the execution of the reference standard described in sufficient detail to permit its replication?				
12. Were the index test results interpreted without knowledge of the results of the reference standard?				
13. Were the reference standard results interpreted without knowledge of the results of the index test?				
14. Were the same clinical data available when test results were interpreted as would be available when the test is used in practice?				
15. Were uninterpretable/intermediate test results reported?				
16. Is it likely that the presence of overfitting was avoided?				

Appendix 2: Publication

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Review Article

Systematic Review: Clinical Metabolomics to Forecast Outcomes in Liver Transplantation Surgery

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Abstract

Liver transplantation is an effective intervention for end-stage liver disease, fulminant hepatic failure, and early hepatocellular carcinoma. Yet, there is marked patient-to-patient variation in liver transplantation outcomes. This calls for novel diagnostics to enable rational deployment of donor livers. Metabolomics is a postgenomic high-throughput systems biology approach to diagnostic innovation in clinical medicine. We report here an original systematic review of the metabolomic studies that have identified putative biomarkers in the context of liver transplantation. Eighteen studies met the inclusion criteria that involved sampling of blood ($n=4$), dialysate fluid ($n=4$), bile ($n=5$), and liver tissue ($n=5$). Metabolites of amino acid and nitrogen metabolism, anaerobic glycolysis, lipid breakdown products, and bile acid metabolism were significantly different in transplanted livers with and without graft dysfunction. However, criteria for defining the graft dysfunction varied across studies. This systematic review demonstrates that metabolomics can be deployed in identification of metabolic indicators of graft dysfunction with a view to implicated molecular mechanisms. We conclude the article with a horizon scanning of metabolomics technology in liver transplantation and its future prospects and challenges in research and clinical practice.

Keywords: forecasting surgery outcomes, metabolomics, biomarkers, liver transplantation, diagnostics, graft dysfunction

Introduction

LIVER TRANSPLANTATION IS THE GOLD STANDARD TREATMENT for end-stage liver disease, fulminant hepatic failure, and early hepatocellular carcinoma (Francoz et al., 2007). Its success has broadened the range of indications for transplantation. This has led to a dramatic increase in demand for donor organs for potential transplant recipients who may benefit significantly from this treatment (Francoz et al., 2007; Perera et al., 2009).

The subsequent disparity between supply and demand is driving many transplant centers to accept donor livers with suboptimal quality for transplantation, which then increases the risk of post-transplant complications (Durand et al., 2008; Perera et al., 2009). These are known as marginal livers. Although they have decreased transplant waiting list mortality (Hashimoto and Miller, 2008), their suboptimal quality is reflected in their inferior recipient outcomes with a higher risk of recipient morbidity and mortality (Mathur et al., 2010). Furthermore, assessment of these organs for transplantation relies heavily on a combination of various factors, including donor medical history, clinical blood test results, and gross appearance of the liver during the organ retrieval procedure.

It is currently not possible to predict the likelihood of a successful post-transplant outcome using a single factor in isolation (Perera et al., 2009). Consequently, criteria for organ selection vary widely between transplant centers (Perera et al., 2009). This has highlighted the urgent need for more objective and accurate methods of graft assessment and selection pre-transplantation.

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Metabolomics is a high-throughput systems biology approach to diagnostic innovation in clinical medicine. It has been applied to the study of donor liver metabolism during donor organ procurement and the transplantation process with the aim of identifying and developing metabolic biomarkers of graft function as objective clinical tests able to forecast graft outcomes before transplantation (Bonneau et al., 2016; Christians et al., 2016; Wishart, 2005). However, despite a number of studies, there are still no validated biomarkers of post-transplantation outcomes available for clinical use.

The aims of this systematic review were to assess these studies, to analyze metabolite perturbations identified during donor liver procurement and transplantation, and to explore their associations with graft quality and post-transplant outcomes. We conclude the article with a horizon scanning of metabolomics technology in liver transplantation and its future prospects and challenges in research and clinical practice.

Clinical and Theoretical Background

The donor liver pathway from organ retrieval to transplantation is outlined in Figure 1. The majority of donor livers are obtained from deceased donors, either after brainstem death (donation after brain death [DBD]) or after cessation of circulation and cardiac death (donation after circulatory death or [DCD]) (Mckeown et al., 2012). The former is considered to be a more ideal source as during DBD, the organ retrieval procedure occurs while the donor's circulation is still intact and therefore the liver remains perfused with warm oxygenated blood up until the point of removal from the donor (Mckeown et al., 2012).

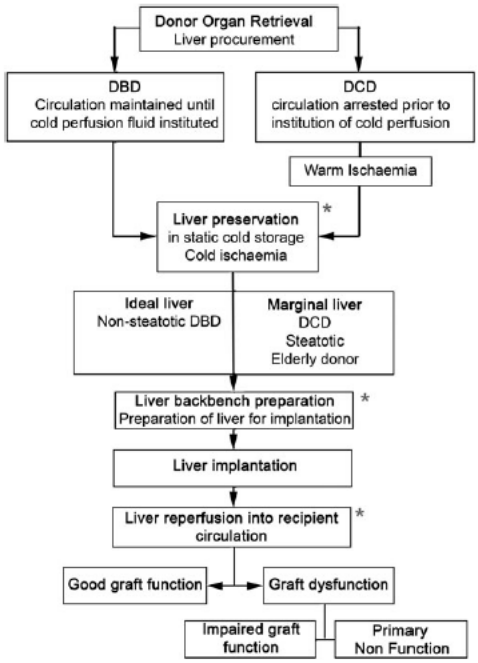


FIG. 1. The donor liver pathway from organ retrieval to transplantation. *Indicates points at which sampling may inform clinical decision making.

In DCD, circulatory arrest must be confirmed before organ retrieval can proceed (Mckeown et al., 2012). This subjects the liver to a period of warm ischemia before removal and exposes the organ to harmful anaerobic metabolism (Monbaliu et al., 2012).

In both cases, following the retrieval procedure, the liver is stored in ice and cold preservation fluid (also known as static cold storage, SCS) for transport to the recipient center. This is maintained up until backbench preparation when the excess tissue surrounding the organ is removed and the vessels and bile duct are prepared for implantation. This preserves the liver in hypothermic conditions to reduce the metabolic requirements of the organ, reduce anaerobic metabolism, and lessen the impact of ischemic damage (Hameed et al., 2017). In the case of DCD, it is well established that the metabolic insult brought on by the period of warm ischemia produces an organ of suboptimal quality for transplantation (Attia et al., 2008; Le Dinh et al., 2012).

Additionally, a number of other factors are known to affect donor liver quality. These include the presence of hepatic steatosis, donor advanced age, and prolonged SCS ischemia (Attia et al., 2008; Le Dinh et al., 2012). These organs have a reduced tolerance to ischemic injury, placing them at a higher risk of post-transplant complications: graft dysfunction, biliary complications, and reduced recipient survival (Le Dinh et al., 2012). Subsequently, the presence of these risk factors during the transplant screening and organ selection process heavily influences the transplant surgeon's decision to utilize the organ for transplantation (Perera et al., 2009).

In the initial period following transplantation, hepatic injury is reflected by a combination of biochemical derangements picked up on clinical blood tests, which reflect a poor functional status of the liver. This injury, also known as *Primary Graft Dysfunction*, occurs over a continuum of varying degrees of severity, the worst of which is *Primary Non-Function* (PNF)—a life-threatening irreversible loss of graft function manifested by biochemical derangements in the blood and circulatory or hemodynamic instability, for which emergency retransplantation is required (Fig. 2) (Le Dinh et al., 2012).

Metabolomics involves the identification and quantification of all metabolites in a biological system through the use of high-throughput analytical technologies (Dunn et al., 2011b; Patti et al., 2012). Metabolites are low-molecular-weight compounds (<2000 Da) that consist of sugars, amino acids, lipids, organic acids, and nucleotides, which drive cellular metabolism (Dunn et al., 2005, 2011b). The quantitative collection of all metabolites in a system under study is defined as the metabolome (Dunn et al., 2005). The human metabolome itself is currently known to consist of more than 100,000 metabolites (Wishart et al., 2018). Its composition is the result of upstream influence from higher levels of cellular function: proteins in the proteome, mRNA in the transcriptome, and DNA in the genome, as well as external stimuli (Fig. 2) (Dunn et al., 2011b).

Starting at the cell's genetic constitution (or genotype), interactions between all these levels encode cellular structure

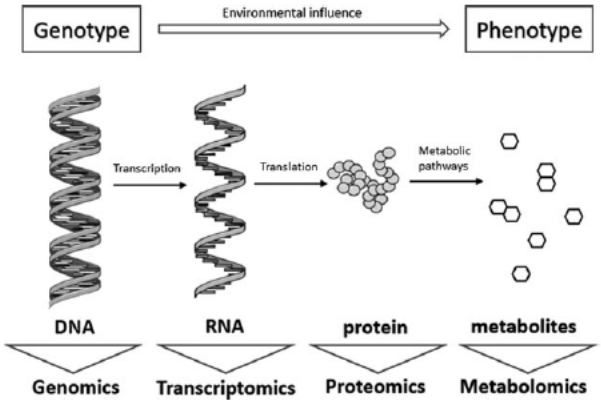


FIG. 2. The "Omics" cascade illustrating the different levels of cell function and their relationship to the "Omics" analytical sciences. Further down the cascade influence from environmental factors (external stimuli) increases. Therefore, the metabolome is more closely related to the biological phenotype than the genome.

function. Additional influence from external stimuli produces its biological phenotype or expressed physical traits (Dunn et al., 2011b). Metabolite flux in a cell is such that changes occur over a matter of seconds or minutes, in contrast to hours or days in the case of proteins and RNA transcription and the relatively static genome (Dunn et al., 2005, 2011b). Therefore, analyzing the metabolome provides a snapshot of changes in cell physiology in real time, making it a sensitive indicator of the biological phenotype (Dunn et al., 2005, 2011b).

Metabolomics research has been primarily driven forward by advances in two analytical platforms—nuclear magnetic resonance spectroscopy (NMR) (Beckonert et al., 2007) and mass spectrometry (MS) (Dunn et al., 2011a) (Table 1). MS is being increasingly employed in metabolomic investigations due to its higher sensitivity, ability to detect thousands of metabolites simultaneously, and its enhanced chemical identification capabilities when coupled with chromatographic separation (Dunn et al., 2011a).

The fundamental goal of metabolomics research is the translation of metabolite data obtained from biological samples into biological knowledge (Fig. 3) (Dunn et al., 2005). The data obtained are heavily influenced by the definition of groups of subjects from which samples will be procured, the type of sample analyzed, the instrument setup, and the method of sample quenching (procedure for halting active metabolic processes in the sample for analysis) (Dunn et al., 2005, 2011b). The initial sample analysis is generally an untargeted one (Table 2)—a broad sweep of all the low-molecular-weight compounds in a sample. Patterns in these data can be used to generate hypotheses focusing on specific metabolites, which can then be tested using more targeted analyses (Dunn et al., 2005, 2011b).

TABLE 1. MAIN DIFFERENCES BETWEEN THE TWO MAJOR METABOLOMIC ANALYTICAL PLATFORMS

	NMR	*MS*
Sample preparation	Minimal/ nondestructive	Destructive
Reproducibility	High	Low
Quantification	Quantitative	Qualitative and quantitative
Relative sensitivity	Low	High
Metabolite coverage	Low	High

NMR, nuclear magnetic resonance spectroscopy; MS, mass spectrometry.

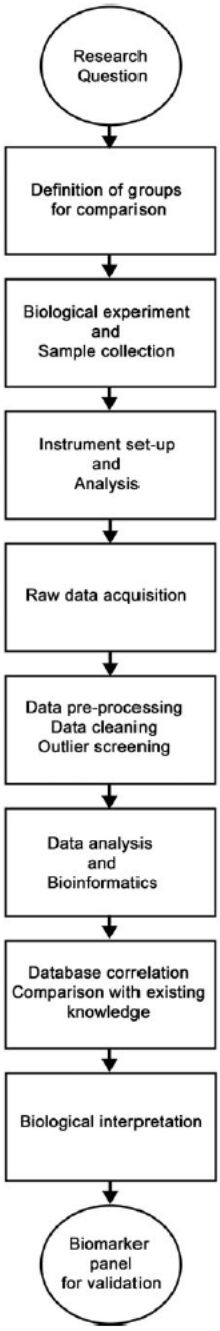
Literature Search and Eligibility Criteria

Studies reporting on the metabolomic findings of transplanted liver grafts were selected according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) protocol (Moher et al., 2009). A literature search was conducted in the OvidSP versions of MEDLINE and EMBASE databases using Medical Subject Heading (MeSH) terms "metabolomics," "liver," and "transplant*" until and including June 10, 2019.

All hits were screened by title and abstract. Inclusion criteria were as follows: (a) human donor liver studies investigating transplanted organs, (b) application of metabolomics analytical techniques, (c) reporting of clinical outcomes, and (d) published in English. Exclusion criteria were animal models; *in vitro* cell culture studies; proteomics, transcriptomics, and genomics; liver disease metabolomics; and nontransplanted donor livers. This systematic review aimed to synthesize all available metabolomics-derived data relating to liver transplantation outcomes; therefore, no studies were excluded for reasons of patient recruitment method.

Data extraction and analysis

Information extracted from each study included: first author, publication year, study design, analytical platform used, sample/population size, sample type methodology, outcomes measured, metabolic data, and inferences. Due to the limited



number of studies available as well as the significant variation in methodology, analytical platforms, multivariate analysis techniques, and reporting of clinical outcomes, a quantitative meta-analysis of the data was not undertaken.

Quality assessment of study methodology

Included study methodology was assessed using the QUADOMICS tool (Lumbreras et al., 2008; Parker et al., 2010), an adaptation of QUADAS (quality assessment tool for diagnostic accuracy studies) (Whiting et al., 2003). The former was designed specifically to take into account the unique challenges faced when appraising studies employing ''-omics''-based diagnostic research (see Supplementary Data for tool template). Studies were listed according to phase of biomarker discovery (Lumbreras et al., 2008), as this influences the number of items in the tool that can be applied to each study. The percentage of applied items, which scored positively in each study, as well as the percentage of each item scored positively across all were taken into account. Single case studies were not included in the quality assessment. The results are presented in Supplementary Table S1.

Results

Search strategy

The PRISMA flowchart for the literature search and selection process is outlined in Figure 4.

Study characteristics

The metabolomic analytical platforms employed in these studies were NMR ($n=7$), MS coupled to liquid chromatography (LC-MS) ($n=3$), direct infusion MS ($n=2$), capillary electrophoresis fingerprinting ($n=1$), and coulometric electrochemical array detection ($n=5$). Analyzed samples included blood ($n=4$), dialysate fluid from liver parenchyma ($n=4$), bile ($n=5$), and liver tissue ($n=5$). Study design included cohort observational studies ($n=11$), case series ($n=5$), and single case reports ($n=2$). Criteria for the diagnosis of graft dysfunction varied between studies. Table 3 outlines the main characteristics of each study, including the main results and inferences.

Quality assessment of included studies

All studies were Phase 1 or exploratory studies of biomarker discovery. Items assessing patient spectrum representation (item 2) and clinical data availability (item 14) were therefore not applicable to these studies. None of the studies included a patient flow diagram detailing the patient selection process and was therefore scored negatively in this regard. One study (6.25%) attempted to avoid data overfitting through the inclusion of an external validation set. For all

FIG. 3. The metabolomic workflow, illustrating the process of the metabolomic experiment from experimental design to data analysis and interpretation. The high sensitivity of metabolomic analytical instruments in detecting metabolites means each step before raw data acquisition must be carefully orchestrated to eliminate bias in the final results.

TABLE 2. MAIN FEATURES OF UNTARGETED AND TARGETED METABOLOMIC ANALYSES

Untargeted analysis	*Targeted analysis*
Global sample screening for a large number of metabolites, relative and not absolute concentrations determined	Identification and quantification of a small number of metabolites
Hypothesis-generating	Hypothesis-testing
Experimental design appropriate for global detection of metabolites in a sample	Experimental design adapted for optimal detection of a subset of metabolites in the metabolome
Metabolite identity not known before data acquisition; the data acquired are used to identify unknown metabolites	Metabolite identity is known before data acquisition; the data acquired are used to confirm the identity

studies, the index test results were interpreted with knowledge of the reference standard outcome (Supplementary Table S1).

Discussion

Application of metabolomics in the assessment of liver function is a challenging endeavor. The liver is involved in a myriad of metabolic pathways (glucose balance, fatty acid metabolism, amino acid metabolism, ammonia detoxification, and bile acid synthesis among others) compounded by ischemia during transplantation. Furthermore, metabolite perturbations in graft dysfunction must be interpreted in the context of the biological sample analyzed. For this reason, the results of included studies will be discussed in this context.

