NORMOTHERMIC MACHINE PERFUSION OF THE LIVER (NMP-L): A NOVEL TECHNIQUE THAT PERMITS THE VIABILITY TESTING OF DONOR LIVERS AND THE TARGETED DELIVERY OF CELLULAR THERAPY.

By Richard Wallace Laing

UNIVERSITY OF BIRMINGHAM

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ABSTRACT

Liver transplantation is a successful treatment for acute and chronic liver failure, yet the field faces challenges in the forms of deteriorating graft quality, increasing demand and more complex logistics. Normothermic machine perfusion is able to preserve donor livers for up to 24 hours and liver function can be objectively assessed during the perfusion process. Criteria that aim to establish donor liver viability have been developed through the perfusion of livers discarded for clinical use. The criteria are based upon metabolic and physiological parameters and have been tested within a clinical pilot study of five transplants using discarded organs. All grafts functioned immediately and all patients survived. Following on from this success, VITTAL (Viability testing and transplantation of marginal livers) – a Wellcome Trust funded trial – was designed and carried out. This saw the transplantation of 22 patients following 31 perfusions of discarded donor livers. The results validate the viability criteria and demonstrate that end-ischemic normothermic machine perfusion enables the safe transplantation of a significant proportion of currently unutilised livers and is associated with increased graft utilisation, extended preservation time and improved logistics. The use of Hemopure, a haemoglobin-based oxygen carrier, was also investigated and has been shown to have logistical, rheological and immunological advantages over packed red cells when used in the perfusion fluid. The final part of this thesis explored the use of machine perfusion devices to deliver cellular therapy to marginal donor livers. The results demonstrate the technique is feasible, with multipotent adult progenitor cells being delivered directly into the target organ allowing them to secrete a host of soluble factors that are known to have anti-inflammatory and immunomodulatory effects.

DEDICATIONS

To my wonderful family and all the patients who risked their lives for the benefit of others.

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I am incredibly proud of what we have achieved – we have furthered the field of transplantation, changed clinical practice and improved the lives of patients. My final thank you is saved for the donors and their families who gave the greatest gift of all and for the patients who selflessly risked their lives in pursuit of better outcomes for others.

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The delivery of stem cell therapy to extended criteria donor human livers using normothermic machine perfusion

British Liver Transplant Group Annual Meeting September 2017

The delivery of stem cell therapy to extended criteria donor human livers using normothermic machine perfusion

British Association for the Study of the Liver Annual Meeting September 2017

Normothermic machine perfusion of the liver.

Presentation of research to date in the Andy Burroughs Award for clinical research session, at the British Liver Transplant Group Meeting in York September 2018

Viability testing and transplantation of marginal livers" - The clinical outcomes of the VITTAL trial.

Presented in the Medawar Session at the British Transplant Society Meeting in Harrogate, March 2019.

Sixteen months of discarded donor livers in the UK - an analysis of donor demographics and factors contributing to organ salvage.

Oral presentation at the British Transplant Society Meeting in Harrogate, March 2019.

Transplantation of discarded livers following viability testing with normothermic machine perfusion: The VITTAL (Viability testing and transplantation of marginal livers) trial outcomes.

Presented in Rising Star Symposium as oral presentation at International Liver Transplant Society (ILTS) Meeting, Toronto, May 2019.

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Normothermic machine perfusion of the liver.

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First author

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Significant contribution to work

Proof of concept: Liver splitting during normothermic machine perfusion

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Viability outcomes for donor livers discarded due to steatosis following extra-corporeal machine perfusion of the liver.

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The impact of temperature on ex-vivo machine perfusion of severely steatotic donor livers.

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The use of a haemoglobin based oxygen carrier in a human liver model of normothermic machine perfusion.

Boteon YL, **Laing RW**, Bhogal R, Neil DA, Smith A, Stephenson BTF, Schlegel A, Hübscher SG, Mirza DF, Afford SC, Mergental H

American Transplant Congress (ATC), American Society of Transplant Surgeons (ASTS), celebrated in Chicago, Illinois, USA, on 29th April–3rd May 2017.

AWARDS ARISING FROM THIS THESIS

Rising Star Award, International Liver Transplant Society, Toronto, May 2019

Medawar Medal for Clinical Research, British Transplant Society, Harrogate, March 2019

Andy Burroughs Award for Research, British Liver Transplant Group, York, 19th September 2018

Clinical Research Network West Midlands Awards "Collaboration in Research". VITTAL trial team, October 2017

Shortlisted for best research – Highly commended. British Liver Transplant Group (BLTG), Annual Meeting, September 2017.

Best research presentation. College of Medical and Dental Sciences, Postgraduate Research Away Day, May 2017.

Travel Award ($1000) from International Liver Transplant Society for abstract entitled "Liver transplantation using grafts from donors after circulatory death: A propensity-matched study from a single centre" presented in Chicago, USA, July 2015

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are some of those with the largest number of interactions and roles in biological processes. 331

LIST OF ABBREVIATIONS

ALT	Alanine transaminase
AKI	Acute kidney injury
AST	Aspartate transaminase
ATP	Adenosine triphosphate
BAR	Balance of risk
BEC	Biliary epithelial cells
BMI	Body mass index
BTS	British Transplantation Society
CD	Cluster of differentiation
CIT	Cold ischemic time
CMV	Cytomegalovirus
COR	Controlled oxygenated rewarming
CRCTU	Cancer Research UK Clinical Trials Unit
DAMPs	Damage-associated molecular pattern molecules
DBD	Donor following brain death
DCD	Donor following circulatory death
DGF	Delayed graft function
DHOPE	Hypothermic oxygenated machine perfusion (portal vein and hepatic artery)
DRI	Donor risk index
eCRF	Electronic case report form
ER	Extraction ratio
ERCP	Endoscopic retrograde cholangiopancreatography
FDA	U.S. Food and Drug Administration
FiO2	Fraction of inspired oxygen

FWIT	Functional warm ischemic time
HA	Hepatic artery
HAT	Hepatic artery thrombosis
HBI	Hypoxic brain injury
HBOC	Haemoglobin-based oxygen carrier
HCC	Hepatocellular carcinoma
H&E	Haematoxylin and eosin
H-R	Hypoxia reoxygenation
HMP	Hypothermic machine perfusion
HOPE	Hypothermic oxygenated machine perfusion (portal vein only)
HRA	Health Research Authority
HSEC	Human sinusoidal endothelial cells
IC	Ischemic cholangiopathy
ICAM-1	Intercellular adhesion molecule-1
ICH	Intracranial haemorrhage
ICU	Intensive care unit
IRI	Ischemia reperfusion injury
ITU	Intensive treatment unit
LFT's	Liver function tests
MAPC cells	Multipotent adult progenitor cells
MELD	Model for end stage liver disease
MFI	Medium fluorescence intensity
MHRA	Medicines and Healthcare Products Regulatory Agency
MRCP	Magnetic resonance cholangiopancreatography
MS	Macrovesicular steatosis

MSC	Mesenchymal stem cells
NASH	Non-alcoholic steatohepatitis
NHS	National Health Service
NHSBT	National Health Service Blood and Transplant
NMP	Normothermic machine perfusion
NMP-L	Normothermic machine perfusion of the liver
NRP	Normothermic regional perfusion
O2ER	Oxygen extraction ratio
PAS	Periodic-acid Schiff
PBC	Primary biliary cirrhosis
PSC	Primary sclerosing cholangitis
PNF	Primary non-function
PSM	Propensity score matching
PV	Portal vein
RBC	Red blood cells
RINTAG	Research, Innovation and Novel Technologies Advisory Group
RIFLE	Risk, injury, failure, loss, end-stage liver disease
ROS	Reactive oxygen species
SCS	Static cold storage
sd	Small droplet
SNMP-L	Subnormothermic machine perfusion of the liver
St. Dev.	Standard deviation
St. diff.	Standardized difference
u-DCD	Unmatched donors following circulatory death
UHBFT	University Hospitals Birmingham NHS Foundation Trust

UK	United Kingdom
UW	University of Wisconsin solution
WIT	Warm ischemic time
VITTAL	Viability testing and transplantation of marginal livers
8-OH-dG	8-hydroxy-2-deoxyguanosine

PART I – LIVER TRANSPLANTATION AND THE USE OF EXTENDED CRITERIA DONORS

CHAPTER 1 - INTRODUCTION

1.1 INTRODUCTION – LIVER TRANSPLANTATION, THE HISTORY OF MACHINE PERFUSION AND THE NEED FOR STRATEGIES TO IMPROVE DONOR QUALITY.