Blood

Singh et al. (2003) investigated recipient serum and urine from a pediatric patient who underwent living-donated liver transplantation, in which a living person donates a portion of their liver. Higher serum glutamine and urea levels, and lower levels of urinary urea were observed following graft dysfunction as a result of portal vein and hepatic artery thrombosis, leading to the patient's death. These findings correlated with conventional clinical blood tests used to monitor for graft dysfunction post-transplant—prothrombin time and serum alanine aminotransferase and aspartate aminotransferase levels. These changes may indicate impairment of the urea cycle in the liver, leading to increased glutamine synthesis and concentration in the blood (Singh et al., 2003).

Serkova et al. (2007) noted distinct metabolic differences between grafts in blood samples procured from a patient who underwent retransplantation for vascular complications, with higher lactate, uric acid, glutamine, methionine, citrate levels, and fatty acid levels, in the first failed transplant detected as early as 2 h postoperatively. This was when routine biochemical tests were still unremarkable (Serkova et al., 2007). Tripathi et al. (2009) observed that lactate and select amino acids (namely alanine, lysine, glutamine, methionine, asparagine, histidine, tyrosine, and phenylalanine) were significantly higher ($p < 0.02$) in a number of patients with postoperative graft failure.

Serum amino acids exhibited an earlier and more consistent increase than routine clinical liver function tests in these patients (Tripathi et al., 2009). Lactate is widely known to be increased in ischemia due to increased anaerobic glycolysis (Serkova et al., 2007). Uric acid, an antioxidant that counters oxidative stress and is the end product of the xanthine pathway, also accumulates under ischemic conditions (Serkova et al., 2007). Since the liver is the primary site of amino acid catabolism, an increase in circulating amino acids potentially indicates reduced metabolism and is therefore reflective of hepatocyte dysfunction (Serkova et al., 2007; Tripathi et al., 2009). Furthermore, liver graft failure results in lipid disturbances and a low circulation concentrations of fatty acids (Serkova et al., 2007).

An investigation into the role of nitric oxide (NO) synthesis inhibition in the assessment of graft recovery post-transplantation (Martin-Sanz et al., 2003) revealed that impaired liver function was associated with higher levels of L-N-monomethylarginine and asymmetric dimethylarginine, both methylated arginine derivatives (NO inhibitors). These were detected early after revascularization of the liver in the recipient circulation when clinical blood tests would have been unremarkable (Martin-Sanz et al., 2003). It was postulated that a reduction in NO, a vasodilator, would impede the hepatic microcirculation and graft perfusion, leading to graft dysfunction (Martin-Sanz et al., 2003).

Parenchymal interstitial fluid

Silva et al. (2005) investigated changes in metabolite concentrations in graft parenchyma by inserting microdialysis catheters and perfusing with dialysate to extract interstitial fluid. High levels of all carbohydrate metabolism intermediaries were observed on reperfusion, normalizing during the first 12 h (Silva et al., 2005). High lactate and low pyruvate levels were initially observed on reperfusion of the liver during transplantation. The concentration difference between these two metabolites decreased with time, indicating recovery of aerobic glycolysis with increasing pyruvate.

A follow-up study found that interstitial lactate was significantly higher during backbench preparation ($p < 0.03$) in those grafts displaying initial poor function (IPF) (Silva et al., 2006). In the same patient cohort, extracellular aspartate was also significantly higher for IPF ($p < 0.05$) (Richards et al., 2007). During backbench preparation of the liver for transplantation, IPF had higher levels of beta-alanine, GABA, glutamine, and threonine ($p < 0.05$) (Richards et al., 2007).

Alanine is involved in the glucose–alanine cycle, a source of nitrogen for the urea cycle and pyruvate for glycolysis. Gamma-aminobutyric acid (GABA) has been implicated in hepatic regeneration, with disruption of GABA homeostatic mechanisms associated with extensive liver injury (Silva et al., 2006). Glutamate plays a role in membrane excitability, and taurine is an intracellular osmolyte, which helps maintain cell volume (Silva et al., 2006). Higher levels of these metabolites in the extracellular interstitial fluid of IPF grafts may therefore indicate disruption of these mechanisms and increased cellular breakdown.

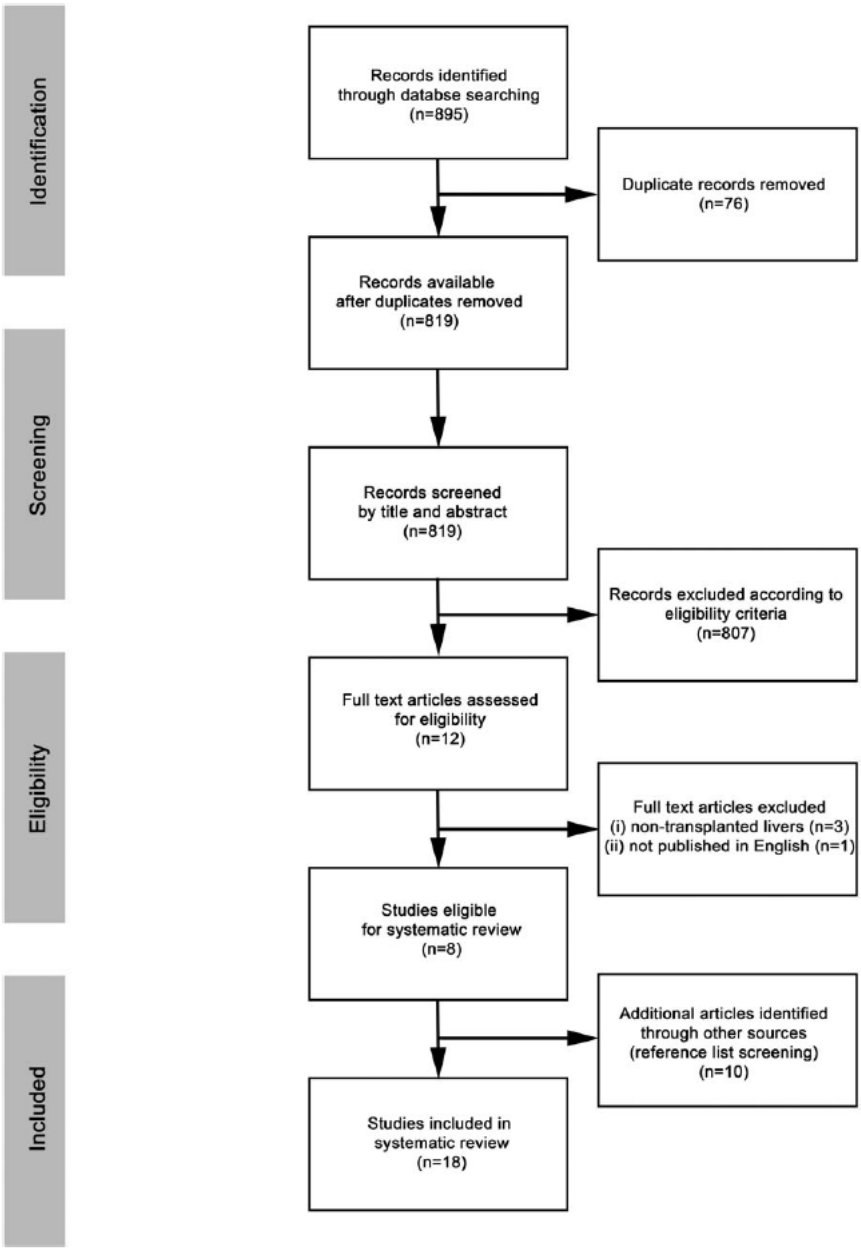


FIG. 4. PRISMA flowchart outlining the search strategy for this systematic review.

Table 3. Main Features and Conclusions of Included Studies

Study	*Analytical platform*	*Study design*	*Population No.*	*Investigated outcomes*	*Measured outcomes*	*Increased metabolites*	*Decreased metabolites*	*Main inferences of significant findings*
Blood metabolomics								
Singh et al. (2003)	^{1}H-NMR	Case study	1 Recipient	Graft dysfunction	Graft dysfunction criteria not stated.	Serum: glutamine and urea	Urine: urea	Urea (ornithine) cycle impairment as a sign of graft failure. Monitoring of recipient serum glutamine levels as a predictor of graft dysfunction.
Martin-Sanz et al. (2003)	HPLC-MS	Prospective cohort	75 Recipients	Graft dysfunction	Grade 1 (n=40): AST <1000 U/L for the first 3 days post-OLT, with good bile production (>40 mL/day) and evidence of coagulation. Grade 2 (n=19): initial AST >1000 U/L but decreased over next 48 h with adequate bile flow and coagulation. Grade 3 (n=12): AST >2500 U/L during first 48 h post-OLT, bile production <40 mL/day, with severe coagulopathy. Grade 4 (n=4): rapidly increasing AST with no bile production and severe coagulopathy.	Methylarginine and dimethylarginine	—	Increased inhibition of nitric oxide synthesis through the action of arginine derivatives related to EAD. MMA and ADMA levels in graft preservation solution as a potential predictor of early liver function.
Serkova et al. (2007)	^{1}H-NMR	Case study	1 Recipient	Graft dysfunction	Graft dysfunction criteria not stated.	Lactic acid, uric acid, citrate, glutamine, and methionine	Fatty acids	Lactate, end product of anaerobic glycolysis, increased in ischemia. Uric acid, end product of xanthine pathway, accumulates during IRI. Lipid dysregulation and decreased circulation of fatty acids associated with graft failure. Amino acid levels as predictor of graft dysfunction. High levels attributed to decreased amino acid metabolism and liver nitrogen metabolism as a result of ischemic injury.
Tripathi et al. (2009)	^{1}H-NMR	Case series	9 Recipients	Graft dysfunction	Mortality: survivors (n=6) vs. nonsurvivors (n=3).	Lactate, alanine, lysine, glutamine, methionine, asparagine, histidine, tyrosine, and phenylalanine	—	Decreased amino acid metabolism and impairment of urea (ornithine) cycle related to graft dysfunction.

(*continued*)

TABLE 3. (CONTINUED)

Study	*Analytical platform*	*Study design*	*Population No.*	*Investigated outcomes*	*Measured outcomes*	*Increased metabolites*	*Decreased metabolites*	*Main inferences of significant findings*
Interstitial fluid/dialysate fluid metabolomics								
Silva et al. (2005)	CEAD/HPLC and fluorometric detection	Prospective cohort	17 Recipients	Graft dysfunction	All patients were reported as having an uneventful postoperative course.	During SCS and post-reperfusion: lactate, pyruvate, glycerol, alanine, glutamate, GABA, and taurine (all with reduction over time)	Arginine (initially decreased post-reperfusion but increased >19 h increased post-transplant)	Increased lactate and pyruvate during backbench preparation—high lactate to pyruvate ratio post-reperfusion as indicator of cell ischemia due to increased anaerobic glycolysis during SCS. Initially high glycerol may be indicative of cell lysis. Reduction in amino acids (GABA, glutamate, taurine, and alanine) over time may reflect recovery of amino acid metabolism. Increased extracellular arginine may be related to Nitric oxide synthase activity.
Silva et al. (2006)	CEAD/HPLC and fluorometric detection	Prospective cohort	15 Recipients	Graft dysfunction	Graft dysfunction as defined by Strasberg et al. (1994): AST >2000 IU/L in the first 24 h ($n=6$) vs. those that did not ($n=9$).	Lactate	—	High lactate in donor graft pre-transplantation as a marker of IRI and development of IPF.
Richards et al. (2007)	CEAD/HPLC and fluorometric detection	Prospective cohort	15 Recipients	IPF	Graft dysfunction as defined by Strasberg et al. (1994).	IPF grafts during procurement: aspartate IPF grafts during SCS: β-alanine, GABA, glutamine, threonine, and arginine	—	Increased extracellular amino acid concentrations in procurement and SCS potentially due to release following hepatocyte death or active release as protective/restorative response.
Perera et al. (2014)	CEAD	Prospective cohort	40 Recipients	Graft dysfunction/ IRI/DBD vs. DCD	Graft dysfunction criteria not stated. DCD ($n=13$), DBD ($n=27$).	DCD: xanthine, uric acid, and kynurenine (SCS) DBD: 3-nitrotyrosine and 4-hydroxy-3-methoxymandelic acid (SCS) DBD and DCD: homovanillic acid and methionine (SCS) PNF graft: reduced glutathione and kynurenine (SCS) and xanthine (post-reperfusion)	—	Xanthine and uric acid (products of the purine catabolism) are modulated by xanthine oxidoreductase, an enzyme implicated in reactive oxygen species generation, aggravated by tissue hypoperfusion. Upregulation of reduced glutathione (cellular antioxidant) as a marker of increased cellular oxidative stress. Kynurenine, as a product of tryptophan metabolism, implicated in mediation of cellular stress response.

(*continued*)

TABLE 3. (CONTINUED)

Study	*Analytical platform*	*Study design*	*Population No.*	*Investigated outcomes*	*Measured outcomes*	*Increased metabolites*	*Decreased metabolites*	*Main inferences of significant findings*
Bile metabolomics								
Melendez et al. (2001)	^{1}H-NMR	Case series	4 Recipients	Graft dysfunction	Graft dysfunction as defined by Clavien et al. (1994): first day AST ≥2000 IU/L or a transient increase in AST ≥1000 IU/L or a persistently high PT >20 (or the equivalent INR >1.4) for at least 3 days. EAD (n=1), PNF (n=1), and good graft function (n=2).	Steatotic livers: PC PGD grafts: persistence of preservation solution component peaks (indicating decreased clearance of solution)	—	Increased PC in steatotic livers suggestive of higher abundance of biliary lipids from increased lipid secretion in bile.
Vilca Melendez et al. (2004)	Reverse ion pair HPLC and GC	Prospective cohort	35 Recipients	Graft dysfunction	Graft dysfunction as defined by Clavien et al. (1994): PGD (n=12), non-PGD (n=23).	Suboptimal graft: total bile acid output rate + CA	Normal liver: CDCA	Higher total bile acid abundance in suboptimal grafts may be due to impaired bile flow. High amount of "less hydrophobic" CA relative to "more hydrophobic" CDCA may indicate impaired water secretion in bile for bile flow.
Papaspyridonos et al. (2008)	CE fingerprinting	Case series	6 Recipients	Graft dysfunction	Graft dysfunction as defined by Clavien et al. (1994): PGD (n=1), non-PGD (n=5).	PGD and steatotic liver: TCA + TDCA	—	Upregulation of tauro-conjugated bile acids associated with liver injury, thereby reflecting metabolic dysfunction in moderately steatotic livers.
Hedaya et al. (2009)	^{1}H-NMR	Prospective cohort	41 Recipients	ACR	Biopsy-proven ACR according to clinical suspicion. ACR (n=12), non-ACR (n=29).	Recipient/donor TCBA ratio ≥0.5 on days 7 and 9, and ≥0.38 on day 11 post-transplantation	—	Recipient and donor TCBA ratios as postoperative predictor of ACR.
Legido-Quigley et al. (2011)	UPLC-MS	Prospective cohort	10 Recipients	Graft steatosis/ graft dysfunction	Graft steatosis by histopathological assessment. Graft dysfunction as defined by Clavien et al. (1994). No PGD livers. Mildly steatotic (n=2), moderately steatotic (n=1), normal (n=7).	Non-steatotic liver: GDCA, GCA, and GCDCA (pre-transplantation). Normal and steatotic livers: TCA and TCDCA (post-transplant), secondary bile acids.	—	Different proportions of bile acids in grafts could be a sign of organ recovery or functional impairment.
Liver tissue metabolomics								
Duarte et al. (2005)	HR-MAS NMR	Case series	6 Recipients	Graft dysfunction/ graft Steatosis	Graft dysfunction as defined by Clavien et al. (1994): PGD (n=1), non-PGD (n=5).	Steatotic grafts (mild and moderate): triglycerides and unsaturated lipids	Good functioning livers: GPC (post-reperfusion) Steatotic livers: amino acids, glucose, and products of nucleotide metabolism	Decreased GPC (a cell membrane-derived phospholipid degradation product) in postoperative good functioning livers attributed to increased cell turnover reflecting and active cellular regeneration. GPC as a potential biomarker of liver function.
Hrydziuszko et al. (2010)	FTCIR-MS CEAD	Case series	8 Recipients	Graft dysfunction	Graft dysfunction as defined by Strasberg et al. (1994): IPF (n=1), non-IPF (n=7).	On reperfusion: urea and urea cycle intermediates Bile acids Formate, orthophosphate, ADP, fumarate, succinate (intermediaries of energy metabolism)	—	Metabolite profile on reperfusion demonstrates recovery of liver metabolic function (increased urea synthesis, bile acid synthesis) and intermediaries of energy metabolism.

(*continued*)

TABLE 3. (CONTINUED)

Study	*Analytical platform*	*Study design*	*Population No.*	*Investigated outcomes*	*Measured outcomes*	*Increased metabolites*	*Decreased metabolites*	*Main inferences of significant findings*
Cortes et al. (2014)	UPLC-MS	Prospective cohort	124 Recipients	Graft dysfunction/ PNF	Graft dysfunction according to Olthoff et al. (2010) classification—at least one of the following criteria for a diagnosis of graft dysfunction as EAD: bilirubin ≥10 mg/dL on day 7; INR ≥1.6 on day 7; AST or ALT >2000 IU/L within the first 7 days. EAD (n=60), IGF (n=64). Model development: EAD (n=48) and IGF (n=48). Model validation: EAD (n=12), IGF (n=12), PNF (n=4).	Lysophosphatidylcholines, lysophosphatidylethanolamines, phosphatidylcholines, phosphatidylethonalmines, sphingomyelins, bile acids, and products of histamine metabolism.	—	Disruption of phospholipid metabolism as a reflection of cell membrane breakdown. Lysophosphatidylcholines are toxic metabolites generated by phospholipase A2-catalyzed phospholipid hydrolysis, implicated hepatocyte apoptosis. Disruption of bile acid homeostasis has been shown to affect bile flow recovery. Increased histamine levels may reflect an adaptive response to ischemic injury in which cytokine release is reduced through activation of the H4 histamine receptor.
Hrydziuszko et al. (2016)	FTCIR MS	Prospective cohort	37 Recipients	Graft dysfunction/ PNF/DBD vs. DCD	Graft dysfunction criteria not stated. DBD (n=27), DCD (n=10). PNF: 1 DBD and 1 DCD.	DCDs and PNF: kynurenine	—	Kynurenine is a product of tryptophan metabolism, implicated in immune modulation. Suboptimal quality of DCD livers due to warm ischemia could be reflected in the higher kynurenine levels. Kynurenine postulated as a potential biomarker of graft dysfunction.
Faitot et al. (2018)	HR-MAS NMR	Prospective cohort	42 Recipients	Graft dysfunction	Graft dysfunction according to Olthoff et al. (2010) classification. EAD (n=7), no EAD (n=35).	Lactate and phosphocholine	—	High lactate is indicative of the anaerobic environment during SCS, potentially acting as a marker of graft tolerance to cold ischemia. High phosphocholine can be linked to cell membrane disruption. It is converted to glycerophosphocholine by the endoplasmic reticulum, therefore sustained high levels could reflect endoplasmic reticulum stress.

ACR, acute cellular rejection; ADMA, asymmetric dimethylarginine; ADP, adenosine diphosphate; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CA, cholic acid; CDCA, chenodeoxycholic acid; CE, capillary electrophoresis; CEAD, coulometric electrochemical array detection; DBD, donation after brainstem death; DCD, donation after circulatory death; EAD, early allograft dysfunction; FTCIR, Fourier transform infrared spectroscopy; GC, gas chromatography; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; GPC, glycophosphocholine; H-NMR, proton nuclear magnetic spectroscopy; HPLC, high-performance liquid chromatography; HR-MAS, high-resolution magic angle spectroscopy; IPF, initial poor function; MMA, monomethylarginine; MS, mass spectrometry; PC, phosphocholine; PGD, primary graft dysfunction; PNF, primary non-function; SCS, static cold storage; TCA, taurocholic acid; TCBA, taurine-conjugated bile acids; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; UPLC, ultra performance liquid chromatography; GABA, gamma-aminobutyric acid.

Perera et al. (2014) observed that interstitial xanthine, uric acid, and kynurenine were found to be increased in DCDs, and 3-nitrotyrosine and 4-hydroxy-3-methoxymandelic acid in DBDs ($p<0.05$). Grafts with PNF demonstrated increased levels of reduced glutathione, a cellular antioxidant, and kynurenine (during SCS) as well as xanthine (post-reperfusion) (Perera et al., 2014). The findings during SCS are of particular significance as they differentiate grafts according to outcome pre-transplantation. Of interest, the investigators identified metabolite changes in amino acid metabolism involving increased levels of activity in the tryptophan/kynurenine pathway in DCDs (Perera et al., 2014).

Kynurenine is an intermediate of tryptophan metabolism and has been implicated in cellular stress mechanisms and inflammatory responses (Perera et al., 2014). Therefore, increased kynurenine production may be related to the longer warm ischemia time of DCDs. Moreover, PNF grafts, in the same study, exhibited a similar metabolic signature to that of DCDs.

Bile

Bile acids are major components of bile and have a significant influence on bile flow and secretion of biliary lipids, such as glycerophosphatidylcholines (Melendez et al., 2001). It has therefore been hypothesized that alterations in bile acid concentrations may reflect graft functional integrity (Melendez et al., 2001; Vilca Melendez et al., 2004).

Vilca Melendenz et al. (2004) found that steatotic livers had a higher abundance of glycerophosphatidylcholines, likely due to increased biliary lipid secretion. Bile acid secretion was slower in grafts with primary graft dysfunction (Melendez et al., 2001).

A further study demonstrated that while there was no difference in bile flow to differentiate steatotic grafts from nonsteatotic grafts, the former had higher bile acid concentrations (Vilca Melendez et al., 2004). The authors postulated that this could be related impairment of canicular bile flow mechanisms in the inferior grafts. Furthermore, donor bile from steatotic grafts pre-reperfusion had a higher proportion of cholic acid and lower proportion of chenodeoxycholic acid with a reversal of proportions in nonsteatotic grafts (Vilca Melendez et al., 2004). Higher proportions of "less hydrophobic" bile acids (such as cholic acid) have been associated with reduced bile flow (Vilca Melendez et al., 2004).

According to Papaspyridonos et al. (2008), a liver exhibiting post-transplant graft dysfunction had higher levels of taurocholic acid and taurodeoxycholic acid when compared with its normal counterparts. The authors attributed this to the moderately steatotic condition of the liver, which translated into reduced bile flow (Papaspyridonos et al., 2008). Similar differences in bile acid composition were observed in pre- and post-transplant bile of steatotic versus nonsteatotic livers (Legido-Quigley et al., 2011). In the setting of living donor transplantation, post-transplant differences in recipient and donor bile acid and taurine-conjugated bile acids ratios were noted between patients with acute cellular rejection and those without (Hedaya et al., 2009).

Tissue

A tissue biopsy is invasive and potentially provides a heterogeneous view of the liver metabolome (especially in poorly perfused livers). However, data obtained from these samples can be correlated directly with histology from the same biopsy. Duarte et al. (2005) demonstrated that metabolomics distinguished grafts with higher degrees of steatosis by revealing higher triglyceride and unsaturated lipid concentrations, together with lower phospholipid levels. A decrease in glycophosphocholine (a phospholipid degradation product), from retrieval to implantation, was also observed in all the livers except one—that which developed primary graft dysfunction, where the metabolite remained constant throughout. This could reflect an increase in cell turnover in a functioning liver as it recovers from the ischemic insult and regenerates (Duarte et al., 2005).

According to a study by Hrydziuszko et al. (2010), the most noticeable tissue metabolic changes on reperfusion were upregulation of the urea cycle (with increased urea and urea cycle intermediates) and increased bile acid levels. In a direct comparison of DBD and DCD metabolic signatures, the most significant finding related to amino acid metabolism involving higher kynurenine levels in DCD grafts ($p<0.05$) in SCS and post-reperfusion. These changes were also noted in the PNF livers (Hrydziuszko et al., 2016).

Cortes et al. (2014) used metabolomics data generated from their study population to develop a validated predictive metabolic model from a molecular biosignature associated with graft dysfunction. The latter was found to have a distinct lipidomic fingerprint consisting of significantly higher levels of a number of phospholipid breakdown products: lysophosphatidylcholines, lysophosphatidylethanolamines, phosphatidylcholines, phosphatidylethonalmines, and sphingomyelins ($p<0.05$) (Cortes et al., 2014). This pattern echoes the lipidomic findings of previous studies since such a biosignature is potentially associated with increased cell membrane lysis and subsequent cell death due to decreased tolerance for the disruption of cellular homeostasis. Additionally, bile acids and products of histidine metabolism were also significantly higher in these livers ($p<0.05$) (Cortes et al., 2014).

Faitot et al. (2018) also assessed the predictive value of metabolites for graft dysfunction. Higher lactate, glutamate, glutamine, alanine, valine, isoleucine, and choline derivatives levels were observed. However, only lactate and phosphocholine gave high accuracy for prediction of graft dysfunction. Receiver operator characteristic curve analysis showed a high predictive value for lactate, and multivariate analysis for graft dysfunction showed that graft lactate content was the only independent predictor ($p=0.046$) (Faitot et al., 2018).

Anticipated challenges and avenues for future metabolomic research

The observation that distinct metabolite signatures associated with graft dysfunction have been found to precede changes in traditional liver function tests, as early as pre-transplantation, justifies the ongoing need for further investment in metabolomic research. Studies included in this systematic review have shown links between changes in donor liver metabolism from organ retrieval to transplantation and graft outcome post-transplantation. The idea that these efforts may lead to the identification of clinically applicable biomarkers of graft viability pre-transplantation remains attractive yet elusive. Although novel metabolic biomarkers for the early detection of graft dysfunction have

been identified, these have yet to proceed beyond the discovery phase (Christians et al., 2016).

There is, as yet, no single metabolite or metabolite panel that has been consistently and robustly observed to be correlated with graft outcome. There are a number of reasons to explain this. As outlined previously, the metabolomic experiment is complex by design and incorporates several preanalytical, analytical, and data-processing steps. These must be tailored according to several factors, chief among which are the characteristics of the population under study, biological sample procurement and processing, the analytical platform available, and the intended outcomes. These should be taken into account early on in the study design, otherwise the investigator may be faced with several pitfalls when interpreting the data.

With this in mind, the main focus of the majority of studies in this review has been the identification of metabolite perturbations and their significance as potential biomarkers rather than external validation of their predictive capabilities for clinical applicability. They are exploratory investigations with small population numbers spanning a variety of sample media and analytical platforms. Furthermore, while analytical platform variety has dramatically extended the field of metabolite discovery, lack of a standardized investigative approach has led to variable results, hindering amalgamation.

Donor liver populations are highly heterogeneous, and there is a multitude of donor-related variables that influence graft quality. Subsequently, data from small population numbers may be riddled with confounding factors and must be interpreted with caution. In the majority of studies, data were generated from convenience sampling rather than a randomized or consecutive patient protocol, further undermining their results. Moreover, the reporting quality of metabolomic data is variable, as evidenced by the risk-of-bias assessment.

Another factor that must be taken into consideration is that different biological samples will yield different results. Of all those described in this review, blood sampling is the least invasive. However, the only sampling moments available are while the liver is being perfused by the donor circulation before preservation or recipient circulation following transplantation. Bile analysis may be useful as it is a direct product of liver metabolism, but its secretion following organ retrieval is variable and limited in quantity (Vilca Melendez et al., 2004).

Extraction of parenchymal interstitial fluid enables analysis of metabolites in closer proximity to, and therefore more representative of, the hepatocellular metabolome. However, current methods of extraction entail mixing of interstitial fluid with dialysate fluid, which itself may interfere with sample analysis (Silva et al., 2005). Tissue biopsies, although invasive, may be the most reliable as these may be procured at any point during organ retrieval, preservation, and transplantation.

It is also worth noting that criteria defining graft dysfunction vary between studies. Classification systems have been developed to improve the standardization of clinical outcomes with respect to the diagnosis of graft dysfunction (Clavien et al., 1994; Olthoff et al., 2010; Strasberg et al., 1994), and these were employed by most studies (as outlined in Table 3). However, the criteria vary between classifications. Furthermore, there were a number of studies that did not state the criteria used. A stronger case is made for data that are reproducible across multiple studies with consistent and objectified outcomes, enhancing clinical validity and utility.

There is therefore a need for a collaborative multicenter approach with standardized analytical as well as clinical protocols to maximize population numbers and increase the validity of relevant and viable data for biomarker discovery. Standardizing thresholds to define clinical outcomes or endpoints will also increase the validity of data generated and facilitate clinical applicability and acceptance of potential metabolic biomarkers.

One final point is that all metabolomic studies described in this systematic review have focused on metabolic changes in donor livers preserved in SCS before transplantation. More recently, a novel technology known as donor liver machine perfusion is being investigated by several research groups and clinical trials as an alternative modality of graft preservation (Laing et al., 2017). The principle behind this technology is that superior preservation of the liver can be achieved through continuous *ex vivo* perfusion with oxygenated and nutrient-rich fluid (Nasralla et al., 2018). It is currently being developed as a tool to either completely replace SCS or compliment it by enabling the functional recovery of the organ following cold storage and before transplantation (Laing et al., 2017).

Both these settings offer a unique opportunity to assess metabolic biomarkers of graft function pre-transplantation. Therefore, the application of metabolomics to this new modality of organ preservation may prove to be an invaluable tool. This potential has been demonstrated in the preclinical setting in a cohort of nontransplanted subnormothermically (21°C) machine-perfused grafts, in which metabolomics was able to differentiate marginal livers with higher degrees of steatosis and longer warm ischemia time (Bruinsma et al., 2016).

The next logical step would be metabolomic analysis of machine-perfused transplanted livers. Such a platform would be of great value, especially to marginal livers rejected for transplantation based on current methods of donor organ selection. It would enable the simultaneous metabolomic analysis of perfusate, bile, and tissue sampling in a controlled *ex vivo* environment to assess graft quality and suitability. Therefore, metabolomics could potentially be used in monitoring liver preservation during perfusion, thus ensuring the optimal environment for graft preservation and metabolic support.

Conclusions

Metabolomics shows promise in developing objective and reliable techniques to metabolically profile liver grafts and develop biomarkers as prognostic markers predictive of graft dysfunction pre-transplantation. However, investigative tools and methodologies must be standardized with multicenter collaborations to enhance the validity of results across multiple patient cohorts in an effort to progress from biomarker discovery to clinical validation and utility.

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Supplementary Material

Supplementary Data
Supplementary Table S1

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Abbreviations Used

ACR = acute cellular rejection
ALT = alanine aminotransferase
AST = aspartate aminotransferase
ADMA = asymmetric dimethylarginine
ADP = adenosine diphosphate
CA = cholic acid
CDCA = chenodeoxycholic acid
CE = capillary electrophoresis
CEAD = coulometric electrochemical array detection
DBD = donation after brain death
DCD = donation after circulatory death
EAD = early allograft dysfunction
FTCIR = Fourier transform infrared spectroscopy
GABA = gamma-aminobutyric acid
GC = gas chromatography
GCA = glycocholic acid
GCDCA = glycochenodeoxycholic acid
GDCA = glycodeoxycholic acid
GPC = glycophosphocholine
H-NMR = proton nuclear magnetic spectroscopy
HPLC = high-performance liquid chromatography
HR-MAS = high-resolution magic angle spectroscopy
INR = international standardized ratio
IPF = initial poor function
IRI = ischemia-reperfusion injury
LC = liquid chromatography
MMA = monomethylarginine
MS = mass spectrometry
NMR = nuclear magnetic resonance spectroscopy
NO = nitric oxide
PC = phosphocholine
PGD = primary graft dysfunction
PNF = primary non-function
PT = prothrombin time
SCS = static cold storage
TCA = taurocholic acid
TCBA = taurine-conjugated bile acids
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Appendix 3: Publication

frontiers in Surgery

ORIGINAL RESEARCH
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Ex situ Normothermic Split Liver Machine Perfusion: Protocol for Robust Comparative Controls in Liver Function Assessment Suitable for Evaluation of Novel Therapeutic Interventions in the Pre-clinical Setting

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Background: *Ex situ* donor liver machine perfusion is a promising tool to assess organ viability prior to transplantation and platform to investigate novel therapeutic interventions. However, the wide variability in donor and graft characteristics between individual donor livers limits the comparability of results. We investigated the hypothesis that the development of a split liver *ex situ* machine perfusion protocol provides the ideal comparative controls in the investigation of machine perfusion techniques and therapeutic interventions, thus leading to more comparable results.