1.1.1 A brief history of transplantation

The concept of transplantation has been around for millennia – the transplantation a leg by the Christian martyrs Cosmas and Damian as depicted in "A Verger's Dream" (Figure 1.1) is just one example of 'successful' transplants that were claimed to have taken place long before the twentieth century(1). It wasn't until the 1900's when the field began to progress. In 1912 Georg Schöne, a scientist working in Paul Ehrlich's laboratory, not only determined that skin homografts (transplanted from another donor) always failed but that subsequent grafts from the same donor failed more rapidly than the first(2). Alexis Carrel, whose work on suturing and transplantation was recognised by the Nobel Prize Committee in 1912, worked extensively on transplantation in the early 1900's(3). He determined that whilst he was able to successfully transplant autografts (tissue from the same subject) using his suturing techniques and observing strict asepsis, homografts (tissue from another donor) always failed. He and a colleague James Murphy then identified that either irradiating recipients or treating them with benzol improved experimental outcomes. During World War II a young zoologist named Peter Medawar started in Glasgow Royal Infirmary working with burn victims. He and plastic surgeon Thomas Gibson reaffirmed the fate of skin homografts, postulating that rejection was likely an immunological event as supported by the "second set phenomenon" previously described by the likes of Schöne and Holman(2, 4, 5).

Figure 1.1 “A Verger's Dream”

A painting by the Master of Los Balbases that probably hung in the Church of Saints Cosmas and Damian in Burgos in northern Spain. It depicts a vision described in a book by Jacobus de Voragine, *Legenda aurea* (The golden legend) in 1275. The vision was received in the Church of Saints Cosmas and Damian, in Rome, by a verger who probably had some venous incompetence causing ulceration in his leg. One night he dreamed that the two saints came and replaced his diseased limb with one from a recently deceased individual. Courtesy of the World Digital Library and the Wellcome Library.

The concept of acquired immunological tolerance, for which Peter Medawar and Frank MacFarlane Burnet won their 1960 Nobel Prize, was the result of serendipity and a culmination of the work of several others including John Hunter, Frank Lillie and Ray Owen(6-8). By

demonstrating that immunological tolerance could be induced in foetal mice and chick embryos, they were able to explain the paradoxical observation that tissue grafts between non-identical twins could be accepted - findings that were critical in the development of transplantation.

In 1954, just over a year after Medawar's landmark paper, Joseph Murray performed the first kidney transplant from an identical twin donor(9). This was the trigger for surgeons around the world to pursue further advances in transplantation – a trend which has continued for the past 60 years. Developments in organ preservation solutions, pharmacological immunosuppression, the concept of brain death, sharing of organs between centres, histocompatibility typing and the transplantation of extrarenal organs drove the field forward during the 'consolidation period'. Pioneers such as René Küss, Roy Calne, and Thomas Starzl took risks that in today's practice would not be considered acceptable, but undoubtedly made transplantation the success that it is today.

1.1.2 Liver transplantation

Liver transplantation (LT) has become a highly successful treatment for end stage chronic liver disease, fulminant hepatic failure and early stage primary liver cancer. Emerging indications for LT include cholangiocarcinoma and colorectal liver metastases in patients with favourable tumoral biology(10, 11). The demand for LT will undoubtedly continue to increase and the statistics are concerning. Deaths from liver disease have soared by 400 percent since 1970 and despite remarkable advances in the treatment of viral hepatitis – Hepatitis C is now a curable disease – the ominous increase in non-alcoholic fatty liver disease (NAFLD) and the ongoing issue of alcohol addiction mean that the incidence of chronic liver disease will continue to rise. Liver disease kills 12,500 people a year in England and the average age of death from liver

disease (59 years), continues to fall(12). Over 600,000 people in the UK have some form of advanced liver disease and 60,000 of these have cirrhosis(13). Liver disease is now the leading cause of death in 35-49 year olds(14) and a report published in the Lancet in 2018 predicted that liver disease is set to overtake coronary heart disease as the leading cause of premature death in the next two years(15).

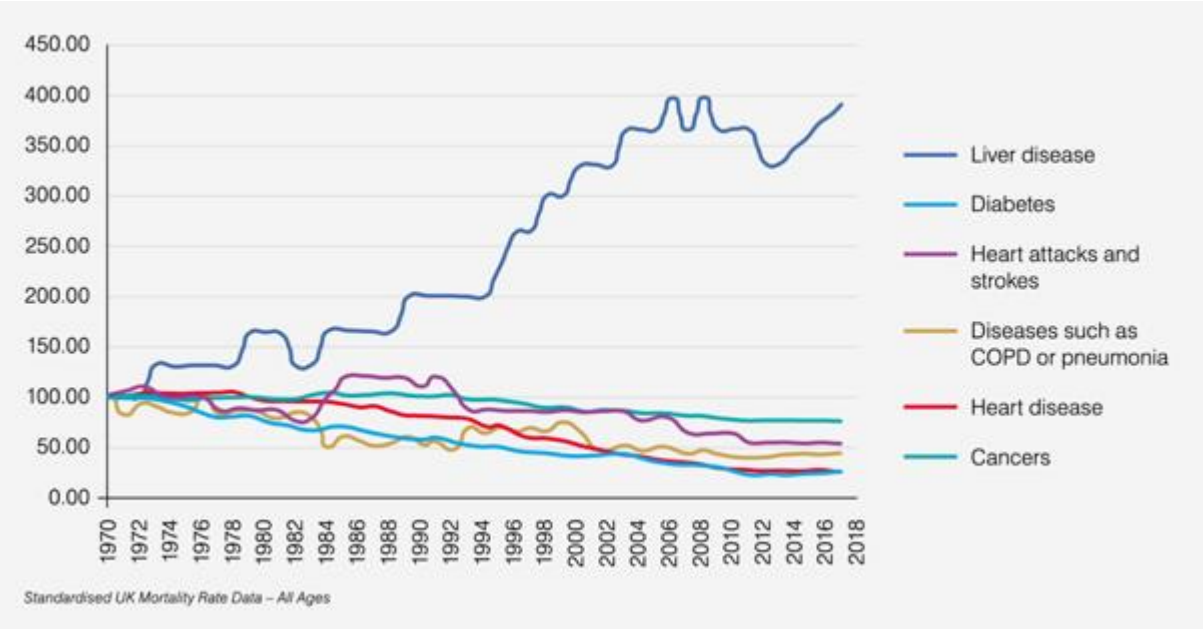

Figure 1.2 The rise in UK deaths related to liver disease compared with the other major diseases(16).

The outcomes for Liver Transplant today are remarkable considering the theory and surgical undertaking. In the 1970's shortly after the introduction of the concept of brain death, over 70% of liver allograft recipients died shortly after surgery. Even in the hands of Starzl, 1-year survival was 33%. In the late 70's however, the tide changed. The introduction of the calcineurin inhibitor cyclosporin revolutionised liver transplantation and between 1979 and 1980, Starzl reported that 11 out of 12 of his transplant patients survived 1 year and shortly after that in Pittsburgh, he reported 70% survival in 40 recipients(17). Over the next ten to fifteen years, graft and patient survival rates improved further through advances in immunosuppression (monoclonal antibodies to T cells and the more potent calcineurin

inhibitor tacrolimus), organ procurement techniques and organ preservation solutions and improvements in organ allocation and post-operative management. In the UK the patient survival rates have gradually increased over the past 20 years varying between 88-95% 1 year and 76-85% 5 year survival for those in receipt of a donor with brain death (DBD) and 81-96% 1 year and 67-82% 5 year survival for those who receive a liver from a circulatory death donor (DCD)(18). Although teams will always continue to strive to improve survival further there are greater challenges that have evolved; how can we safely respond to the increasing incidence of liver disease requiring transplantation from a limited donor pool? how can we achieve similar outcomes using livers that are from an increasingly elderly and co-morbid population? These challenges were addressed in NHSBT's 2020 vision document which set out a detailed strategy to increase the number of potential donors, improve donor conversion rates, increasing utilisation and improving survival(19).

1.1.3 The use of "marginal" or "extended criteria donors"

An increasing number of transplants are carried out using "marginal" or "extended criteria" grafts, procured from elderly donors with multiple co-morbidities such as obesity and metabolic syndrome (20). There is also an increasing reliance on the use of DCD livers which are exposed to a period of warm ischemia. These livers are significantly more susceptible to cold storage-related injury, which increases the risk of post-reperfusion syndrome, ischaemia reperfusion injury, graft failure and recipient morbidity and mortality. The change in demographics has been stark even over the past eight years with a reduction in the proportion of donors aged 19-49 from 42 to 35% and an increase in the proportion of donors over the age of 60 from 30 to 48% between 2010 and 2018. In terms of body habitus, the proportion of donors with a BMI >30 has increased from 20 to 29% and those with a BMI of between 20 and 29 has reduced from 73 to 66%(18). In 2018, of the 934 DBD livers that were initially offered

for transplantation, 856 were retrieved and 762 were transplanted (82% of those offered). Of the 588 DCD's, 257 were retrieved and 186 were transplanted (32% of those offered) – this is in contrast to 1043 DCD kidneys that were used which equates to 83% of those initially offered for transplantation. The number of donor livers has increased since 2010 with 637 potential DBD donors, 567 livers retrieved and 524 transplanted (82% of those offered) and 373 potential DCD's, 145 retrieved and 100 transplanted (27% of those offered). Over an eight-year period there has only been a marginal increase (5%) in the proportion of DCD livers that are considered transplantable (likely due to an increase in the size of the donor pool) and the proportion of transplantable DBD's has remained static, which as discussed, is testament to the transplant teams as the pool of donors from which they can be selected has become more marginal(18, 21).