Methods: Four discarded human donor livers were surgically split following identification and separation of right and left inflow and outflow vessels. Each lobe, on separate perfusion machines, was subjected to normothermic perfusion using an artificial hemoglobin-based oxygen carrier solution for 6 h. Metabolic parameters as well as hepatic artery and portal vein perfusion parameters monitored.

Results: Trends in hepatic artery and portal vein flows showed a general increase in both lobes throughout each perfusion experiment, even when normalized for tissue weight. Progressive decreases in perfusate lactate and glucose levels exhibited comparable trends in between lobes.

Conclusion: Our results demonstrate comparability between right and left lobes when simultaneously subjected to normothermic machine perfusion. In the pre-clinical setting, this model provides the ideal comparative controls in the investigation of therapeutic interventions.

Keywords: normothermic, liver function, organ preservation, split liver technique, machine perfusion

INTRODUCTION

The main purpose of *ex situ* donor liver machine perfusion has been the development of superior modality of organ preservation to conventional static cold storage as well as a method to assess organ viability prior to transplantation (1). In the United Kingdom, between April 2018 and March 2019, 15% of donor livers retrieved were not transplanted, representing a significant pool of potentially viable grafts (2) A recent randomized controlled clinical trial demonstrated that normothermic machine liver perfusion (NMLP) reduced discard rates of donor organs when compared to static cold storage, without jeopardizing transplant outcomes (3, 4). A number of perfusion devices are currently available. The principal components include a blood reservoir, centrifugal pump, oxygen concentrator, heat exchanger and a circuit which continuously pumps perfusate through the liver via the organ's inflow vessels (hepatic artery and/or portal vein) and recirculates this following drainage from the inferior vena cava (1). These systems allow for extraction of perfusate for blood gas analysis, thus enabling real-time monitoring of oxygen and carbon dioxide levels, acid-base homeostasis as well as glucose levels (1) All these parameters have been described as markers for organ viability during machine perfusion (5). Several pre-clinical and clinical non-randomized studies have investigated different perfusion modalities with variations in perfusion temperature, perfusate composition, perfusion duration, vessel cannulation as well as other technical considerations (1).

The technology provides a unique opportunity for assessing liver metabolic and synthetic function. This has been recognized by pre-clinical studies investigating the effect of novel therapeutic interventions on machine perfused donor livers (6, 7). However, donor livers available for research in machine perfusion have been, so far, in short supply. Furthermore, the inherent variation in donor characteristics and liver quality that exist between different livers have limited their capacity as suitable comparative controls and, therefore, the interpretability of data from small series of whole organ perfusions (8).

Splitting of the donor liver is a well-established strategy used in transplantation to increase organ availability by allowing the same organ to be "shared" between two recipients (9). The surgical technique splits the donor organ into two separate independently-functioning units (9). A pre-clinical study by Huang et al. reported that, during *ex situ* subnormothermic machine perfusion, split livers demonstrated comparable perfusion and functional characteristics, providing a controlled comparison between split lobes, thus allowing each liver to act as its own internal control (10). However, this has yet to be demonstrated in a normothermic machine perfusion model. NMLP has been shown to enable the functional assessment and viability testing of donor livers prior to transplantation in a near-physiological environment (5, 11). In the context of scientific research, this makes NMLP a promising pre-clinical platform for the investigation of therapeutics and mechanistic studies. We therefore sought to develop a normothermic machine perfusion protocol for the application of the split liver model to pre-clinical research. Our hypothesis was that individual lobes from the same liver would recover and function similarly to one another when subjected to *ex situ* end-ischaemic NMLP. In addition to providing more liver units for perfusion experiments, the protocol enables each liver to act as its own internal control, thus eliminating the inherent heterogeneity of whole organ perfusions.

Abbreviations: BD, Bile duct; DBD, Donor after Brainstem Death; DCD, Donor after Cardiac Death; CHA, Common Hepatic Artery; LHV, Left Hepatic Vein; LPV, Left Branch of Portal Vein; LL, Left Lobe; MHV, Middle Hepatic Vein; NHSBT, National Health Service Blood and Transplant; NMLP, Normothermic Machine Liver Perfusion; PAS, Periodic Acid Schiff; PV, Portal Vein; RHA, Right Hepatic Artery; RHV, Right Hepatic Vein; RL, Right Lobe; RPV, Right Branch of Portal Vein; T0, Start of Perfusion; T6, End of Perfusion after 6 h; VC, Vena Cava.

MATERIALS AND METHODS

Study Design

This study was designed to investigate and compare the performance of right and left lobes from the same human liver. Each lobe has their own inflow and outflow vessels as well as bile drainage, during *ex situ* end-ischaemic NMLP on separate perfusion devices, thereby demonstrating their suitable utilization as comparative controls in the pre-clinical setting. The primary endpoints were assessment of liver function and evaluation of perfusion parameters. The perfusion machines used for this study both were Liver Assist devices (Organ Assist, Groningen, The Netherlands).

Donor Liver Source and Selection

Four donor livers were included in this study, resulting in a total of eight perfusions. All donor livers included in this study were originally retrieved with the primary intention for transplantation as per the policy of the National Health Service Blood and Transplant (NHSBT). The organs were subsequently rejected for transplantation by all UK liver transplant centers and, following that, were offered nationally for research by NHSBT. The authors had no influence in the process of declining donor livers. This was done by the transplant surgeons at each center. Informed consent for research use of donor organs was obtained by specialist nurses in organ donation from the donor's next of kin during informed consent for organ donation. Authorisation for research was mediated by each centre's specialist nurse in organ donation. All methods described were performed in accordance with NHSBT guidelines and regulations. Study ethical approval was obtained from the London-Surrey Borders National Research Ethics Service (Reference Number 13/LO/1926) and the NHSBT ethics committee (Reference Number 06/Q702/61). No organs used in this study originated from executed prisoners.

Donor liver exclusion criteria for this study were: gross macroscopic appearance indicative of moderate or severe steatosis; asymmetric or poor perfusion demonstrated during organ retrieval; presence of hepatic malignancy; organ subjected to machine perfusion prior to being discarded.

Liver Splitting Protocol

During transportation to our center, the organs were preserved in University of Wisconsin fluid at hypothermic temperatures in ice as per current standard clinical practice for static cold storage in the United Kingdom. Donor livers were split while in cold storage prior to commencement of perfusion in order to ensure that both lobes were subjected to similar ischaemic times and assessed simultaneously when placed in the perfusion devices. Upon receipt by our center, the liver was initially cleared of excess tissue in order to assess the quality of the organ. This also allowed for assessment of the organ's outflow, with respect to the hepatic veins and post-hepatic inferior vena cava, as well as the inflow, in terms of the hepatic arterial tree and portal vein (**Figure 1**). Since the perfusion device allows for open drainage of the perfusate from the organ's outflow via the post-hepatic inferior vena cava, the latter was incised in order to create two separate patches with direct visualization of left, middle and right hepatic veins (**Figure 1**). This was the first step in determining the line of demarcation for parenchymal division. For the purposes of this study, we sought to divide right and left halves of the liver along Cantlie's line—an extrapolated line, used when planning right or left hepatectomies (liver resections), extending from the middle of the post-hepatic inferior vena cava across the diaphragmatic (superior) surface of the liver to the point where the fundus of the gallbladder on the inferior surface typically contacts the antero-inferior margin of the liver (**Figure 2**). This approximates the plane of the middle hepatic vein and demarcates the right and left lobes of the liver.

Once the vena cava was opened, the liver hilum was cleared further of excess tissue in order to identify the left and right branches of the portal vein and hepatic artery, respectively (**Figure 1**). Particular attention was paid to identification of the arterial branch supplying segment 4 of the liver as this would influence the level of division of the branches during splitting. The left and right hepatic ducts were also identified to ensure that each half would have adequate biliary drainage (**Figure 1**). A cholecystectomy was performed, and the cystic duct identified and ligated. Following satisfactory identification of all relevant anatomy, the vessels and bile duct were divided as follows. The hepatic arterial tree was divided such that the main trunk was retained by the left lobe of the liver with the right lobe keeping the right hepatic branch, anatomy-permitting. The right lobe retained the main trunks of the portal vein and bile duct with the left halve keeping its respective branches. At this point, parenchymal division was performed and completed along Cantlie's line (**Figure 2**).

If vessel length and diameter was deemed to be inadequate for safe cannulation of either lobe, particularly with respect to the isolated branches, then a decision was made to reconstruct the vessels to enable proper positioning of the cannulas for perfusion. This was done by fashioning a conduit using native vessels for extra length in one of two ways: (1) resecting the distal portion of the main arterial or venous trunk retained by either lobe, which was then anastomosed onto the branch of the other; (2) utilizing the length of donor iliac vessels for the reconstruction (received with the donor liver for the purposes of arterial or venous reconstruction if required in the setting of transplantation) (**Figure 2**). Which technique was adopted was dependent on the conduit length required as well as the caliber of the vessels to be anastomosed together. The hepatic arteries were cannulated with 10—14 F cannulas. Each portal vein was cannulated with a 24 F cannula.

Ex-situ Perfusion Protocol

Both perfusion devices were primed with our previously described (12) perfusion protocol using Hemopure [HBOC-201, hemoglobin glutamer-250 (bovine); HBOC-201, Hemoglobin Oxygen Therapeutics LLC, Cambridge, MA] instead of packed red blood cells as the oxygen carrier (**Supplementary Material**). The former is a polymerised bovine hemoglobin-based acellular oxygen carrier of low immunogenicity and an oxygen-carrying capacity similar to that of human hemoglobin at normothermic temperatures. Its efficacy as an alternative to blood-based machine perfusion fluid has been demonstrated in pre-clinical and clinical studies (12, 13).

The pre-perfusion weight of each lobe was recorded post-splitting. The arterial and portal venous supplies were cannulated with each lobe positioned inside the organ reservoir such that the open drainage from the hepatic veins could be visualized directly. Perfusion was commenced at 36–37°C with oxygenated pulsatile flow and non-pulsatile flow in the hepatic artery and PV, respectively. Perfusion of each lobe was commenced within five to 15 min of each other (**Figure 3**). Perfusion pressures and flow parameters were monitored continuously. An epoprostenol infusion pump was connected to each perfusion circuit and commenced at an initial rate of 4 ml/h. The rate of prostaglandin infusion was adjusted according to the flow readings to maintain physiological parameters. Hepatic artery pressures were maintained at 60–70 mm Hg while portal vein pressures were maintained at 10 mmHg. Oxygen supply was adjusted in order to maintain a perfusate oxygen partial pressure >10 kPa in the arterial circuit. Serial perfusate samples were analyzed in real-time using the Cobas b 221 point of care system (Roche Diagnostics, USA) Blood Gas Analyser in order to monitor metabolic parameters including oxygen partial pressures, lactate and glucose levels. These parameters have been previously described as appropriate methods of monitoring liver function and viability during the perfusion process (5). Each lobe underwent NMLP for a total of 6 h. For histological analysis, core needle biopsies from each lobe were obtained at the beginning and end of perfusion, fixed in formalin and embedded in paraffin. These biopsies were subsequently stained with haematoxylin and eosin for conventional examination and periodic acid schiff (PAS) for glycogen content and distribution.

Statistical Analysis

Data analysis was carried out using Prism 7 (GraphPad Inc., CA). Continuous data at each timepoint was compared using Wilcoxon signed-rank test. Statistical significance was set at $p<0.05$. Data is presented as per lobar mass as well as following normalization of pre-perfusion lobar weight per gram of tissue.

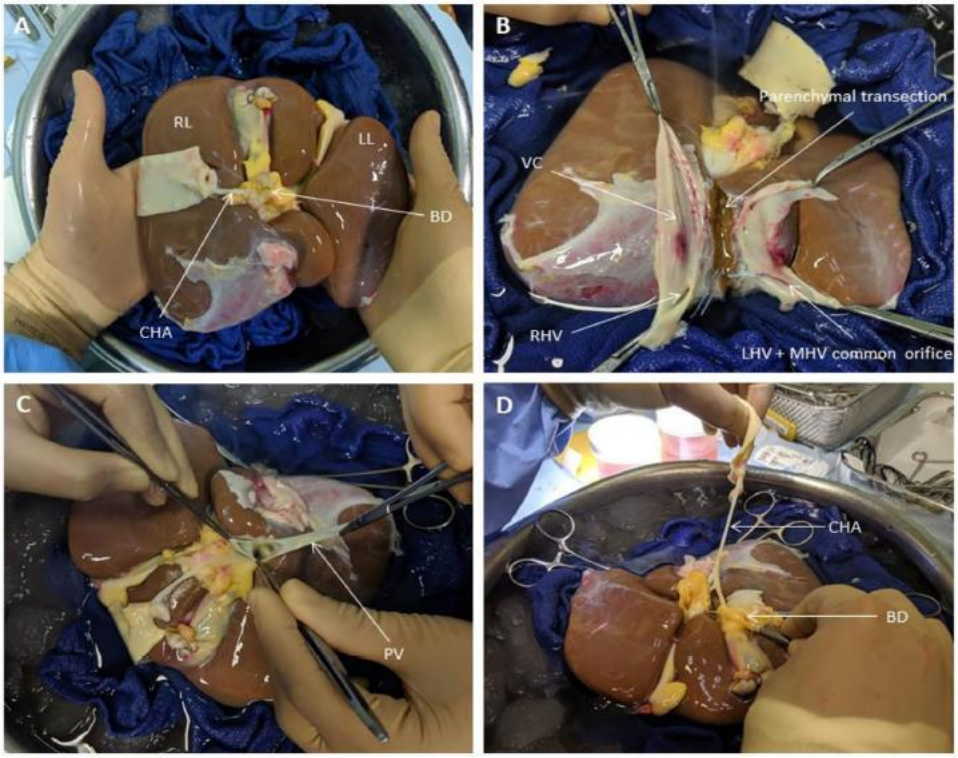

FIGURE 1 | Liver Splitting Procedure: **(A)** Inspection of liver hilum **(B)** Opening inferior vena cava to identify hepatic venous drainage **(C)** Identification of portal vein branches **(D)** Identification of hepatic arterial branches and hepatic duct transection. BD, bile duct; CHA, common hepatic artery; LHV, left hepatic vein; LL, left lobe; MHV, middle hepatic vein; PV, portal vein; RL, right lobe; RHV, right hepatic vein; VC, vena cava.

RESULTS

Donor Liver Characteristics

Four livers were included in the study. Information relating to donor characteristics and liver characteristics can be found in (**Table 1**). Right lobe mass was consistently significantly higher than their left lobe counterparts.

Assessment of Liver Function

Lactate levels were comparable at the start of perfusion, T0, and decreased significantly in all lobes up until the end of the perfusion experiment, T6 (**Figure 4**). Trends in perfusate lactate clearance were similar in both lobes. When normalized for pre-perfusion tissue weight, perfusate lactate levels tended to be higher in the left lobe. However, the rate of reduction in lactate levels was observed to be similar in both lobes across all perfusion experiments. Interestingly, in two of the four livers, an increase in perfusate lactate was observed in both lobes at the same timepoint during the perfusion experiment. Perfusate glucose levels were initially high and trended downwards during the course of the experiment. Lactate and glucose levels were comparable at all timepoints (**Figure 4**).

Perfusion Hemodynamics

Hepatic artery and portal venous flows were relatively consistent in both left and right lobes with a trend toward an increase in flow rate as the perfusion experiment progressed (**Figure 5**). Differences in flow rates for hepatic artery and portal vein were statistically insignificant across all time points.