1.1.4 Risks of using "marginal" or "extended criteria" donor livers

The risks associated with the use of extended criteria donor organs are well documented and the factors that can contribute to inferior organ quality and denote their classification can be seen in Table 1.1.

Table 1.1 University Hospital Birmingham NHS FT criteria for marginal DBD livers and extended criteria (ECDCD) donor livers. Adapted from British Transplantation Society Guidelines.

Marginal DBD	**Extended Criteria DCD**
Age > 80	Age >50 years
ICU stay with ventilation > 5 days	Weight >100kg
Deranged LFT's above 3 x norm	FWIT >20 mins
Estimated CIT > 12 hours	CIT >8 hours
Moderate or severe macrovescicular steatosis (> 30 %) or/and donor BMI > 30	>15% Steatosis
	ICU stay >5 days

ICU, Intensive Care Unit; LFT, Liver function test; CIT, Cold ischemic time; BMI, Body mass index; FWIT, Functional warm ischemic time

Donor parameters that are recognised as impacting upon patient outcome include age, BMI – which can be indicative of steatosis, DCD donation, split or whole graft, prolongation of cold ischaemia, prolonged ICU stay and donor instability, elevated donor transaminases due to e.g. out of hospital cardiac arrest and prolonged warm ischemia (>30 minutes) in DCD donation(22). Several of these are included in the Donor Risk Index (DRI) which was developed using North American data and later validated using European data(23, 24). Other groups have developed their own risk scores including the UK Donor liver index, the UK DCD Score and the Balance of Risk Score which also recognises recipient factors(25-27).

The risks of using these grafts include delayed graft function (DGF) and early allograft dysfunction (EAD), terms which are used interchangeably and refer to a form of primary graft dysfunction which has been reported in up to 39% of transplant recipients(28). It is characterised by derangement in biochemical markers of function and metabolism such as serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) levels, prothrombin time (PT), international normalized ratio (INR) and serum lactate. It is associated with reduced graft and patient survival, prolonged intensive care unit (ICU) stays and increased

postoperative morbidity and mortality(29-33). The variables, ranges and cut-offs vary between studies however the most widely used definition is that from Kim Olthoff which classifies EAD as the presence of one or more of the following variables; serum bilirubin levels ≥10mg/dL and/or INR ≥1.6 on postoperative day 7; serum AST or ALT levels >2,000IU/L at any time within the first 7 days of surgery(30).

Primary non-function (PNF) is a more severe manifestation of graft dysfunction and although a consensus definition remains to be established, a patient is unable to survive without urgent re-transplantation(34). The most helpful definition of PNF is that from Uemura, which states that PNF is graft function that results in liver re-transplantation or death in the absence of other causes of failure such as hepatic artery or portal vein thrombosis and builds upon the definition by Makowka by extending the time period to 7 days(35, 36). Both EAD and PNF are related to the metabolic capacity of the graft and have associations with ischaemia reperfusion injury and recipient immunological insult to the graft for example in hyperacute rejection(37). Primary non-function rates are thankfully low and are generally accepted as occurring in less than 5% of cases (0.9 to 7.2%), however the impact of this complication is so severe that the majority of marginal DBD's and extended criteria DCD's are rejected due to the significant risk of PNF.

Ischaemia reperfusion injury (IRI) is an inevitable metabolic consequence of the classical preservation process, where oxygen supply is interrupted for a period of time(38). Clinically, its manifests as hepatocyte injury and can cause EAD, acute kidney injury and intrahepatic biliary stricturing known as ischaemic-type biliary lesions (ITBL) through injury to the biliary endothelium(39, 40). Liver IRI is triggered by the period of warm ischaemia prior to preservation in DCD donation and physiological instability caused by the process of brain

death. It is then propagated by the oxygen-free cold storage period and again on re-warming(41). The type and degree of injury invariably differs depending on the type of donation and quality of graft for example in severely steatotic livers. During the initial ischaemic phase, mitochondria produce reactive oxygen species (ROS) in response to hypoxia and lack of substrates. Steatotic livers are known to release higher levels of ROS which induce oxidative stress and initiate damage to parenchymal cells such as hepatocytes and hepatic sinusoidal endothelial cells (HSEC)(42). When oxygen supplies are restored, the mitochondria – deplete of ATP – induce a further release of inflammatory cytokines and chemokines which activate neutrophils and macrophages resulting in cellular damage(43). This is exacerbated by lipid peroxidation, ROS and damage-associated molecular patterns (DAMPS)(44). This complex cascade is mediated by other molecules and processes such as autophagy which limits ROS production and hypoxia-inducible factors (HIF) which influence neutrophil viability, macrophage activation and tissue recovery(45-47). The challenges associated with the use of these organs are clear, Chapter 2 investigates the use of DCDs and ECDCDs at University Hospital Birmingham and strategies that can be used to maximise successful transplantation if such organs(48).

1.1.5 Maximising the use of high-risk donor livers

Several studies have highlighted that due to the subjective nature of graft assessment, high risk livers are generally underutilised. Utilisation varies between country as well as between units and is affected by unit size and surgeon experience(20, 49, 50). To respond to the projected demands on the service, the 2020 strategy document from NHSBT's set out plans to ensure that "as many organs as possible are used; retrieval surgeons will have a better range of options for preserving organs; transplant surgeons will have more information and guidance to help them decide which organs can be safely and effectively transplanted; there will be greater

consistency in the acceptance of offers of organs"(19). Machine perfusion as a form of organ preservation has the potential to address all of these points.

1.1.6 Machine perfusion

The history of organ perfusion can be traced back to Carrel and Lindberg and their first "Model T" glass cardiac perfusion device in the 1930's whilst Brettschneider and Starzl first attempted machine perfusion of the liver in the late 1960's(49, 50). It has developed into several different modalities all of which now deliver oxygen; hypothermic machine perfusion (using either portal vein alone [HOPE] or artery and portal vein [DHOPE]), normothermic machine perfusion (NMP-L) which simulates normal physiological conditions, subnormothermic (SNMP-L) which operates at temperatures just below physiological temperatures and controlled rewarming (COR). In terms of timing, MP can occur in-situ at the time of retrieval in the form of normothermic regional perfusion (NRP) in DCD donation (which aims to re-instate physiological conditions permitting a period of organ recovery prior to cold storage), immediately following procurement until transplantation as normothermic machine preservation or "back to base" as an end-ischemic modality in the form of normothermic machine perfusion or hypothermic machine perfusion following cold-storage prior to transplantation.

1.1.7 Hypothermic machine perfusion

Following on from a significant body of pre-clinical animal work(51-54), in 2009, a clinical trial investigating the use of non-oxygenated HMP by Guarrera *et al* demonstrated a reduction in post-transplant transaminase and bilirubin levels and was the first to demonstrate safety and efficacy of HMP(55). A subsequent trial demonstrated safety of transplanting these organs following HMP even if they had previously been declined(56). In 2014, the group from Zurich

showed that hypothermic oxygenated MP via the portal vein (HOPE) enabled them to achieve outcomes with DCD grafts that were at least comparable (and in some cases better) to a group of matched DBD recipients in terms of post-operative transaminase levels (as a surrogate for liver injury), ICU stay and costs during admission(57). A larger matched cohort study from the same group demonstrated that DCDs treated with end-ischaemic HOPE significantly reduced peak post-transplant ALT, biliary complications and improved 1-year graft survival when compared to a matched cohort of SCS-stored DCD grafts(58). The results from randomised trials examining the use of D-HOPE and HOPE are awaited, although unfortunately they are looking at different grafts and different end-points so questions are likely to remain.

1.1.8 Normothermic machine perfusion

The current standard of donor liver preservation is based on static cold storage (SCS) (59). Organs are flushed and cooled with chilled preservation solution (University of Wisconsin [UW] solution is used most commonly in the UK) and ice is packed around the organs. After procurement, the organ is placed in preservation fluid-filled sterile plastic bags and stored in an ice-box prior to transplant. The aim is to reduce metabolic activity and cellular swelling. Anaerobic metabolism results in depletion of adenosine tri-phosphate (ATP) which leads to influx of free calcium and activation of phospholipases. Despite reducing metabolism approximately 12-fold, destruction of cellular integrity still occurs. As discussed, the energy-deplete mitochondria trigger a complex cascade of cellular and molecular events that initiate a series of immunological processes leading to cellular injury. Static cold storage is therefore unable to reverse the injury sustained during the retrieval, causes further injury due to the process of cooling, limits preservation time and inhibits physiological assessment prior to transplantation.