Liver Histology

Overall histological analysis showed that both liver lobes behaved in a similar manner throughout the perfusions. Lobar architecture was well-preserved in both pre- and post-perfusion

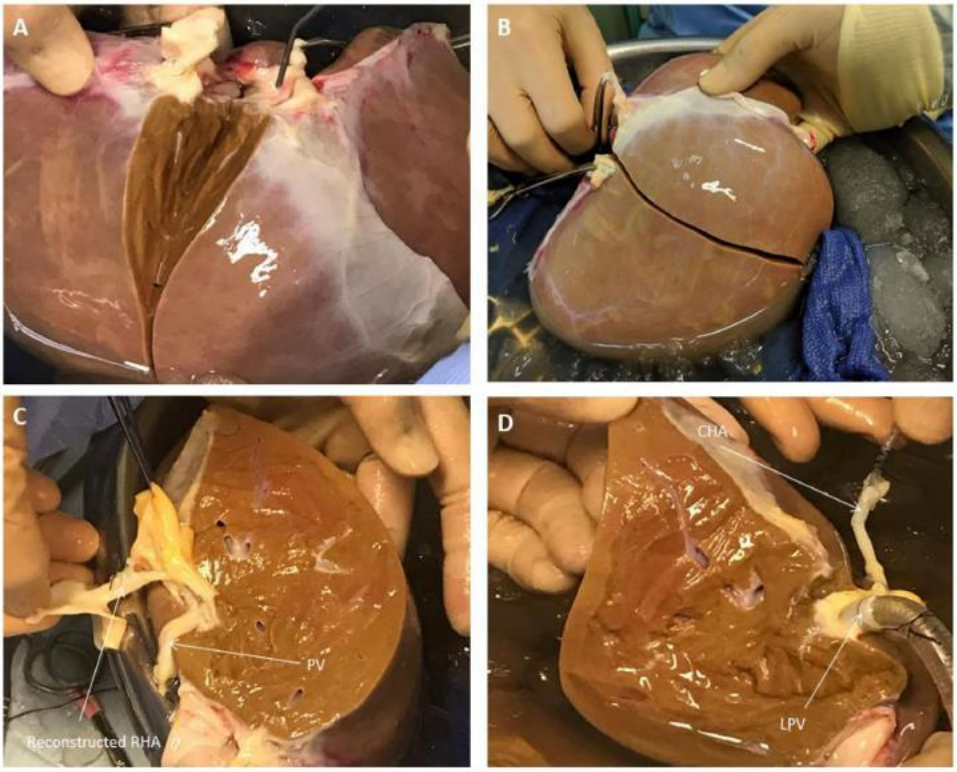

FIGURE 2 | Liver Splitting Procedure: **(A)** Parenchymal transection across gallbladder bed **(B)** Parenchymal transection complete **(C)** Reconstruction of hepatic artery using donor celiac artery trunk **(D)** Cannulation of hepatic artery and portal vein branches. CHA, common hepatic artery; LPV, left portal vein; PV, portal vein; RHA, right hepatic artery.

biopsies. Some variable centrilobular necrosis was observed at both time points. However, this was present to the same degree before and during NMLP. All biopsies were PAS positive with staining showing a range of intensity from mild to strong and ranging from even distribution throughout the parenchyma to patchy. Whatever the variability between liver, each of the paired lobes showed strong similarity, with no change throughout the perfusion. In addition, patterns of histology did not correlate with any parameters of functional assessment. Only one liver showed evidence of significant macrovesicular steatosis which did not change between lobes or throughout the perfusion (**Figure 6**).

DISCUSSION

Several studies have demonstrated that NMLP enables the functional recovery of discarded donor livers and provides a window of opportunity for viability assessment prior to transplantation. However, results are compounded by small sample size as a consequence of the limited availability of discarded donor livers for research, further compounded by the inherent differences between donor livers. This limits comparability between groups of individual livers.

Splitting of the donor liver is a well-known procedure in liver transplantation to optimize the use of grafts (9). Human split liver machine perfusion data in the normothermic setting is limited to case reports (14, 15). With this in mind, we sought to adapt the split liver technique to the normothermic machine perfusion model with the aim of providing a more robust perfusion protocol with each liver providing its own internal control. Our results show that split livers recover functionality and perform similarly to each other when subjected NMLP. The development of a split liver model to *ex situ* end-ischaemic NMLP, therefore, has the potential to provide a platform for suitable comparative controls for the investigation and assessment of therapeutic

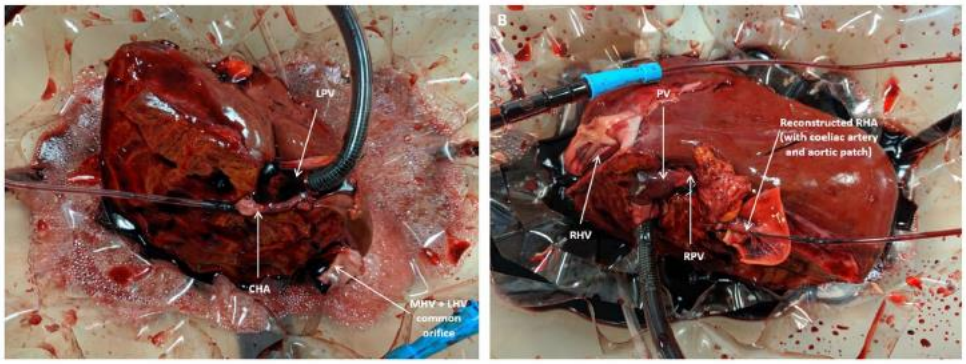

FIGURE 3 | Split lobe perfusion: **(A)** Left lobe **(B)** Right lobe. CHA, common hepatic artery; LPV, left portal vein; MHV, middle hepatic vein; PV, portal vein; RHA, right hepatic artery; RPV, right portal vein.

TABLE 1 | Donor liver demographics and characteristics.

	Liver 1	Liver 2	Liver 3	Liver 4
Donor age (years)	31	75	57	73
Gender	Male	Male	Female	Female
DBD/DCD	DCD	DBD	DCD	DBD
Cold ischaemia time (min)	902	799	1,108	1,026
Weight (g)				
Right lobe	715	1,120	957	739
Left lobe	488	851	851	363

DBD, donor after brainstem death; DCD, donor after cardiac death.

interventions prior to being subjected to the rigor of a clinical trial. Research published previously by our group allowed for an initial four-hour window of NMLP before a full viability assessment of the perfused organ is carried out (11). This period is important in the setting of split liver machine perfusion for two reasons. First and foremost, it will allow for the functional recovery of both lobes following a variable period of cold ischaemia. Secondly, it will enable the investigator to assess and monitor differences in the parameters and performance of the two lobes prior to therapeutic intervention.

The model described in the paper was developed solely for experimental purposes without considerations for clinical adoption. The inflow portal venous anatomy was without variations which allowed us to develop a standardized approached used in all livers. As the outflow from the liver was on free drainage to the reservoir, the site of caval division did not have any impact on function. In view of the open circuit perfusion system, the liver outflow veins (including segmental veins draining into the middle hepatic vein) did not require any reconstruction as the blood was freely drained and collected in the device reservoir. As such, hepatic veins anatomy and inferior vena cava accessories did not influence the surgical technique or the perfusion parameters. The situation would be different if a closed circuit was being used or if clinical use of these segmental grafts was under consideration.

The incorporation of NMLP to the split liver model has the benefit of enabling liver functional assessment under near-physiological conditions. The use of an artificial acellular hemoglobin-based perfusion fluid also has its advantages. Unlike blood products (derived from various donors), it has low immunogenicity and may therefore be of greater value in the study of liver-specific immune cell populations and mechanistic studies (12). Secondly, while the utility of blood products as perfusion fluid is limited to physiological temperatures, Hemopure can be deployed as a perfusion fluid under hypothermic conditions (12). Subsequently, our proposed split liver model can be applied to a range of perfusion temperatures.

It must be noted that there are limitations to the design of this perfusion model. First, splitting of the liver requires considerable surgical expertise and increases the cold ischaemia time beyond what is usual for the preparation of a whole liver for machine perfusion. Secondly, due to the liver's anatomy, the right lobe is invariably larger than the left lobe and therefore they cannot be split into entities of equal mass without compromising blood supply. Furthermore, the shorter and smaller caliber of hepatic artery and portal vein branches makes them much more difficult to cannulate. We attempted to circumvent this issue by retaining the main trunk of the vessel with one lobe while performing vascular reconstructions on the other, using donor iliac vessels or larger caliber sections from the proximal celiac trunk or splenic artery (if included in the specimen). In all experiments this provided the additional length for safe cannulation. However, this technique further increases the cold ischaemia time of the liver and would also require the appropriate surgical expertise for a robust reconstruction that would not compromise lobar perfusion. All these aforementioned factors may lead to differences in the perfusion characteristics of each lobe and must be considered. Finally, not all livers can be split. This is often a result of variations in arterial anatomy due to

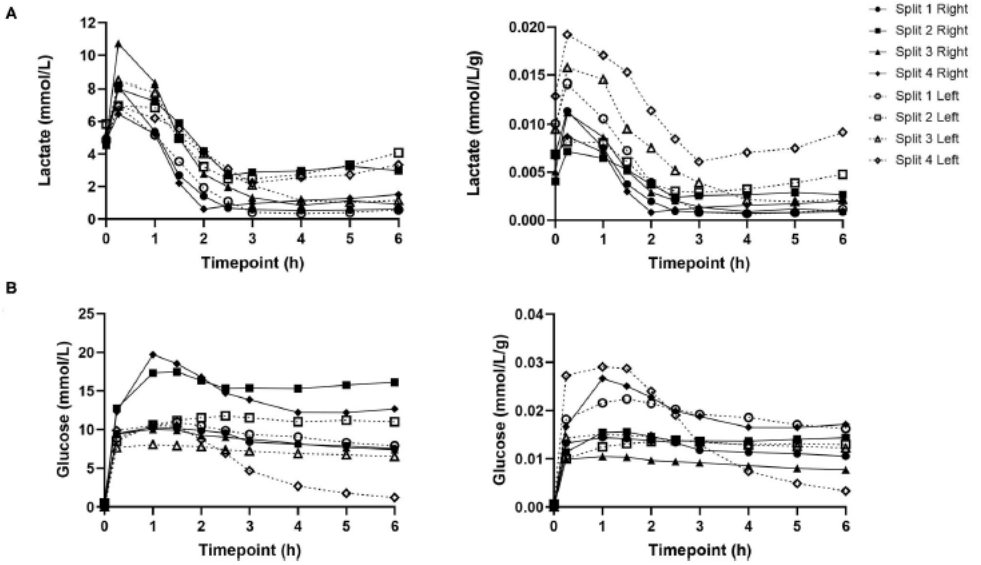

FIGURE 4 | Perfusate lactate **(A)** and glucose **(B)** levels for each individual split lobe over 6 h of end-ischaemic normothermic machine perfusion.

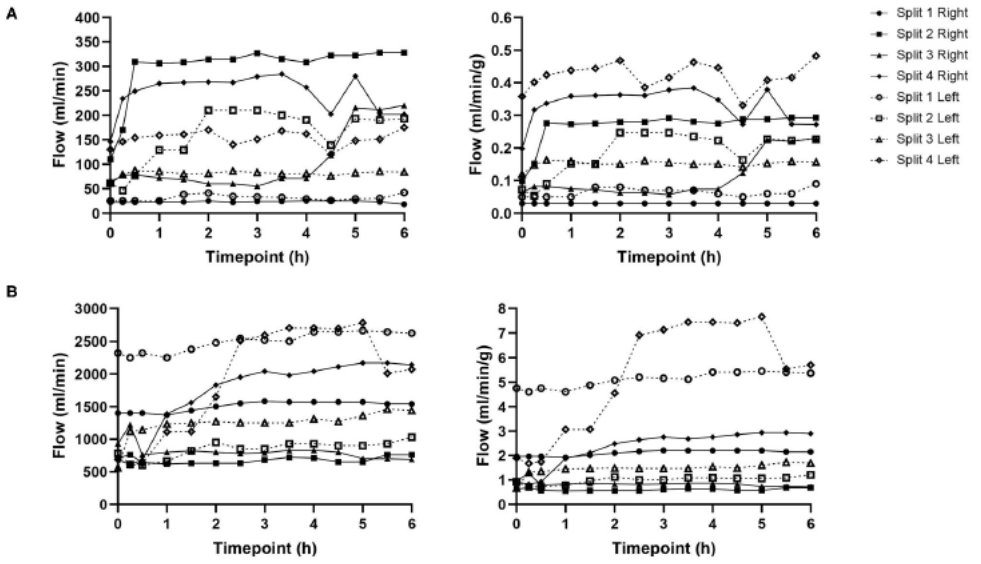

FIGURE 5 | Hepatic artery **(A)** and portal vein **(B)** flows for each individual split lobe over 6 h of end-ischaemic normothermic machine perfusion.

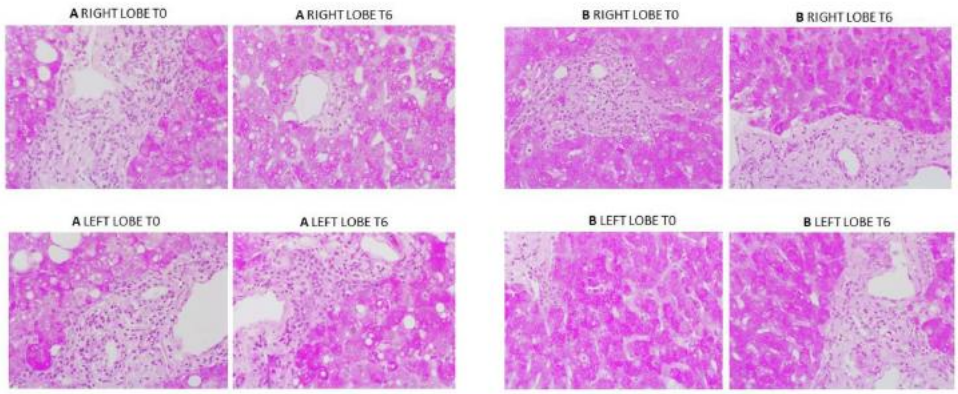

FIGURE 6 | PAS staining from two representative perfusion experiments. **(A)** (from liver case number 2) shows mild to moderate macrovesicular steatosis, portal inflammatory cell infiltration and patchy PAS staining which was the same at commencement and end of perfusion. **(B)** (from liver case number 4) shows a liver with evenly distributed PAS staining throughout. Again, a degree of portal inflammation was seen. **T0**: pre-perfusion, **T6**: end of perfusion.

branching patterns which would compromise the inflow of a segment of the lobe if divided.

We acknowledge that the data presented in this manuscript is from a small number of human donor livers. Nevertheless, the results of this proof-of-concept study indicate that trends in the functional recovery and metabolic parameters between lobes were very similar and may therefore prove useful in the assessment of responses to therapeutic interventions in the pre-clinical normothermic setting. Additionally, this model addresses the variability that exists between individual donor livers by enabling the same donor liver to be placed in the treatment and control groups. This also maximizes use of resources as one donor liver for pre-clinical studies.

Other benefits observed during these experiments came from the use of a perfusion machine with an open circuit. These included: facilitated manipulation of the graft for direct visualization, direct visualization of and access to graft outflow and superior access to graft for direct therapeutic interventions when compared to closed circuit alternatives.

CONCLUSION

Liver splitting is a feasible model for providing comparative controls for pre-clinical normothermic machine perfusion research. Further work should involve the optimisation of this protocol to minimize the need for surgical expertise when preparing the liver for machine perfusion as well as its application to other perfusion modalities, namely, hypothermic and sub-normothermic perfusion. This novel split liver model can be tested for cellular therapies to investigated cellular phenotype and lineage changes and future pharmacological interventions of donor liver before implantation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the London-Surrey Borders National Research Ethics Service (Reference Number 13/LO/1926) and the NHSBT Ethics Committee (Reference Number 06/Q702/61). Informed consent for research use of donor organs was obtained by specialist nurses in organ donation from the donor's next of kin during informed consent for organ donation.

AUTHOR CONTRIBUTIONS

JA, YB, LW, and SA designed the study and established the methodology. JA, LW, DO-B, and VR conducted the experiments. JA and LW collected the data. DM and HM supervised the liver splitting procedure. JA performed the data analysis and drafted the manuscript. SA and GR reviewed the histological specimens. YO, MP, HM, and DM contributed to critical revision and editing of the final manuscript. All authors approved the final version of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsurg.2021.627332/full#supplementary-material

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Conflict of Interest: JA is a clinical research fellow at the Queen Elizabeth Hospital in Birmingham and employed by University Hospitals Birmingham.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix 4: Results (Chapter 4)

Table 14. Lipid relation significant results from one-way measures ANOVA (q<1x10-10) after correction for multiple testing. Results to determine metabolite features whose abundance changed at the three sampling points (t0.25, t2 and t4). The mean fold change comparing 0.25 to 2, 0.25 to 4 and 2 to 4 hours. The mean fold change when comparing 0.25 to 2, 0.25 to 4 and 2 to 4 hours were calculated by dividing the normalised peak area for each time point for each liver separately and then calculating the mean fold change for all livers combined with the 95% confidence interval.