Normothermic machine perfusion of the liver (NMP-L) combats these limitations by aiming to maintain the organ at the body's natural temperature whilst providing oxygen, nutrition and the essential substrates necessary for adequate cellular metabolism. Providing this environment enables us to extend our storage period and possibly test the organs physiological parameters. A phase I study carried by Oxford University in conjunction with King's College Hospital London and University Hospitals Birmingham NHS Foundation Trust (UHBFT) recruited 20 patients into a phase 1 study which used normothermic machine perfusion from the point of retrieval to preserve the donor liver prior to transplantation. They then retrospectively matched (1:2) these patients with a historic cohort demonstrating a significant reduction in post-transplant peak AST and concluded the procedure was feasible and safe when perfusing livers considered transplantable using current conventional donor acceptance criteria (60). In 2016, Selzner *et al* showed that grafts preserved by NMP-L and Steen solution (FDA approved) had lower liver transaminase levels in the first few days after transplantation compared with those preserved using SCS although the results did not reach statistical significance (likely due to the small numbers included)(61). The largest and most influential clinical trial to date has been the COPE trial (Consortium for Organ Preservation in Europe). In this multi-centre randomized control trial (to which the team at UHB contributed 50% of the cases), 220 adult DBD and DCD donors considered transplantable as per current guidelines were randomised to NMP-L (120) or SCS (100). NMP-L was associated with a 50% reduction in peak AST (used as a surrogate for liver injury(62)), in spite of a 54% longer mean preservation time. There was also a 50% reduction in organ discard with no differences in biliary complications or graft or patient survival(63). The experiences from this trial enabled us to see how a transplantable liver performed on the machine and from this stemmed a body of research using livers that had been rejected for transplantation based on a perceived high risk of use, or sometimes purely for logistical purposes or the presence of malignancy in the donor. The variation in acceptance

rates and reasons for rejection of donor livers varies dramatically across the country(64), something that NHSBT wanted to address. The reasons for rejection are usually subjective; macroscopic assessment of steatosis without biopsy, perceived donor risk factors, prolonged CIT in conjunction with donor comorbidities etc. The livers perfused normothermically behave similarly to those in-vivo – they are metabolically active and perform similar functions in terms of bile production, protein production and glucose and lactate metabolism. Therefore, the team are able to objectively assess liver function in real time and decide on whether they feel the graft has the metabolic capacity to support the intended recipient. The Part II of this thesis (Chapters 3-7) investigates this theory and examines the ability of NMP-L to assess liver "viability" thereby allowing surgeons to safely transplant livers that have previously been considered too high risk for use and therefore discarded.

1.1.9 Taking machine perfusion forward

Normothermic machine perfusion maintains livers in a physiological state for a significant period of time prior to transplantation. Total preservation time in the COPE trial extended up to 24 hours in some cases. There are several technical considerations that need to be taken into account when the perfusion time is extended in this way. The perfusion fluid uses donor blood matched to the intended recipient as the oxygen carrier which is of course physiological, but is subjected to shear forces in the centrifugal pumps and tubing and undergoes haemolysis. It is also a precious resource, requires refrigeration, complicates logistics and can be associated with immunological phenomena and blood-borne infectious transmission. It also only acts as an effective oxygen carrier at physiological temperature and pH which limits the possible modalities of use. Chapter 8 looks at ways the perfusion fluid can be manipulated to improve upon the limitations of packed red cells.

The other advantage of having an ex-vivo human liver model is the ability to deliver therapeutics to donor livers prior to transplantation. In 2013, Van Raemdonck *et al* proposed that NMP-L could be used as a way of delivering cellular therapy to livers prior to transplantation(65). Mesenchymal stem cells (MSC) have been shown to have powerful immunomodulatory, anti-apoptotic, angiogenic and cytoprotective properties, making them of interest in the field of transplantation.(66) Recent data suggests they help coordinate the production of factors responsible for regeneration and repair by monocytes and macrophages, suppress the localization of inflammatory eosinophils and inhibit the damaging effects of neutrophils without affecting their phagocytic or chemotactic functions(67-70). With respect to transplantation, the most beneficial actions of MSC are not due to in-situ regeneration through differentiation into mature cells but through paracrine actions within the tissue(71). MSC have been shown to ameliorate the ischaemic-reperfusion injury by inhibiting H_2O_2-induced apoptosis and promoting hepatocyte proliferation(72) and they may be able to prevent or treat allogeneic rejection following transplantation, through secretion of IL10, IL-4 and IL-5(73).

Multipotent adult progenitor cells (MAPC® cells) have been proposed as an immune-active treatment for a wide variety of conditions(74). They belong to the family of MSC but show a higher proliferative capacity and a broader differentiation potential(75). They are derived from bone-marrow and meet the formal criteria for designation as stromal stem cells as they are plastic-adherent and express CD73, CD90, and CD105, in the absence of the hematopoietic markers CD14, CD34, CD45, and HLA-DR(76). They have been shown in animal models to treat graft versus host disease and in a porcine and human lung model of machine perfusion to reduce cold-storage related ischemic injury and modulate the immune cell population(77-82). Not only can they impair the induction of CD8+ cytotoxic T-lymphocyte function and supress

T-lymphocyte proliferation(83), but MAPC cells and related mesenchymal stem cells (MSC) have been shown to reduce ischaemia reperfusion injury (IRI) and reduce the inflammatory response in solid organs(74, 81, 84, 85). Given the increasing demands on the transplant service and the gradual reduction in organ quality, Chapter 9 examines the potential advantages of delivering cellular therapy to marginal grafts using machine perfusion.

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CHAPTER 2 - THE USE OF EXTENDED CRITERIA DONORS

2.1 LIVER TRANSPLANTATION USING GRAFTS FROM DONORS AFTER CIRCULATORY DEATH: A PROPENSITY SCORE–MATCHED STUDY FROM A SINGLE CENTRE.

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Authors: Laing RW[1,3], Scalera I[1], Isaac J[1,2], Mergental H[1], Mirza DF[1,2], Hodson J[1], Wilkin R[1,3], Perera MT[1,2], Muiesan P[1,2]

Affiliations:

[1] University Hospitals Birmingham NHS Foundation Trust, Department of liver surgery, United Kingdom

[2] Birmingham Children's Hospital NHS Foundation Trust, Department of liver surgery, United Kingdom

[3] NIHR Liver Biomedical Research Unit, University Hospitals Birmingham, United Kingdom

2.2.1 Abstract

The use of livers from donors following circulatory death (DCD) is increasing, but concerns regarding outcomes following use of marginal donors exist. To compare outcomes in transplants using DCD and DBD (donation following brain death), a propensity match was performed on 973 patients with chronic liver disease and/or malignancy who underwent primary whole liver transplant between 2004 and 2014 at University Hospital Birmingham. Primary endpoints were overall graft and patient survival. Secondary endpoints included post-operative, biliary and vascular complications. Over 10 years, 234 transplants were carried out using DCD grafts. Of the 187 matched DCD's, 82.9% were classified as "marginal" as per British Transplant Society Guidelines. Kaplan-Meier analysis of graft and patient survival found no significant differences for either outcome between the paired DCD and DBD patients ($p=0.162$ and 0.519 respectively). AST was significantly higher in DCD recipients until 48 hours post-transplant ($p<0.001$). The incidences of acute kidney injury and ischemic cholangiopathy were greater in DCD recipients (32.6% vs. 15% [$p<0.001$] and 9.1% vs. 1.1% [$p<0.001$] respectively). With appropriate recipient selection, the use of DCD's, including those deemed marginal, can be used safely and produce outcomes comparable to those seen when using DBD grafts in similar recipients.

2.2.2 Introduction

Liver transplantation is the only curative option for patients with end-stage liver disease, irrespective of aetiology. Liver disease is the 5th biggest cause of death in the United Kingdom (UK) and the mortality rate continues to increase(1). In the past decade, the number patients on the active UK liver transplant register has more than doubled (253 in 2004 to 611 patients in 2015)(2, 3) and in response, there has been a ten-fold increase in the number of transplants using grafts from circulatory death donors (DCD) (13 in 2003 to 177 in 2015)(2, 3). Last year in the UK, 15% of patients died or were removed from the liver transplant waiting list(3) – a proportion of whom may have been saved had an appropriate donor become available.

Donation following brain death has been the preferred practice in countries that use deceased donation since the Harvard criteria were introduced in 1968, as it permits oxygenation of the organ until the point of preservation(4). In the late 1980's, interest in DCDs grew due to the increasing demand for organs. Following long-term success with kidney transplants using DCD grafts(5), specialists turned their attention to the use of DCD liver grafts, with outcomes benefiting from decades of improved preservation methods, immunosuppression and surgical techniques. However, DCD organs are still used judiciously and many factors are taken into account to minimise the likelihood of an adverse outcome.

In the UK, virtually all DCD retrievals are from controlled donors (Maastricht III)(6), enabling the retrieval team to closely monitor the functional warm ischemic time (FWIT) – the point at which oxygen saturations fall below 80% or systolic blood pressure below 50mmHg until aortic perfusion occurs(7). Organ ischemia triggers a complex cascade of cellular and molecular events, including the release of pro-inflammatory mediators and chemotaxis of cell types that

initiate progressive immunological processes. During the reperfusion phase, “the reflow paradox” promotes infiltration of the tissues by leucocytes and cellular injury occurs through a series of pathways that include lipid peroxidation and the creation of reactive oxygen species(8). The FWIT increases the recipient’s risk of post-reperfusion syndrome(9), primary non-function (PNF), delayed graft function (DGF)(10-12), ischemic cholangiopathy (IC)(13-16), and acute and chronic kidney disease(17). The cost of DCD transplants can also be 50% higher – IC for example, is associated with a higher readmission rate, multiple invasive procedures and in some cases retransplantation(18-21).

Between April 2013 and March 2014 University Hospitals Birmingham NHS Foundation Trust performed 189 liver transplants in 171 patients, 44 of which were performed using DCD grafts. It has a very active DCD programme, utilising over 80% of the DCD grafts that are offered(22). In 2014, a meta-analysis by O’Neil concluded that DCD transplantation was associated with an increase in biliary complications, IC, graft loss and mortality(23). Our aim was to investigate whether this statement was applicable to our patient population and as such, present the largest single-center study of its kind.

2.2.3 Materials and Methods

University Hospitals Birmingham NHS Trust approved this study (CARMS-02246). Adult patients (>16 years of age) who underwent primary orthotopic liver transplantation between July 2004 and July 2014 were initially included. Paediatric transplants, recipients of living donors, split livers, machine-perfused grafts, domino grafts or multiple organs were excluded, as were patients with a primary aetiology of acute liver failure (as they would be less likely to receive a DCD graft). The hospital transplant database is maintained prospectively and contains information on the donor and recipient, the retrieval process, peri-operative period, complications and follow-up.

During the retrieval process, most teams in the UK use aortic and portal perfusion to flush the graft effectively (with the only exception in DBD retrievals where the pancreas and small bowel are also being procured). The preferred preservation fluid regimen for procurement without pancreas is 3-4 litres of heparinised Marshall's solution (a low-viscosity solution) via the aorta under 200mmHg pressure (which results in superior organ washout than gravity-alone perfusion(24, 25)), 1 litre of University of Wisconsin solution (UW) under gravity via the portal vein and an additional UW back-table flush through the artery and portal vein. During DCD procurement the gallbladder is opened after vascular perfusion, the bile duct is then divided and then flushed via the gallbladder opening, as well as on the back table. Donor FWIT is generally limited within the UK to 30 minutes for DCD liver procurement. Cold ischemic time (CIT) is defined as the time between cold aortic perfusion and re-perfusion at implantation via either the portal vein or hepatic artery.

Primary endpoints were overall graft and patient survival. Secondary endpoints included relevant post-operative complication rates within 90 days, incidence of post-operative acute

kidney injury (AKI), ventilator duration, length of ITU stay, length of hospital stay, biliary complications (cholangitis, leak, IC, anastomotic stricture) and vascular complications (hepatic artery stenosis and hepatic artery thrombosis) over the follow-up period. AKI was defined as peak serum creatinine ≥2.0–2.9 times baseline and therefore included the "Risk, Injury, Failure, Loss and End-stage kidney disease" (RIFLE) categories. IC was defined as non-anastomotic biliary strictures in the presence of a patent hepatic artery, confirmed on magnetic resonance cholangiopancreatography (MRCP) or endoscopic retrograde cholangiopancreatography (ERCP) by one of two consultant specialist radiologists. The donor risk index (DRI) and balance of risk score (BAR) were also calculated for the matched recipients.

Propensity score matching (PSM) was used to match patients receiving DCD livers to those receiving DBD livers. PSM is a recognized method of balancing covariates in two groups in order to reduce selection bias(26). In our analysis, we included all donor and recipient variables of clinical relevance to the post-transplant outcome measures in the propensity score model, namely: donor age and BMI, days on ventilator, CIT, recipient age and BMI, recipient primary diagnosis and MELD (Supplementary Table S2.1). A total of 187 DCD recipients were successfully matched to DBD recipients using these criteria, with the remaining 47 DCD recipients excluded from the matched analysis. Year of transplant was not used as a variable because its inclusion reduced the number of matched pairs. Additional information regarding the PSM process can be found as supplementary material.

Comparisons between organ types in the unmatched data were performed using t-tests for continuous factors, and Fisher's exact tests for categorical variables. After matching, normally distributed continuous variables and non-parametric continuous variables were compared using the paired *t*-test and Wilcoxon signed rank test respectively. McNemar's test was used to

compare categorical data. Survival was estimated using Kaplan-Meier plots with log-rank test for differences, and adjusted survival was determined using Cox proportional hazard analyses. Data were analyzed using SPSS v21® (© IBM Corporation). Continuous variables were expressed as mean and standard deviation (SD), or median and interquartile range (IQR) as appropriate.

2.2.4 Results

2.2.4.1 Donor and recipient characteristics

973 patients underwent primary whole liver transplantation for chronic liver disease between July 2004 and July 2014, of whom 234 (24.0%) received DCD and 739 (76.0%) DBD organs. All patients had at least 90 days follow-up. The mean donor age was 50.1, 52.4% were male and the mean BMI was 26.6. Donor cause of death was consistent with national data(22). The mean recipient age was 53.1 years, 65.3% were male and the mean BMI was 27.5. The most common causes of chronic disease were alcoholic cirrhosis (25.9%), hepatitis C cirrhosis (21.2%), primary biliary cirrhosis (PBC) (12.9%) and primary sclerosis cholangitis (PSC) (10.5%). The mean MELD score was 16, which is in keeping with previous results from our centre(17) (Tables 2.1 and 2.2). The number of transplants using DCD grafts increased from 1 in 2004 to 49 in 2014 (Figure 2.1). As per the British Transplantation Society (BTS) Guidelines (2013), 83.8% of the 234 DCD grafts were classified as “marginal” and 41.0% fulfilled 2 or more of the following criteria which define marginality; age > 50, weight >100kg, ICU stay > 5 days, FWIT > 20 minutes, CIT > 8 hours and >15% steatosis(27).

Table 2.1 Demographics of whole data and associated standardised differences

	Total		**DCD**		**DBD**		**St. Diff.**
n	**973**		**234**		**739**		
Donor Factors							
Age	50.1	(14.9)	49.1	(16.6)	50.4	(14.3)	0.084
Sex*							
Male	510	(52.4%)	132	(56.4%)	378	(51.2%)	0.104
Female	463	(47.6%)	102	(43.6%)	361	(48.8%)	0.104
BMI*	26.6	(4.9)	25.2	(4.0)	27.0	(5.0)	0.398
Virology							
CMV +ve	477	(49.0%)	113	(48.3%)	364	(49.3%)	0.020
Hepatitis B +ve	27	(2.8%)	4	(1.7%)	23	(3.1%)	0.092
Hepatitis C +ve	13	(1.3%)	2	(0.9%)	11	(1.5%)	0.055
Days on ventilator*	2.4	(3.5)	2.1	(3.4)	2.5	(3.5)	0.116
Cause of death							
Cerebrovascular accident	636	(65.4%)	124	(53.0%)	512	(69.3%)	0.339
Head injury	115	(11.8%)	38	(16.2%)	77	(10.4%)	0.171
Cardiac arrest	67	(6.9%)	28	(12.0%)	39	(5.3%)	0.240
Malignancy	23	(2.4%)	4	(1.7%)	19	(2.6%)	0.062
Other	132	(13.6%)	40	(17.1%)	92	(12.4%)	0.133
Location of donor							
Local	129	(13.3%)	33	(14.1%)	96	(13.0%)	0.032
Regional	213	(21.9%)	60	(25.6%)	153	(20.7%)	0.116
National	631	(64.9%)	141	(60.3%)	490	(66.3%)	0.125
Retrieval Team							
Birmingham	696	(71.5%)	149	(63.7%)	547	(74.0%)	0.224
Other	277	(28.5%)	85	(36.3%)	192	(26.0%)	0.224
DCD FWIT (mins)	21	(15-25)	20.6	(6.8)	-	-	-
CIT (hrs)*	8.3	(2.3)	7.1	(1.6)	8.7	(2.4)	0.784
Marginal DCD**			201	(83.8%)			
>1 Marginal feature			127	(41.0%)			
Recipient Factors							
Age*	53.1	(10.6)	55.3	(9.3)	52.5	(10.9)	0.276
Sex*							
Male	635	(65.3%)	148	(63.2%)	487	(65.9%)	0.056
Female	338	(34.7%)	86	(36.8%)	252	(34.1%)	0.056
BMI*	27.5	(5.1)	26.7	(4.9)	27.7	(5.2)	0.198
MELD*	16	(5.7)	13.8	(4.7)	16.2	(5.8)	0.455
HCC present	266	(27.3%)	88	(37.6%)	178	(24.1%)	0.295
Recipient Diagnosis*							
Alcohol-related cirrhosis	252	(25.9%)	63	(26.9%)	189	(25.6%)	0.030
Hepatitis C cirrhosis	206	(21.2%)	54	(23.1%)	152	(20.6%)	0.061
Primary biliary cirrhosis	126	(12.9%)	41	(17.5%)	85	(11.5%)	0.171
PSC	102	(10.5%)	22	(9.4%)	80	10.8%)	0.046
NASH	61	(6.3%)	17	(7.3%)	44	(6.0%)	0.052
Hepatitis B cirrhosis	42	(4.3%)	11	(4.7%)	31	(4.2%)	0.024
Other	184	(18.9%)	26	(11.1%)	158	(21.4%)	0.282