Adjusted P-value	**Fold change**						**Metabolite annotation**	**Metabolite/lipid class**
	0.25hours/2 hours		**0.25 hours/4 hours**		**2 hours/4 hours**			
	mean	**CI (+/- 95%)**	**mean**	**CI (+/- 95%)**	**mean**	**CI (+/- 95%)**		
1.08E-10	0.69	0.10	0.67	0.13	0.95	0.08	Acetylcarnitine	Acyl carnitine
5.31E-14	1.69	0.32	3.72	1.33	1.98	0.29	Hexanoylcarnitine	
2.94E-13	0.60	0.13	0.52	0.14	0.84	0.04	Benzoylcarnitine	
2.56E-14	0.69	0.09	0.61	0.09	0.88	0.04	Hydroxyhexanoycarnitine	
2.15E-11	0.62	0.11	0.63	0.12	0.99	0.04	Octenoylcarnitine	
3.38E-17	0.40	0.11	0.31	0.12	0.72	0.05	decatrienoylcarnitine	
2.65E-13	0.62	0.13	0.49	0.12	0.76	0.05	O-sebacoylcarnitine	
1.88E-17	0.49	0.10	0.41	0.09	0.84	0.06	3-hydroxy-cis-5-octenoylcarnitine	
7.32E-13	0.64	0.08	0.63	0.08	0.99	0.07	Tiglylcarnitine	
2.89E-14	0.44	0.08	0.46	0.10	1.03	0.09	Octanoylcarnitine	
6.27E-20	2.22	0.40	2.73	0.48	1.24	0.09	Butenylcarnitine	
8.71E-12	1.71	0.32	2.74	0.58	1.57	0.13	hydroxydecanoyl carnitine	
3.91E-28	3.71	0.53	5.47	1.08	1.47	0.13	Octadecadienoyl carnitine	
2.91E-24	1.66	0.16	2.94	0.34	1.77	0.14	Carnitine	

1.57E-11	1.52	0.23	2.34	0.47	1.56	0.20	Decenoylcarnitine	
8.94E-15	2.93	1.19	9.96	4.82	3.10	0.64	Fumarycarnitine	
9.57E-25	0.68	0.04	0.64	0.04	0.94	0.03	Cer(d40:1(OH))	Ceramide
4.13E-28	0.60	0.04	0.52	0.04	0.88	0.03	Cer(d38:0(OH))	
1.02E-10	0.82	0.06	0.77	0.06	0.93	0.03	Cer(d43:2)	
1.40E-17	0.91	0.04	0.89	0.06	0.98	0.04	Cer(d42:2(OH))	
3.01E-24	0.63	0.04	0.53	0.05	0.84	0.04	Cer(d42:2)	
4.13E-28	0.45	0.04	0.38	0.05	0.84	0.04	Cer(d38:1(OH))	
4.13E-28	0.55	0.04	0.44	0.04	0.79	0.04	Cer(d40:1)	
1.41E-24	0.63	0.04	0.52	0.05	0.83	0.04	Cer(d42:3)	
4.17E-27	0.32	0.05	0.25	0.05	0.78	0.04	Cer(t35:1(2OH))	
4.26E-30	0.57	0.04	0.45	0.04	0.80	0.04	Cer(d41:2)	
3.04E-27	0.54	0.04	0.47	0.05	0.86	0.04	Cer(d39:1(OH))	
1.35E-26	0.65	0.04	0.54	0.04	0.83	0.04	Cer(t39:0)||Cer(d41:1)	
6.80E-19	0.86	0.04	0.78	0.06	0.90	0.04	Cer(t41:1(2OH))	
1.70E-25	0.68	0.04	0.57	0.05	0.84	0.04	Cer(d41:1)	
4.22E-32	0.39	0.04	0.29	0.03	0.74	0.04	Cer(d38:1)	
1.34E-19	0.87	0.04	0.81	0.06	0.93	0.04	Cer(d42:1(OH))	
4.22E-32	0.45	0.04	0.34	0.03	0.76	0.04	Cer(d39:1)	
5.43E-31	0.49	0.04	0.39	0.04	0.79	0.05	Cer(d40:2)	
4.45E-19	0.86	0.04	0.80	0.07	0.93	0.05	Cer(d40:0(OH))	
6.93E-32	0.33	0.04	0.25	0.04	0.73	0.05	Cer(d34:1)	
4.22E-32	0.33	0.04	0.25	0.04	0.74	0.05	Cer(d36:1)	
9.83E-23	0.65	0.05	0.53	0.06	0.82	0.05	Cer(t42:1(OH))	
6.46E-28	0.39	0.05	0.37	0.06	0.92	0.05	Cer(t32:0)	
4.11E-13	0.94	0.08	0.82	0.09	0.87	0.06	Cer(t37:1(2OH))	
4.13E-28	0.49	0.04	0.37	0.04	0.75	0.06	Cer(t41:1(2OH))||Cer(d39:1)	
5.73E-13	0.93	0.07	0.91	0.11	0.96	0.06	Cer(d43:1)	
5.27E-10	0.84	0.07	0.70	0.09	0.82	0.06	Ganglioside GM3 (d34:1)|	
7.49E-16	0.79	0.06	0.74	0.09	0.92	0.07	Cer(d44:2)	

7.04E-13	0.61	0.06	0.54	0.09	0.86	0.08	GlcCer(t34:1(2OH))	
2.81E-10	1.49	0.20	2.20	0.80	1.34	0.19	Cer(d47:2)	
3.97E-20	0.38	0.11	0.19	0.06	0.54	0.08	LysoPC(20:4)	Lysoglycerophospholipid
4.55E-23	0.37	0.07	0.24	0.06	0.67	0.09	LysoPE(20:4)	
7.35E-13	0.68	0.12	0.49	0.08	0.77	0.09	LysoPE(18:1)‖hydroxyoctadecenoylcarnitine	
1.73E-21	0.40	0.08	0.28	0.06	0.74	0.10	LysoPE(18:2)‖hydroxylinoleoylcarnitine‖Ubiquinone-4	
5.78E-16	0.66	0.14	0.46	0.09	0.77	0.10	LysoPE(18:1)‖LysoPC(15:1)	
2.72E-22	0.36	0.08	0.25	0.07	0.76	0.10	LysoPE(22:6)	
4.29E-13	0.61	0.18	0.40	0.13	0.67	0.10	LysoPC(18:2)‖LysoPE(20:2)	
3.62E-10	0.71	0.12	0.53	0.12	0.77	0.11	LysoPC(20:5)	
3.09E-21	0.37	0.09	0.25	0.06	0.73	0.11	LysoPE(18:2)	
1.03E-16	0.44	0.16	0.25	0.10	0.59	0.14	LysoPC(22:6)‖LysoPC(20:3)	
2.10E-10	0.86	0.19	0.70	0.17	0.86	0.14	LysoPE(20:2)‖LysoPC(18:2)	
1.47E-10	0.73	0.23	0.43	0.13	0.65	0.18	LysoPC(20:3)	
5.86E-20	0.74	0.04	0.66	0.05	0.90	0.03	PC(34:2)‖PE(37:2)‖PA(39:3)	Glycerophospholipid
3.65E-19	0.75	0.04	0.67	0.05	0.88	0.03	PC(34:1)‖PE(37:1)	
9.10E-18	0.86	0.04	0.71	0.05	0.82	0.03	PC(40:6)‖PE(43:6)	
4.16E-22	0.77	0.04	0.63	0.05	0.81	0.03	PC(36:2)‖PE(39:2)	
1.63E-11	0.96	0.06	0.99	0.08	1.02	0.03	PS(41:4)	
1.64E-17	0.57	0.06	0.53	0.06	0.92	0.04	PA(38:1)‖PC(34:1)‖PE(37:1)	
2.57E-14	0.87	0.07	0.77	0.10	0.88	0.04	PS(39:1)	
2.01E-24	0.55	0.05	0.45	0.06	0.79	0.04	PC(30:0)‖PE(33:0)‖PA(35:1)	
1.07E-16	0.76	0.06	0.62	0.06	0.81	0.04	PS(O-39:1)‖PS(P-39:0)‖PG(O-38:2)‖PG(P-38:1)‖PC(40:6)	
1.88E-16	0.75	0.07	0.64	0.10	0.84	0.04	PC(34:0)‖PE(37:0)	
2.79E-15	0.80	0.08	0.69	0.10	0.84	0.04	PI(35:0)‖PS(42:8)	
4.85E-20	0.79	0.04	0.63	0.05	0.79	0.04	PA(40:2)‖PC(35:1)‖PE(38:1)	
3.96E-12	0.88	0.05	0.78	0.05	0.89	0.04	PA(40:0)‖PC(34:0)‖PE(39:3)	
7.24E-16	0.74	0.09	0.64	0.11	0.85	0.04	PC(34:2)‖PE(37:2)	
5.46E-15	0.84	0.07	0.74	0.09	0.86	0.04	PC(38:3)‖PE(41:3)‖PS(44:8)	

2.69E-18	0.65	0.06	0.56	0.07	0.84	0.04	PS(O-37:1)||PS(P-37:0)||PG(O-36:2)||PG(P-36:1)||PC(38:6)	
1.09E-23	0.61	0.05	0.47	0.06	0.75	0.04	PC(33:1)||PE(36:1)||PA(38:2)	
2.90E-11	0.93	0.08	0.92	0.09	1.00	0.04	PS(39:2)	
2.06E-15	0.80	0.07	0.69	0.10	0.85	0.04	PS(41:5)	
1.49E-15	0.83	0.07	0.69	0.09	0.82	0.04	PI(41:3)	
2.32E-14	0.77	0.10	0.67	0.12	0.85	0.04	PC(36:4)||PE(39:4)||PS(39:3)	
4.36E-11	0.92	0.07	1.02	0.08	1.10	0.04	PS(37:1)	
1.21E-16	0.65	0.06	0.57	0.08	0.87	0.04	PG(O-36:2)||PG(P-36:1)	
1.83E-13	0.72	0.07	0.63	0.08	0.85	0.04	PG(O-38:2)||PG(P-38:1)||PI(39:3)	
1.39E-13	0.67	0.08	0.64	0.09	0.94	0.04	PI(38:4)	
8.79E-16	0.77	0.08	0.63	0.11	0.80	0.04	PC(38:6)||PE(41:6)||PG(39:5)	
6.89E-13	0.84	0.08	0.76	0.11	0.88	0.05	PS(39:2)	
1.38E-14	0.86	0.06	0.75	0.09	0.85	0.05	PS(41:3)	
3.16E-17	0.60	0.07	0.54	0.07	0.88	0.05	PI(38:6)||PS(42:8)	
3.44E-15	0.63	0.08	0.59	0.08	0.92	0.05	PE(44:12)||PI(38:7)||PE(42:9)	
1.16E-11	0.91	0.06	1.00	0.09	1.10	0.05	PS(39:3)	
4.86E-12	0.89	0.08	0.97	0.09	1.08	0.05	PS(43:6)||PS(O-38:3)||PS(P-38:2)	
4.18E-17	0.57	0.10	0.46	0.13	0.76	0.05	PS(35:0)||PC(31:1)||PE(34:1)	
1.36E-22	0.56	0.06	0.43	0.08	0.75	0.05	PC(32:2)||PE(35:2)||PA(37:3)	
1.47E-17	0.48	0.08	0.35	0.09	0.70	0.05	PA(38:3)||PC(33:2)||PE(36:2)	
2.94E-16	0.65	0.09	0.61	0.12	0.92	0.05	PI(36:4)	
1.37E-10	1.01	0.07	0.94	0.09	0.92	0.05	PE(P-40:7)	
4.57E-17	0.55	0.11	0.45	0.13	0.76	0.05	PC(31:1)||PE(34:1)	
3.30E-16	0.64	0.10	0.60	0.12	0.92	0.05	PC(37:6)||PE(40:6)	
1.97E-15	0.69	0.09	0.68	0.12	0.95	0.05	PC(39:6)||PE(42:6)||PI(38:4)	
5.25E-22	0.54	0.07	0.42	0.08	0.74	0.05	PC(30:1)||PE(33:1)	
1.24E-14	0.71	0.10	0.71	0.13	0.97	0.05	PI(34:2)	
7.24E-16	0.69	0.10	0.55	0.13	0.76	0.05	PS(41:7)	
3.29E-12	0.90	0.06	0.99	0.09	1.10	0.05	PS(37:0)	

7.28E-11	1.31	0.11	1.38	0.14	1.05	0.05	PC(P-32:0)||PE(O-35:1)||PA(O-37:2)	
1.44E-14	0.63	0.07	0.57	0.08	0.89	0.05	PI(40:6)||PS(44:8)	
3.50E-13	0.84	0.05	0.74	0.05	0.89	0.05	PC(36:3)||PE(39:3)||PA(40:0)	
3.30E-20	1.49	0.09	1.60	0.12	1.07	0.05	PC(P-36:3)||PC(O-36:4)	
1.15E-13	0.68	0.08	0.54	0.09	0.77	0.05	PC(35:3)||PE(38:3)	
2.20E-11	1.32	0.10	1.40	0.13	1.06	0.05	PA(O-37:2)||PA(P-37:1)||PC(O-32:1)||PC(P-32:0)||PE(O-35:1(9Z))||PE(P-35:0)	
5.65E-15	0.76	0.09	0.62	0.14	0.79	0.05	PS(38:1)||PC(34:2)||PE(37:2)	
8.27E-11	0.75	0.09	0.63	0.10	0.81	0.05	PC(36:1)||PE(39:1)||PA(40:1)	
2.04E-16	0.64	0.10	0.51	0.13	0.75	0.05	PS(39:4)||PC(35:5)||PE(38:5)	
9.03E-16	0.65	0.09	0.66	0.12	0.98	0.05	PI(40:6)	
1.23E-20	0.43	0.06	0.31	0.08	0.68	0.06	PC(32:1)||PE(35:1)||PS(35:0)	
4.06E-15	0.71	0.09	0.71	0.12	0.97	0.06	PI(38:4)	
4.85E-17	0.60	0.08	0.43	0.09	0.70	0.06	PC(35:4)||PE(38:4)||PG(37:3)	
4.74E-11	1.35	0.08	1.32	0.12	0.98	0.06	PC(P-36:4)	
5.44E-12	0.65	0.08	0.51	0.10	0.77	0.06	PG(O-34:0)||PC(36:4)||PE(39:4)	
2.60E-21	0.60	0.06	0.43	0.08	0.69	0.06	PS(O-35:0)||PG(O-34:1)||PC(36:5)||PE(39:5)	
2.95E-20	0.56	0.07	0.39	0.09	0.66	0.06	PG(42:8)||PS(41:7)	
4.77E-11	1.22	0.07	1.42	0.12	1.15	0.06	PI(44:1)	
8.52E-15	0.72	0.07	0.58	0.09	0.77	0.06	PC(28:0)||PE(31:0)||PA(33:1)	
8.40E-12	0.56	0.09	0.54	0.11	0.92	0.06	PE(42:2)	
2.06E-20	0.37	0.07	0.26	0.08	0.65	0.06	PC(31:0)||PE(34:0)||PA(36:1)	
4.62E-17	0.50	0.08	0.35	0.10	0.66	0.06	PA(42:1)	
5.39E-17	0.50	0.08	0.35	0.10	0.66	0.06	PC(35:2)||PE(38:2)||PA(42:6)	
8.67E-15	0.75	0.09	0.78	0.13	1.00	0.06	PI(36:1)	
3.61E-13	0.83	0.08	0.74	0.12	0.87	0.06	PC(36:3)||PE(39:3)||PG(37:2)	
2.36E-17	0.56	0.08	0.40	0.09	0.68	0.06	PC(36:5)||PE(39:5)||PS(39:4)	
6.51E-13	0.68	0.07	0.62	0.09	0.88	0.06	PA(P-42:2)||PI(34:2)	
1.31E-16	0.55	0.12	0.43	0.15	0.72	0.06	PS(35:1)||PC(31:2)||PE(34:2)	
2.95E-20	0.58	0.06	0.41	0.09	0.67	0.06	PG(O-34:1)||PG(P-34:0)||PG(42:8)	