Values expressed as mean (standard deviation) or number (percentage) as appropriate.
* Variables used in propensity matching process
** Marginal as described by British Transplantation Society UK Guidelines 2013

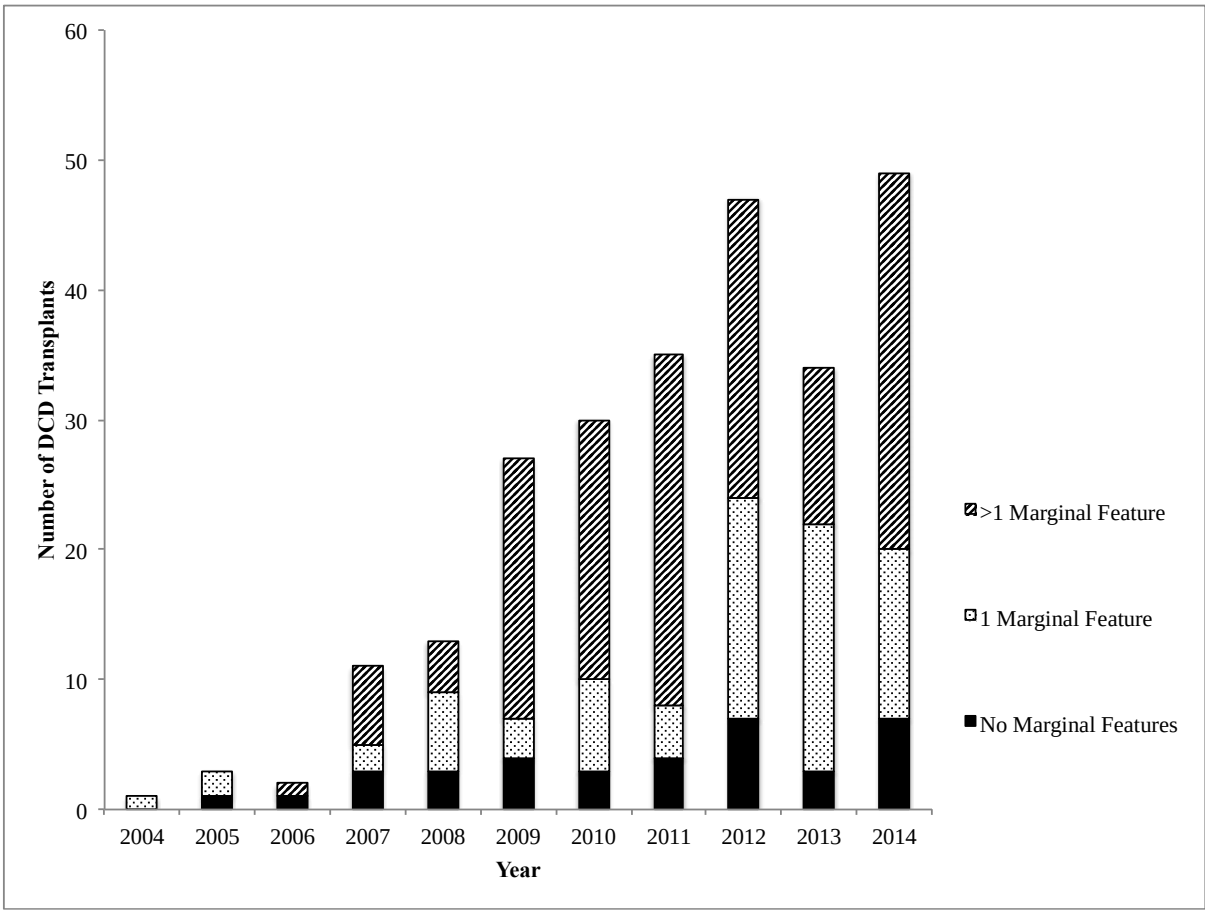

Figure 2.1 Number of transplants using DCD donors at UHB and proportions of marginal donors.

Following the PSM, 187 pairs of patients were closely matched with the majority of variables found to have standardised differences <0.100 (Table 2.2). 82.9% of matched DCD were classified as marginal as previously described. There was a trend towards a higher BAR score in the DCD group (4.88 vs. 4.40 p=0.053) and DRI was significantly higher for these recipients (2.82 vs. 1.80 p<0.001). However, this difference was lost when graft type was removed from the DRI equation (factor of 0.411), resulting in means of 1.87 vs. 1.80 (p=0.077). 47 DCD recipients were not matched to DBD recipients (u-DCD). Their demographics and outcomes are presented in tables 2.2-2.4 for comparison. The PSM process does not specify why a match cannot be performed for a particular case, however on analysis of all u-DCD's, it is likely that a lower MELD score prohibited a successful match to a DBD recipient. With respect to the

demographics of this particular subset, they were otherwise very similar to the matched DCD cohort (Table 2.2).

Table 2.2 Demographics of propensity-matched groups and associated standardised differences

	DCD		***DBD***		***St. Diff***	***u-DCD***	
n	**187**		**187**			**47**	
Donor Factors							
Age	49.4	(16.2)	47.7	(14.7)	0.110	48.3	(18.0)
Sex*							
Male	102	(54.5%)	106	(56.7%)	0.044	30	(63.8%)
Female	85	(45.5%)	81	(43.3%)	0.044	17	(36.2%)
BMI*	25.5	(4.1)	25.4	(4.7)	0.023	24.1	(3.6)
Virology							
CMV +ve	94	(50.3%)	105	(56.1%)	0.116	19	(40.4%)
Hepatitis B +ve	3	(1.6%)	6	(3.2%)	0.105	1	(2.1%)
Hepatitis C +ve	2	(1.1%)	3	(1.6%)	0.043	-	-
Days on ventilator*	2.2	(3.5)	2.3	(2.5)	0.033	2.1	(3.2%)
Cause of death							
Cerebrovascular accident	100	(53.5%)	115	(61.5%)	0.162	24	(51.1%)
Head injury	30	(16.0%)	31	(16.6%)	0.016	8	(17.0%)
Cardiac arrest	22	(11.8%)	7	(3.7%)	0.306	6	(12.8%)
Malignancy	4	(2.1%)	4	(2.1%)	0.000	-	-
Other	31	(16.6%)	30	(16.0%)	0.016	9	(19.1%)
Location of donor							
Local	24	(12.8%)	30	(16.0%)	0.091	9	(19.1%)
Regional	49	(26.2%)	35	(18.8%)	0.178	11	(23.4%)
National	114	(61.0%)	122	(65.2%)	0.087	27	(57.4%)
Retrieval Team							
Birmingham	115	(61.5%)	160	(85.6%)	0.568	34	(72.3%)
Other	72	(38.5%)	27	(14.4%)	0.568	13	(27.7%)
DCD FWIT (mins)	20	(7)				22	(7)
CIT (hrs)*	7.3	(1.6)	7.4	(2.0)	0.094	6.3	(1.4)
Marginal DCD	155	(82.9%)				41	(87.2%)
>1 Marginal feature	75	(40.1%)				23	(48.9%)
Recipient Factors							
Age*	54.8	(9.7)	55.2	(10.0)	0.041	57.5	(7.6)
Sex*							
Male	119	(63.6%)	188	(59.9%)	0.076	29	(61.7%)
Female	68	(36.4%)	75	(40.1%)	0.076	18	(38.3%)
BMI*	26.9	(4.9)	26.9	(4.8)	0.000	26.1	(4.7)
MELD*	14.0	(4.8)	13.7	(4.4)	0.065	10.7	(5.4)
HCC present	67	(35.8%)	57	(30.5%)	0.113	21	(44.7%)
Recipient Diagnosis*							
Alcohol-related cirrhosis	47	(25.1%)	43	(23.0%)	0.049	16	(34.0%)
Hepatitis C cirrhosis	48	(25.7%)	42	(22.5%)	0.075	6	(12.8%)
Primary biliary cirrhosis	29	(15.5%)	38	(20.3%)	0.125	12	(25.5%)
PSC	17	(9.1%)	17	(9.1%)	0.000	5	(10.6%)
NASH	12	(6.4%)	10	(5.3%)	0.047	5	(10.6%)
Hepatitis B cirrhosis	9	(4.8%)	9	(4.8%)	0.000	2	(4.3%)
Other	25	(13.4%)	28	(15.0%)	0.046	1	(2.1%)
Risk Stratification					**p-value****		
DRI	2.82	(0.64)	1.80	(0.34)	<0.001	2.72	(0.61)
DRI minus donor type	1.87	(0.42)	1.80	(0.34)	0.077	1.81	(0.41)
BAR	4.88	(2.66)	4.40	(2.49)	0.053	3.7	(2.7)