7.27E-10	0.90	0.09	0.98	0.14	1.06	0.06	PI(34:1)	
1.39E-19	0.55	0.07	0.42	0.08	0.75	0.06	PA(39:4)||PC(34:3)||PE(37:3)	
7.61E-13	0.79	0.09	0.85	0.14	1.04	0.06	PI(36:2)	
1.51E-10	0.51	0.06	0.45	0.07	0.89	0.06	PC(39:6)||PE(42:6)|||PI(38:3)	
3.62E-13	0.78	0.09	0.84	0.13	1.05	0.07	PC(37:4)||PE(40:4)||PI(36:2)	
1.93E-19	0.40	0.08	0.28	0.11	0.64	0.07	PA(38:0)||PC(34:3)||PE(37:3)	
1.31E-14	0.64	0.08	0.56	0.10	0.84	0.07	PG(O-34:3)||PG(P-34:2)||PS(O-34:1)||PS(P-34:0)	
1.57E-11	0.50	0.06	0.45	0.07	0.89	0.07	PE(44:9)||PI(38:4)||PC(39:6)|PE(42:6)	
2.08E-29	1.88	0.15	2.52	0.24	1.34	0.07	PC(P-34:2)||PC(O-34:3)||PE(O-35:0)	
1.74E-14	0.70	0.35	0.44	0.16	0.69	0.07	PS(37:2)||PC(33:3)||PE(36:3)	
8.14E-19	1.55	0.11	1.76	0.14	1.15	0.09	PC(P-36:0)||PC(O-36:1)	
7.98E-10	1.58	0.22	2.01	0.34	1.25	0.10	PA(O-38:6)||PA(P-38:5)||PE(P-36:4)	
1.40E-15	1.55	0.16	2.18	0.30	1.37	0.10	PA(O-38:4)||PA(P-38:3)||PE(P-36:2)||PE(O-36:3)	
7.62E-15	0.78	0.09	0.55	0.13	0.70	0.10	PC(36:0)||PE(39:0)	
9.83E-10	1.64	0.08	2.32	0.20	1.42	0.10	PS(44:9)	
3.79E-12	1.59	0.20	2.34	0.39	1.42	0.10	PA(O-38:3)||PA(P-38:2)||PC(P-33:1)||PE(P-36:1)||PE(O-36:2)	
5.59E-10	0.74	0.09	0.77	0.16	0.97	0.11	PC(31:2)||PE(34:2)||PA(34:1)	
9.50E-13	1.18	0.11	1.75	0.22	1.47	0.12	PG(O-34:0)||PS(41:6)||PG(42:7)	
4.20E-14	1.16	0.10	1.83	0.25	1.55	0.12	PG(42:7)||PS(41:6)	
1.30E-14	1.23	0.09	1.85	0.26	1.47	0.13	PI(37:2)	
9.11E-18	1.70	0.18	2.68	0.34	1.60	0.17	PG(O-36:0)||PS(43:6)	
9.27E-16	1.23	0.09	2.22	0.37	1.74	0.18	PS(39:4)||PG(40:5)	
1.11E-10	1.20	0.09	1.72	0.23	1.46	0.18	PG(O-36:1)||PG(P-36:0)||PC(38:5)||PE(41:5)	
3.61E-10	1.61	0.30	2.67	1.30	1.42	0.21	PC(P-38:0)||PC(O-38:1)||PE(P-41:0)||PA(O-41:0)	

Appendix 5: Results (Chapter 4)

Table 15. Lipid-related metabolite features which are statistically significant (q<0.05) when comparing livers which (1) did and (2) did not meet the viability criteria for perfusate samples collected at 0.25 hours

Adjusted P-value	Fold Change (Criteria not met/criteria met)	Metabolite annotation	Metabolite/lipid class
0.047577	3.14	Tetradecenoylcarnitine	Acyl carnitine
0.04339	3.79	O-(17-carboxyheptadecanoyl)carnitine	
0.019383	4.21	3-hydroxydecanoyl carnitine||6-Keto-decanoylcarnitine	
0.029306	4.83	Hexadecanedioic acid mono-L-carnitine ester	
0.016029	5.22	Tetradecanoylcarnitine	
0.026289	5.39	hydroxydodecenoylcarnitine	
0.020548	5.96	hydroxyoctadecenoylcarnitine	
0.0072239	6.23	Octadecadienoyl carnitine	
0.0028911	7.21	Hexadecenoylcarnitine	
0.011537	7.31	Palmitoylcarnitine	
0.0029435	9.64	Octadecenylcarnitine	
0.0045031	10.19	13-carboxytridec-5-enoylcarnitine	
0.016816	13.22	2-Methylbutyroylcarnitine||Isovalerylcarnitine||Pivaloylcarnitine||Valerylcarnitine	
0.020965	1.53	Cer(d47:2)	Ceramides
0.019746	0.11	GlcCer(d34:1)	
0.041906	4.52	Cer(d36:2)	
0.042968	4.25	Cer(d38:2)	
0.025207	6.00	GlcCer(d36:2)	
0.039208	8.43	Cer(m42:2)||Cer(d43:2(2OH))	
0.017991	0.25	Cer(d52:2(34OH))	
0.043913	1.80	Cer(d48:0(30OH))	

0.04226	3.26	Cer(d40:2)	
0.020512	3.28	Cer(d36:1)	
0.045424	3.33	Cer(d36:0)	
0.011905	4.63	Cer(m36:1)	
0.0052454	5.51	Cer(m42:1)	
0.043544	5.83	Cer(d42:2)	
0.022591	1.56	PC(P-38:0)||PC(O-38:1)||PE(P-41:0)||PA(O-41:0)	Glycerophospholipid
0.019789	1.77	PC(P-38:0)||PC(O-38:1)||PC(44:8)	
0.044241	2.05	PIM1(32:1)	
0.026579	2.08	PC(P-38:2)||PC(O-38:3)||DG(46:3)	
0.019789	4.45	PI(36:3)	
0.0093895	5.82	PC(39:7)||PE(42:7)	
0.018175	6.64	PI(32:1)	
0.00016688	9.11	PC(37:6)||PE(40:6)	
0.027853	0.21	PA(21:0)	
0.047297	1.72	PC(P-38:3)||PC(O-38:4)	
0.040656	2.15	PI(36:4)||PS(40:7)	
0.047555	2.20	PG(35:2)||PS(34:1)	
0.021856	2.31	PE(44:9)||PI(38:4)	
0.040656	2.40	PI(38:7)	
0.047297	2.70	PC(P-40:3)||PC(O-40:4)||PI(36:3)	
0.021856	2.73	PS(36:2)||PG(35:0)	
0.018181	2.74	PE(38:6)||PC(35:6)	
0.045963	2.85	PA(37:2)||PC(31:2)||PE(34:2)	
0.011537	3.28	PA(41:4)||PC(35:4)||PE(38:4)	
0.028076	3.45	PI(41:3)	
0.017155	3.67	PG(41:2)||PC(40:4)	
0.0094687	4.21	PG(38:8)||PS(38:6)	
0.024308	4.40	PI(36:4)	
0.013274	4.58	PA(46:6)	

0.0085959	5.14	PG(34:4)||PS(34:2)	
0.0019342	20.11	PS(36:1)||PG(36:3)	
0.039208	2.46	PC(39:5)||PE(42:5)||PI(38:3)	
0.043056	2.48	PC(31:1)||PE(34:1)||PA(34:0)	
0.029424	2.65	PC(39:6)||PE(42:6)||PI(38:4)	
0.032023	2.69	PC(39:6)||PE(42:6)	
0.042267	2.91	PC(33:1)||PE(36:1)||PA(36:0)	
0.044517	2.99	PC(32:5)||PE(35:5)||PG(34:2)	
0.020042	3.32	PC(33:4)||PE(36:4)||PA(36:3)	
0.015827	3.43	PE(40:9)||PS(40:8)||PG(38:8)	
0.036575	3.70	PC(37:4)||PE(40:4)||PI(36:2)	
0.029052	3.90	PC(31:2)||PE(34:2)||PA(34:1)	
0.04289	4.06	PC(37:7)||PE(40:7)	
0.022689	4.16	PS(36:4)||PC(32:3)||PE(35:3)||PG(34:2)	
0.017322	4.35	PI(34:2)	
0.0048743	4.71	PI(34:3)	
0.020042	4.89	PS(38:3)	
0.015827	4.91	PI(34:1)	
0.012491	5.19	PE(40:9)||PC(35:6)	
0.020042	5.49	PC(37:3)||PE(40:3)||PI(36:1)	
0.030078	5.57	PC(29:1)||PE(32:1)||PA(32:0)	
0.029167	5.88	PC(31:3)||PE(34:3)||PA(34:2)	
0.043544	0.52	PI(33:2)||PS(40:5)	
0.026808	0.64	PS(40:5)||PI(33:2)	
0.022944	1.70	PE(44:9)||PI(40:7)	
0.024119	1.91	PC(35:4)||PE(38:4)||PA(41:4)	
0.029925	2.06	PG(43:1)	
0.013013	2.22	PC(44:9)	
0.031464	2.24	PI(38:6)||PS(42:8)	
0.011905	2.25	PI(38:4)	

0.010461	2.48	PE(40:9)||PA(42:10)	
0.011084	2.55	PC(33:4)||PE(36:4)	
0.014278	2.63	PC(35:4)||PE(38:4)|PA(39:1)	
0.014797	2.68	PS(40:6)	
0.015479	2.71	PA(36:3)||PC(31:2)||PE(34:2)	
0.018937	2.79	PI(36:2)	
0.02361	2.88	PE(39:7)	
0.015703	2.92	PG(O-35:2)||PG(P-35:1)||PC(37:6)||PE(40:6)||PA(44:10)	
0.043101	3.00	PA(43:6)||PC(37:6)||PE(40:6)||PS(O-36:1(9Z))||PS(P-36:0)	
0.013913	3.05	PI(40:6)||PS(44:8)	
0.010809	3.16	PG(39:4)||PS(40:6)	
0.02435	3.29	PE(42:10)||PC(37:7)||PI(34:2)	
0.031464	3.37	PI(35:3)	
0.022577	3.45	PI(36:5)	
0.02195	3.48	PC(37:7)||PE(40:7)||PI(34:2)	
0.015479	3.95	PS(41:4)||PC(42:9)	
0.011905	4.10	PS(38:6)||PG(38:8)	
0.01766	5.09	PA(42:8)||PC(37:7)||PE(40:7)||PI(34:2)	
0.011905	5.15	PE(44:12)||PI(38:7)||PS(42:8)	
0.0036469	5.22	PS(38:6)	
0.0025061	5.91	PI(40:6)	
0.032886	5.93	PI(34:4)	
0.0036469	6.80	PI(40:5)||PS(44:7)	
0.018283	7.25	PI(40:4)	
0.003761	7.95	PI(36:1)	

Appendix 6: Results (Chapter 4)

Table 16. Lipid-related metabolites features which are statistically significant (q<0.05) when comparing livers which (1) Did and (2) Did not meet the transplantation criteria for perfusate samples collected at 2 hours.

Adjusted P-value	Fold Change (Criteria not met/criteria met)	Metabolite annotation	Metabolite/lipid class
0.020066	2.86	Propionylcarnitine	Acyl carnitine
0.01144	7.91	Carnitine	
0.0063645	0.05	3-hydroxynonanoyl carnitine	
0.049018	0.25	3-Hydroxyhexanoyl carnitine	
0.00096118	0.30	Butyrylcarnitine||Isobutyrylcarnitine	
0.013472	0.48	Decadienoylcarnitine	
0.012701	0.51	Isovalerylcarnitine||Pivaloylcarnitine||Valerylcarnitine	
0.024302	2.46	hydroxydodecenoylcarnitine	
0.030182	2.75	O-(17-carboxyheptadecanoyl)carnitine	
0.010202	2.84	Tetradecanoylcarnitine	
0.019077	3.10	3-hydroxytridecanoyl carnitine	
0.014427	3.34	O-[(9Z)-17-carboxyheptadec-9-enoyl]carnitine	
0.032205	3.50	Hexadecanedioic acid mono-L-carnitine ester	
0.0023724	4.40	Hexadecenoylcarnitine	
0.0037443	4.79	Octadecadienoyl carnitine	
0.0014723	5.37	Octadecenylcarnitine	
0.025451	6.94	Palmitoylcarnitine	
0.0069688	9.18	2-Methylbutyroylcarnitine||Isovalerylcarnitine||Pivaloylcarnitine||Valerylcarnitine	
0.00078999	17.99	3-hydroxyhexadecadienoylcarnitine	
0.00075442	21.96	hydroxyoctadecenoylcarnitine	
0.026243	0.30	Gamma-linolenyl carnitine	
0.046644	1.56	Cer(d47:2)	Ceramides

0.015261	0.18	PE-Cer(d40:3)	
0.012265	0.30	PE-Cer(d38:2)	
0.0081522	0.32	GlcCer(d34:1)	
0.0031685	0.23	GlcCer(d34:1(2OH))	
0.0031685	4.57	GlcCer(d36:2)	
0.0040389	0.33	Cer(d52:2(34OH))	
0.032385	0.65	PE-Cer(d35:2)	
0.027694	1.80	Cer(d41:2)	
0.018984	1.88	Cer(d48:0(30OH))	
0.0073603	1.89	Cer(d40:2)	
0.0057662	1.98	Cer(d42:3)	
0.01381	2.06	Cer(d40:1)	
0.019362	2.07	Cer(d38:2)	
0.0065816	2.18	Cer(d36:1)	
0.013547	2.48	Cer(m42:1)	
0.033237	2.59	Cer(d36:2)	
0.020441	2.62	Cer(d34:2)	
0.0025842	4.73	Cer(m36:1)	
0.017042	0.32	PC(35:3)||PE(38:3)	Glycerophospholipid
0.012135	0.46	PC(35:2)||PE(38:2)	
0.018056	0.50	PC(34:1)||PE(37:1)	
0.027737	0.52	PC(36:4)||PE(39:4)	
0.021362	0.71	PC(38:4)||PE(41:4)	
0.014245	0.77	PG(25:0)	
0.032898	2.10	PI(36:3)	
0.046749	2.53	PG(P-42:2)	
0.0072307	2.56	PI(40:6)	
0.031935	2.83	PC(P-38:0)||PC(O-38:1)||PC(44:8)	
0.0060163	3.07	PC(39:7)||PE(42:7)	
0.01023	3.29	PI(41:4)||PG(44:7)	

0.026206	0.46	PS(O-36:2)||PS(P-36:1)||PG(O-36:3)||PG(P-36:2)	
0.0013547	2.10	PI(38:4)	
0.044105	2.13	PI(38:7)	
0.027517	2.29	PC(P-38:3)||PC(O-38:4)||PI(36:3)	
0.0041255	2.32	PI(36:4)||PS(40:7)	
0.0020766	2.34	PE(44:10)||PI(36:2)	
0.025819	2.42	PIP2(36:1)	
0.0016293	2.52	PG(41:2)	
0.0027168	2.54	PI(36:4)	
0.0078912	2.63	PI(36:3)||PC(38:1)	
0.00079924	2.64	PE(44:9)||PI(38:4)	
0.0058925	2.67	PG(41:2)||PC(40:4)	
0.012027	2.67	PI(41:3)	
0.0019812	2.80	PI(36:2)||PC(39:7)||PE(40:7)	
0.0014448	3.02	PC(35:4)||PE(38:4)	
0.00079924	3.11	PI(39:2)||PC(42:4)	
0.00075442	3.27	PI(34:2)||PC(37:7()||PE(40:7)	
0.0073986	3.67	PG(35:2)||PS(34:1)	
0.0041173	3.67	PI(34:1)	
0.026758	4.03	PA(12:0)	
0.015268	4.78	PI(36:5)||PG(39:2)||PS(40:7)	
0.0016779	5.67	PG(38:8)||PS(38:6)	
0.0048973	5.85	PS(36:2)||PG(35:0)	
0.0012564	6.92	PG(34:4)||PS(34:2)	
0.028413	0.34	PS(O-35:2)||PS(P-35:1)	
0.0079488	0.35	PG(40:8)	
0.048373	1.96	PC(O-35:2)||PC(P-33:1)||PE(O-36:2)||PE(P-36:1)||PA(O-36:1)||PA(P-36:0)	
0.042276	1.96	PC(P-31:1)||PE(O-34:2)||PE(P-34:1)||PA(O-34:1)||PA(P-34:0)	
0.04912	2.21	PC(O-33:2)||PC(P-33:1)||PE(O-36:2)||PE(P-36:1)||PA(O-36:1)||PA(P-36:0)	
0.01382	2.44	PE(O-38:5)||PE(P-38:4)||PA(O-38:4)||PA(P-38:3)	

0.02694	2.60	PE(O-38:5)||PE(P-38:4)	
0.012486	2.81	PC(31:1)||PE(34:1)||PA(34:0)	
0.0072117	2.85	PC(39:6)||PE(42:6)	
0.0056571	2.85	PC(39:6)||PE(42:6)||PI(38:4)	
0.019264	2.94	PC(39:5)||PE(42:5)||PI(38:3)	
0.0053586	3.05	PC(33:4)||PE(36:4)||PA(36:3)	
0.013212	3.09	PI(38:3)	
0.025065	3.10	PS(38:4)||PC(35:5)||PE(38:5)	
0.026413	3.17	PI(35:2)	
0.0031685	3.19	PE(40:9)||PS(40:8)||PG(38:8)	
0.013101	3.24	PC(31:2)||PE(34:2)||PA(34:1)	
0.011097	3.32	PC(33:1)||PE(36:1)||PA(36:0)	
0.041059	3.35	PE(O-40:5)||PE(P-40:4)||PA(O-40:4)||PA(P-40:3)	
0.019264	3.54	PC(32:5)||PE(35:5)||PG(34:2)	
0.0031685	3.71	PI(34:2)	
0.003508	3.86	PC(37:4)||PE(40:4)||PI(36:2)	
0.0086169	3.89	PI(39:6)	
0.025073	4.30	PC(31:3)||PE(34:3)||PA(34:2)	
0.0054794	4.41	PC(39:5)||PE(42:5)||PI(40:6)	
0.025187	4.43	PE(40:9)||PC(35:6)	
0.016476	4.56	PC(37:7)||PE(40:7)	
0.0032155	5.16	PI(35:1)	
0.010905	5.27	PC(29:1)||PE(32:1)||PA(32:0)	
0.011097	5.32	PG(40:7)||PA(42:7)	
0.02706	5.48	PC(33:5)||PE(36:5)	
0.0017187	5.87	PC(37:3)||PE(40:3)||PI(36:1)	
0.0017187	5.89	PI(36:1)	
0.0034098	6.22	PG(O-33:0)||PS(38:6)||PE(38:7)||PG(36:6)	
0.0017187	6.95	PC(37:5)||PE(40:5)	
0.0017187	7.78	PC(46:3)	

0.0073752	8.43	PS(36:4)||PC(32:3)||PE(35:3)||PG(34:2)	
0.0017187	9.00	PI(32:1)	
0.0034306	10.00	PC(33:3)||PE(36:3)	
0.030705	0.21	PC(42:7)	
0.022845	0.23	PE(O-34:3)||PE(P-34:2)	
0.035614	0.28	PC(37:0)||PE(40:0)||PA(44:4)	
0.0079735	0.29	PS(O-40:6)||PS(P-40:5)	
0.0037796	0.29	PA(O-35:0)	
0.024828	0.33	PS(O-34:2)||PS(P-34:1)||PC(35:4)||PE(38:4)	
0.0072377	0.34	PI(35:0)	
0.024133	0.40	PC(P-18:1/P-18:1)	
0.036413	0.46	PA(38:9)	
0.039412	0.64	PA(O-36:0)	
0.017133	0.65	PC(37:2)||PE(40:2)	
0.0079735	1.96	PI(38:6)	
0.0067405	2.14	PE(44:12)||PI(38:7)	
0.0049777	2.24	PE(44:12)||PI(36:4)	
0.0044707	2.29	PI(38:6)||PS(42:8)	
0.027153	2.30	PI(38:5)||PG(41:2)	
0.0049777	2.32	PS(41:4)||PI(36:4)||PG(39:1)	
0.0044026	2.33	PI(36:4)||PG(39:1)||PS(40:6)	
0.025553	2.38	PI(40:7)||PG(43:4)	
0.0021246	2.54	PC(44:9)	
0.0068787	2.56	PG(43:1)	
0.029925	2.64	PG(37:2)||PS(36:1)	
0.0022821	2.66	PE(40:9)||PA(42:10)	
0.0025842	2.69	PC(33:4)||PE(36:4)	
0.011564	2.74	PC(35:4)||PE(38:4)||PA(41:4)	
0.036652	2.85	PG(O-31:1)||PG(P-31:0)||PC(33:5)||PE(36:5)	
0.0049748	2.85	PA(36:3)||PC(31:2)||PE(34:2)	

0.0027785	2.97	PC(35:4)||PE(38:4)|PA(39:1)	
0.02252	3.01	PA(40:8)||PE(38:7)	
0.0021246	3.04	PC(35:6)||PE(38:6)	
0.0035727	3.09	PG(O-35:2)||PG(P-35:1)||PC(37:6)||PE(40:6)||PA(44:10)	
0.0025842	3.10	PI(36:2)||PS(401:4)	
0.0021246	3.13	PE(42:10)||PC(37:7)||PI(34:2)	
0.023349	3.16	PI(36:3)||PS(40:5)	
0.0040389	3.20	PA(43:6)||PC(37:6)||PE(40:6)||PS(O-36:1(9Z))||PS(P-36:0)	
0.0021246	3.20	PC(37:7)||PE(40:7)||PI(34:2)	
0.0021246	3.21	PI(40:6)||PS(44:8)	
0.020154	3.23	PC(33:1)||PE(36:1)	
0.009845	3.46	PE(42:10)||PA(44:11)||PS(O-36:2)||PS(P-36:1)	
0.0022821	3.51	PC(37:6)||PE(40:6)	
0.0042522	3.94	PE(39:7)	
0.0056059	4.42	PA(42:8)||PC(37:7)||PE(40:7)||PI(34:2)	
0.00425	4.53	PS(40:6)	
0.0021246	4.86	PS(40:6)||PG(40:8)	
0.0054632	4.96	PS(38:6)||PG(38:8)	
0.0048046	5.23	PS(36:1)||PG(36:3)	
0.0021246	5.72	PS(38:6)	
0.0028417	5.73	PI(40:5)||PS(44:7)	
0.0021246	6.88	PI(34:4)	
0.020454	6.95	PE(40:9)||PI(34:4)	

Appendix 7: Results (Chapter 4)

Table 17. Lipid-related metabolite features which are statistically significant (q<0.05) when comparing livers which (1) did and (2) did not meet the viability criteria for perfusate samples collected at 4 hours.

Adjusted P-value	**Fold Change (Criteria not met/criteria met)**	**Metabolite annotation**	**Metabolite/lipid class**
0.029323	2.09	hydroxydecanoyl carnitine	Acyl carnitine
0.038393	3.17	O-(13-carboxytridecanoyl)carnitine	
0.0076804	8.26	hexadecanedioic acid mono-L-carnitine ester	
0.020927	0.14	PE-Cer(d36:1(2OH))	Ceramides
0.012135	0.21	GlcCer(d34:1(2OH))	
0.029125	0.37	GlcCer(d40:2)	
0.025625	3.00	GlcCer(d36:2)	
0.038487	0.28	PE-Cer(d37:1(2OH))	
0.046041	0.53	Cer(d52:2(34OH))	
0.0049771	0.54	PE-Cer(d35:2)	
0.010211	1.56	Cer(d42:2)	
0.005762	1.73	Cer(d40:2)	
0.017256	1.77	Cer(d41:2)	
0.0047457	1.84	Cer(d42:3)	
0.0069719	1.89	Cer(d44:2)	
0.023407	1.91	Cer(d38:2)	
0.0071954	1.95	Cer(d36:1)	
0.032101	2.03	Cer(d34:1)	
0.004175	2.13	Cer(d40:1)	
0.0035648	2.17	Cer(d48:0(30OH))	

0.017763	2.55	Cer(d36:2)	
0.0094549	2.76	Cer(d34:2)	
0.033833	2.85	Cer(t18:1(6OH)/18:0(2OH))	
0.011501	3.20	Cer(m36:1)	
0.019985	3.41	Cer(d38:3)	
0.0077826	0.19	PS(O-35:2)||PS(P-35:1)	Glycerophospholipid
0.0036725	0.24	PC(O-39:0)||PE(O-42:0)	
0.029984	0.27	PS(43:6)	
0.036805	0.31	PI(O-41:0)	
0.021159	0.32	PC(28:0)||PE(31:0)||DG(39:3)	
0.0016302	0.33	PG(40:8)	
0.022687	0.35	PC(39:8)||PE(42:8)||PS(35:2)||PG(36:0)	
0.025481	0.35	PS(44:9)	
0.043981	0.37	PI(37:0)	
0.020228	0.39	PS(35:2)||PG(36:0)	
0.039426	0.47	PS(43:6)||PC(39:7)||PE(42:7)	
0.02511	0.50	PI(O-42:1)	
0.039426	0.50	PS(40:3)||PC(36:4)||PE(39:4)||PG(38:3)	
0.031658	0.52	PIM1(35:0)	
0.039426	0.52	PS(39:2)||PC(35:3)||PE(38:3)	
0.035378	0.54	PA(46:7)	
0.042797	0.57	PC(36:3)||PE(39:3)||PG(37:2)	
0.031252	1.98	PC(P-31:1)||PE(O-34:2)||PE(P-34:1)||PA(O-34:1)||PA(P-34:0)	
0.02418	2.18	PC(O-35:2)||PC(P-33:1)||PE(O-36:2)||PE(P-36:1)||PA(O-36:1)||PA(P-36:0)	
0.031252	2.21	PI(36:4)	
0.012135	2.34	PE(O-38:5)||PE(P-38:4)||PA(O-38:4)||PA(P-38:3)	
0.023535	2.36	PE(40:9)||PS(40:8)||PG(38:8)	
0.048659	2.36	PA(37:0)||PG(36:0)	
0.045197	2.41	PC(33:1)||PE(36:1)||PA(36:0)	

0.0087901	2.61	PC(39:6)||PE(42:6)	
0.0077826	2.63	PC(39:6)||PE(42:6)||PI(38:4)	
0.0080457	2.73	PE(O-38:5)||PE(P-38:4)	
0.046593	2.81	PC(29:1)||PE(32:1)||PA(32:0)	
0.039426	2.90	PC(32:5)||PE(35:5)||PG(34:2)	
0.026355	2.93	PC(39:5)||PE(42:5)||PI(38:3)	
0.039426	2.96	PC(37:7)||PE(40:7)	
0.020228	2.98	PI(38:3)	
0.0053157	3.05	PI(34:2)	
0.033444	3.20	PC(33:5)||PE(36:5)	
0.026868	3.20	PA(32:4)	
0.0055444	3.21	PI(36:2)	
0.008981	3.78	PE(40:9)||PC(35:6)	
0.032275	3.92	PC(31:3)||PE(34:3)||PA(34:2)	
0.017805	3.93	PE(O-40:5)||PE(P-40:4)||PA(O-40:4)||PA(P-40:3)	
0.0040173	4.00	PI(32:1)	
0.0068438	4.00	PI(40:4)	
0.0057054	4.10	PC(39:5)||PE(42:5)||PI(40:6)	
0.0053157	4.83	PI(35:1)	
0.0010935	4.99	PC(37:3)||PE(40:3)||PI(36:1)	
0.0013037	5.23	PE(P-36:4)||PE(O-36:5)||PS(P-38:6)	
0.0010935	6.16	PC(37:4)||PE(40:4)||PI(38:5)	
0.0025715	6.35	PG(O-33:0)||PS(38:6)||PE(38:7)||PG(36:6)	
0.0010935	6.94	PC(37:7)||PE(40:7)||PS(42:9)	
0.0023804	10.53	PS(36:4)||PC(32:3)||PE(35:3)||PG(34:2)	
0.00071187	11.74	PC(33:3)||PE(36:3)	
0.0010231	11.94	PC(37:5)||PE(40:5)	
0.0033352	0.24	PE(O-34:3)||PE(P-34:2)	
0.040088	0.25	PS(P-38:4)||PG(P-38:6)||PS(O-38:5)	
0.0094548	0.26	PS(O-40:6)||PS(P-40:5)	

0.0075714	0.31	PIM2(36:0)	
0.0034426	0.36	PA(O-35:0)	
0.035236	0.38	PG(O-38:6)||PG(P-38:5)||PS(O-38:4)||PS(P-38:3)	
0.035492	0.51	PC(P-38:2)||PC(O-38:3)||PG(O-42:6)||PA(O-40:0)||PE(44:9)	
0.022534	0.58	PG(O-38:3)||PG(P-38:2)	
0.032597	0.59	PG(O-36:3)||PG(P-36:2)||PC(38:7)||PE(41:7)	
0.0093345	0.62	PC(37:2)||PE(40:2)	
0.013314	1.80	PI(38:6)	
0.0033347	2.23	PE(44:12)||PI(38:7)	
0.0026933	2.27	PI(38:5)||PG(41:2)	
0.00326	2.27	PE(44:12)||PI(36:4)	
0.00326	2.36	PS(41:4)||PI(36:4)||PG(39:1)	
0.0026757	2.36	PI(38:6)||PS(42:8)	
0.012842	2.39	PG(41:2)||PS(42:7)||PI(38:5)	
0.010574	2.41	PI(36:4)||PG(39:1)||PS(40:6)	
0.040202	2.46	PA(40:8)||PE(38:7)	
0.0022267	2.48	PE(44:9)||PI(40:7)	
0.004133	2.50	PC(33:4)||PE(36:4)	
0.0098328	2.51	PA(36:3)||PC(31:2)||PE(34:2)	
0.0048139	2.60	PE(40:9)||PA(42:10)	
0.037328	2.64	PG(O-31:1)||PG(P-31:0)||PC(33:5)||PE(36:5)	
0.00071425	2.65	PI(38:4)	
0.004068	2.66	PC(35:6)||PE(38:6)	
0.021996	2.67	PC(33:1)||PE(36:1)	
0.00096084	2.68	PC(44:9)	
0.005126	2.74	LysoPC(18:0)||LysoPE(21:0)	
0.0044153	2.83	PC(35:4)||PE(38:4)|PA(39:1)	
0.0070435	2.88	PI(38:3)||PG(41:0)	
0.0049111	2.95	PG(O-35:2)||PG(P-35:1)||PC(37:6)||PE(40:6)||PA(44:10)	
0.016755	3.05	PA(43:6)||PC(37:6)||PE(40:6)||PS(O-36:1(9Z))||PS(P-36:0)	

0.0074502	3.07	PI(36:3)||PS(40:5)	
0.00083557	3.10	PC(37:7)||PE(40:7)||PI(34:2)	
0.0033352	3.15	PC(37:6)||PE(40:6)	
0.00067255	3.26	PI(40:6)||PS(44:8)	
0.0014837	3.27	PI(36:2)||PS(401:4)	
0.0029347	3.29	PI(37:5)||PS(44:8)	
0.004068	3.33	PE(42:10)||PC(37:7)||PI(34:2)	
0.010476	3.40	PG(37:2)||PS(36:1)	
0.009628	3.50	PE(39:7)	
0.0035352	3.64	PG(43:1)	
0.008728	3.66	PC(35:4)||PE(38:4)||PA(41:4)	
0.00954	3.69	PG(38:6)||PS(38:4)	
0.00061688	4.10	PG(37:4)||PS(38:6)	
0.0026757	4.20	PE(42:10)||PA(44:11)||PS(O-36:2)||PS(P-36:1)	
0.005126	4.20	PI(36:5)	
0.0052259	4.36	PA(42:8)||PC(37:7)||PE(40:7)||PI(34:2)	
0.0027072	4.98	PI(40:6)	
0.00031247	5.40	PI(36:1)	
0.0035352	5.44	PE(40:9)||PI(34:4)	
0.0014575	5.97	PS(38:6)||PG(38:8)	
0.0011198	5.97	PI(40:5)||PS(44:7)	
0.0018194	5.99	PS(40:6)||PG(40:8)	
0.00013637	6.25	PS(38:6)	
0.001257	6.58	PS(40:6)	
0.001257	7.22	PI(34:4)	
0.00061688	8.37	PS(40:4)||PG(40:6)	
0.00021373	9.41	PS(36:1)||PG(36:3)	

Appendix 8

VITTAL clinical trial perfusate solution

Starting perfusate:

- Packed red blood cells
- Colloid solution
- Heparin to prevent thrombosis in the circuit.
- Sodium bicarbonate pH buffer
- Calcium gluconate
- Antibiotic Gentamicin

Constant infusions:

- Parenteral nutrition solution containing amino acids and glucose
- Insulin for perfusate glucose control
- Heparin for anticoagulation.
- Sodium taurocholate 2 % solution in isotonic saline
- Prostacyclin – a vasodilator

Appendix 9

Ex Situ Normothermic Split Liver Machine Perfusion: Protocol for Robust Comparative Controls in Liver Function Assessment suitable for the evaluation of novel therapeutic interventions in the preclinical setting

Matherials and Method - Perfusate fluid components

- 3 units (250mL/unit) HBOC-201
- 1000 mL 5% w/v human albumin solution (Alburex 5, CSL Behring GmbH, Germany)
- 10,000 IU heparin (Wockhardt, UK)
- 30mL 8.4 % sodium bicarbonate 8.4% (B. Braun Medical Limited, UK)
- 10 mL 10% calcium gluconate
- 500 mg vancomycin (Wockhardt, UK)
- 60 mg gentamicin (Cidomycin, Sanofi, UK)
- 50 mL 10% v/v Aminoplasmal (B.Braun Medical Limited, UK)
- 0.2 mL Cernevit (Baxter Healthcare Ltd., UK)
- 0.1 mg phytomenadione (Konakion, Roche Products Ltd, UK)
- Epoprostenol (Flolan, GlaxoSmithKline, UK, 2 µg/ml) continuous infusion at 4 - 8 mL/hour