* Variables used in propensity matching process ** Paired t-test

2.2.4.2 Post-operative course, outcome and complications

There was no significant difference between the paired DCD and DBD recipients with respect to the post-operative course (Table 2.3). AST was not normally distributed, therefore the values were logged and reported as geometric means. The resulting values were significantly higher in DCD recipients until 48 hours post-transplant (2-tailed t-test $p<0.001$), returning to a level similar to that seen in DBD recipients at day 5 (Figure 2.2).

Table 2.3 Post-operative course and outcomes for matched groups and unmatched DCDs.

	DCD		***DBD***		***p-value***	***u-DCD***	
Post-operative course*							
Operating time (hrs)	4.8	(4.0-5.7)	4.9	(4.3-6.0)	0.104	4.9	(4.1-5.9)
Days ventilated	1	(1-2)	1	(1-2)	0.331	1	(1-2)
Days in ITU	3	(2-6)	2	(2-4)	0.066	3	(1-5)
Length of stay (days)	10	(7-15)	10	(7-15)	0.870	7	(9-17)
Estimated graft survival							
< 30-days	90.4%	(0.022)	93.6%	(0.018)		95.7%	(0.029)
<1-year	82.7%	(0.028)	86.1%	(0.025)		95.7%	(0.029)
*Overall Graft Survival***					0.166		
Estimated patient survival							
< 30-days	94.1%	(0.017)	96.3%	(0.014)		97.9%	(0.021)
< 1-year	87.6%	(0.025)	88.8%	(0.023)		95.6%	(0.030)
*Overall Patient survival***					0.519		

Values expressed as median (interquartile range), number (percentage) or percentage (standard error) as appropriate.
*Wilcoxon Signed Rank Test
**Log Rank (Mantel-Cox)
Graft survival includes all deaths as well as patients who require re-transplantation.

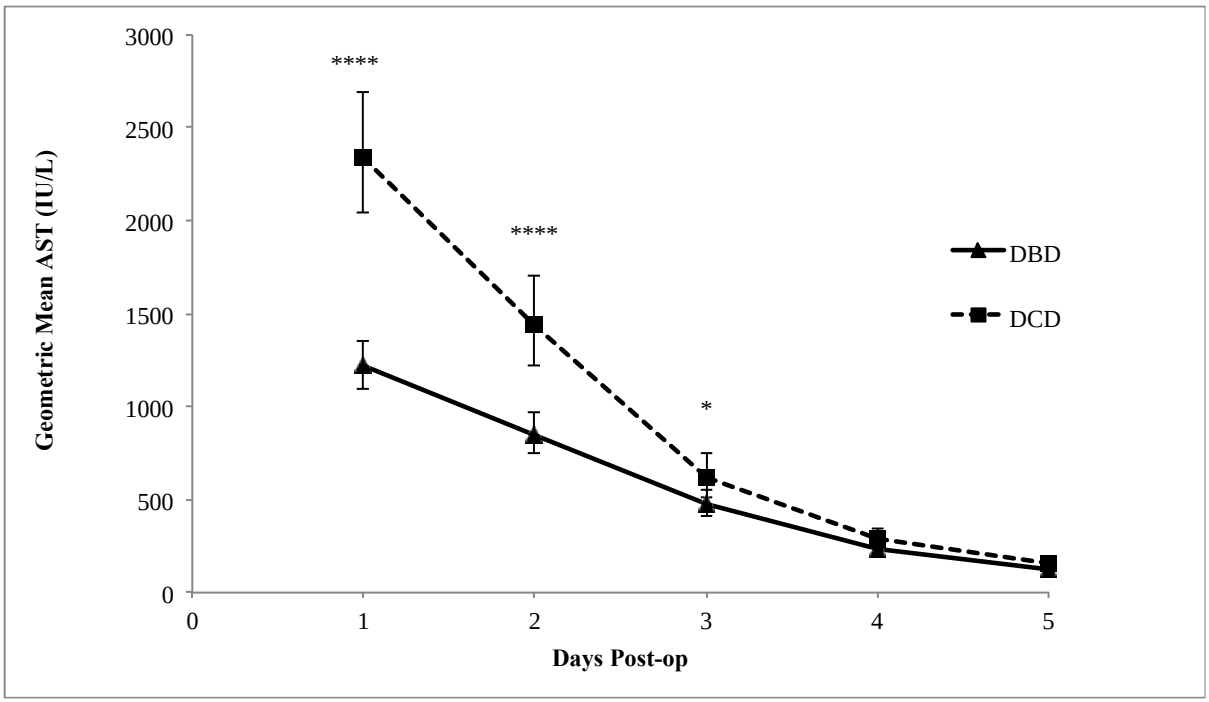

Figure 2.2 Chart of geometric mean post-operative AST.

(187 patients in each matched group, number available for analysis DCD n=101, DBD n=173)

****p<0.001, *p=0.030.

Kaplan Meier analysis of overall graft and patient survival found no significant differences for either outcome between the paired DCD and DBD patients (p=0.162 and 0.519 respectively). A stratified cox regression returned a hazard ratio for mortality of 1.16 (95% CI: 0.68 – 2.01, p=0.579) for DCD, relative to DBD patients (Figures 2.3 and 2.4). Supplementary Table S2.2 contains the aetiology of re-transplantation (re-graft) and death for matched DCD and DBD recipients as well as unmatched DCD recipients. For all matched recipients, the most common causes of death were recurrence of HCC (23.5%), sepsis (18.0%), pulmonary complications (13.5%), HAT (11.2%) and cardiac complications (10.1%). The primary causes of graft loss and death within the first 30 days were PNF (DCD n=5 [2.6%] resulting in 2 deaths; DBD n=2 [1.1%]) and HAT for all matched recipients. After 1 year, recurrence of HCC accounted for most deaths.

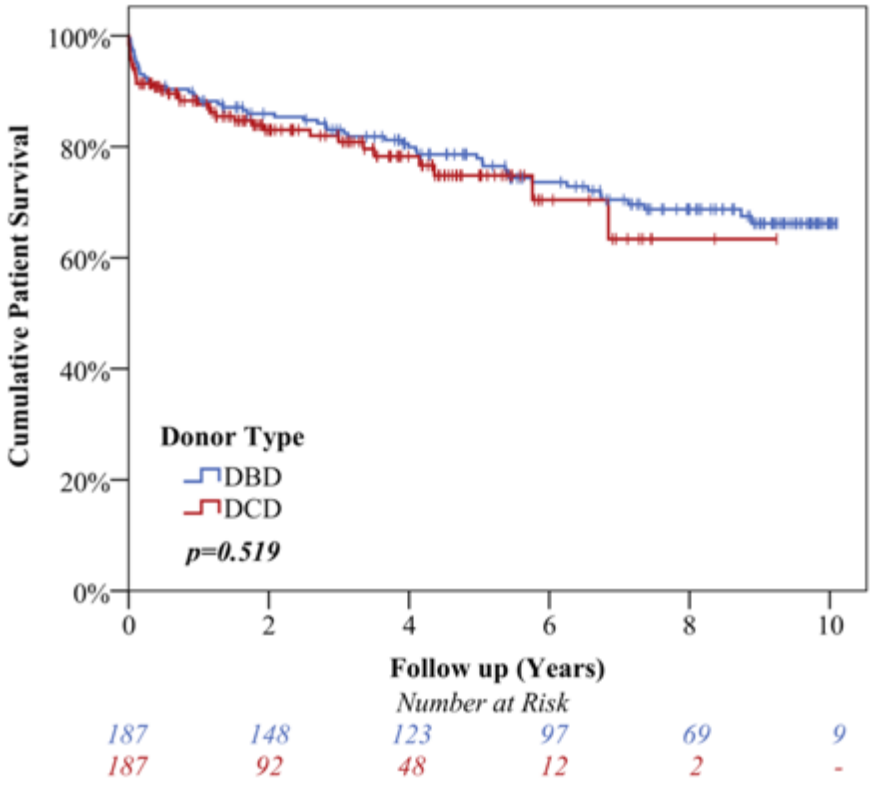

Figure 2.3 Kaplan-Meier curve of patient survival.

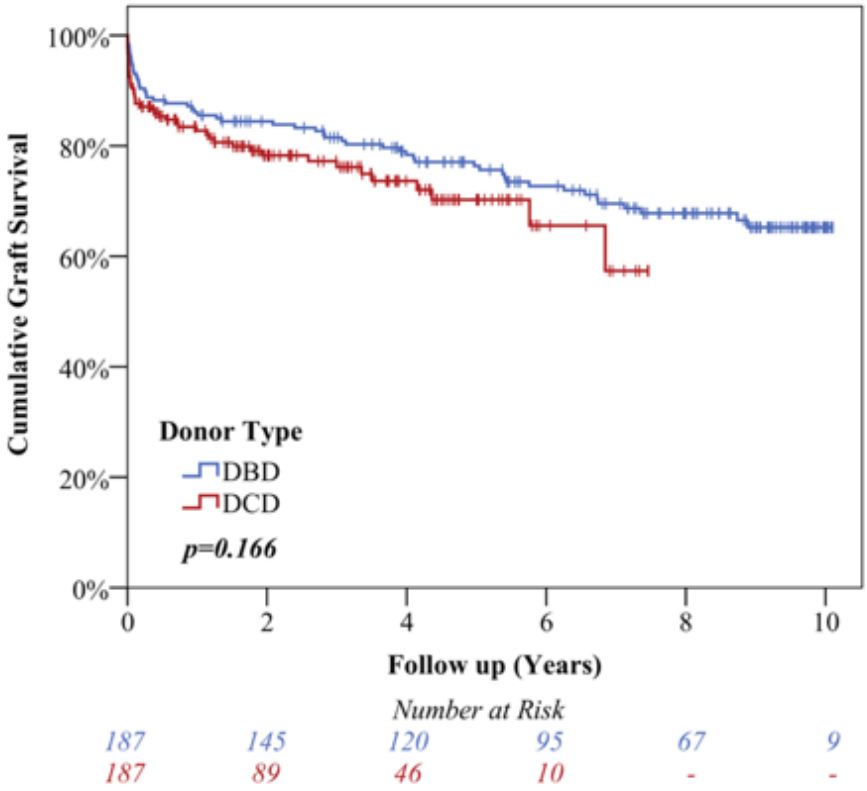

Figure 2.4 Kaplan-Meier curve of graft survival.

The incidence of AKI was significantly greater in DCD recipients (32.6% vs. 15.0% p<0.001) and there was a trend in the same group towards a higher incidence of post-op bleeding (12.8% vs. 7.0% p=0.080). On further analysis of renal function, there was no difference in urea or creatinine between matched recipients one year post-transplant (Table 2.4), however, irrespective of graft type, patients who required short-term filtration went on to have elevated levels of urea and creatinine at 1 year (filtration vs no filtration; urea (St. dev.) 10.2 (3.3) vs 8.0 (2.5) p<0.001; creatinine (Std. dev.) 124.6 (33.1) vs 105.6 (27.7) p<0.001). There was a significantly higher incidence of IC in DCD recipients (9.1% vs. 1.1% p<0.001) with similar rates of cholangitis, bile leak, anastomotic biliary stricture, hepatic artery stenosis and hepatic artery thrombosis (Table 2.4).

Table 2.4 Post-operative complications for matched groups and unmatched DCDs

	DCD		**DBD**		**p-value**	**u-DCD**	
<90-day post-op complications*							
Cardiac complication	16	(8.6%)	11	(5.9%)	0.405	2	(4.3%)
Post-op bleeding	24	(12.8%)	13	(7.0%)	0.080	1	(2.1%)
Respiratory complication	20	(10.7%)	29	(15.6%)	0.188	3	(6.4%)
Post-transplant diabetes	11	(5.9%)	18	(9.6%)	0.230	2	(4.3%)
Acute kidney injury	61	(32.6%)	28	(15.0%)	**<0.001**	5	(10.6%)
Renal function 1-year post-transplant							
Urea (mmol/L)	8.0	(2.6)	8.7	(3.0)	0.847	8.2	(2.2)
Creatinine (mmol/L)	105	(48)	115	(30)	0.763	102	(25)
Biliary complications**							
Cholangitis	8	(4.3%)	9	(4.8%)	0.791	-	-
Bile leak	9	(4.8%)	5	(2.6%)	0.270	1	(2.1%)
Ischemic cholangiopathy	17	(9.1%)	2	(1.1%)	**<0.001**	5	(10.6%)
Anastomotic stricture	27	(14.4%)	23	(12.2%)	0.289	6	(12.8%)
Vascular complications**							
Hepatic artery stenosis	5	(2.7%)	2	(1.1%)	0.180	-	-
Hepatic artery thrombosis	9	(4.8%)	6	(3.2%)	0.416	3	(6.4%)
Combined	14	(7.5%)	8	(4.3%)	0.148	3	(6.4%)

*Reported as rates at 90 days, with p-values from McNemar Test.
**Reported as Kaplan-Meier *estimated* overall rates with p-values from Log Rank (Mantel-Cox) tests of all available follow up.

2.2.5 Discussion

This retrospective propensity-matched study using data from the largest single-centre DCD cohort in the literature has demonstrated similar graft and patient survival following transplant with DCD and DBD grafts. With the exception of IC and AKI, we have also demonstrated similar post-operative complication rates.

PSM is an accepted method of estimating the effect of a treatment by attempting to reduce bias due to confounding variables(26, 28). We performed a 1:1 match, as this is the most commonly accepted form of this technique, which allowed us to determine the impact of receiving a DCD graft. Despite supposedly resulting in increased precision, cohort studies matching at ratios of 1:n>1 have been shown to result in somewhat higher levels of bias(29, 30). Any bias introduced by year of transplant, which was excluded from the PSM process, is expected to be minimised by the fact that the number of DCD transplants performed during the early years of the DCD program were small and as techniques for the utilisation of DCDs improved, numbers increased.

Despite their use remaining controversial, the transplant community must continue to maximise the pool of DCD grafts in order to respond to the increasing incidence of chronic liver disease. There is a mixed picture in the literature with studies arising from early registry data showing up to 30% graft failure(10, 11) whereas smaller high-volume single centre studies have demonstrated similar graft and patient survival(9, 31, 32). A recent meta-analysis demonstrated higher incidence of biliary complications, decreased 1-year graft survival and 3-year patient survival in DCD recipients. They did, however, comment on significant unexplained differences in effect size between centres(23) – a sentiment echoed by Callaghan *et al* in 2013 in their UK cohort study(33). Our data demonstrates similar graft and patient survival in a

matched cohort of 'low-risk' recipients, albeit with a weak trend towards reduced graft survival in the DCD cohort.

AST levels within the first 5 days following transplant reflect the damage at a hepatocellular level. In 2012, the Trust Biochemistry department changed their policy on the testing of AST and began using ALT as the standard transaminase in transplant patients. This meant that 46% of our DCD cohort was excluded from the AST analysis (compared to 9% of DBDs). In spite of this, we were still able to demonstrate a significant difference between AST levels within the first 48 hours post-transplant (Figure 2.2). Of note, average peak ALT was also higher in DCD recipients. Leithead *et al* were the first to show that peak AST was the only variable associated with the development of AKI(17). They also demonstrated that ischemia-reperfusion injury was strongly related to post-operative AKI in DBD recipients(34). Although AST is also released from damaged renal tissue, peak AST has been shown to correlate strongly with histological grading of hepatic injury(35). Peak AST was higher in DCD recipients and in terms of early complications, AKI was the only complication found to differ significantly between the two organ types ($p<0.001$). There was a trend towards more post-operative bleeding in DCD recipients, which could be an indicator of inferior graft function and disordered clotting cascades ($p=0.080$). Transplants for HCC or PSC in recipients with lower MELD scores tend to take less time than transplants in patients with higher MELD scores (such as those with ALD and recurrent spontaneous bacterial peritonitis). In these cases, the full extent of post-reperfusion coagulopathy may occur following abdominal closure and therefore we advocate a hemostatic pause before completing the biliary anastomosis to allow for this in such situations.

When considering late complications, De Olivera *et al* demonstrated levels of IC not seen previously in the literature (2.5% incidence in DCD cohort) and hypothesised that it was due

to a policy of only accepting grafts exposed to <30 minutes of warm ischemia and restricting CIT to 8 hours(36). A balance must be reached, as stringent selection criteria will significantly reduce the number of available organs. Our data shows a rate of IC in DCD recipients of 9.1% compared to 1.1% in DBD recipients ($p<0.001$) and anastomotic biliary stricture rates of 14.4% and 12.2% for DCD and DBD recipients ($p=0.289$). These are consistent with a large body of literature(13-15, 37, 38). Patients with symptoms or liver function tests indicative of IC were imaged using MRCP. If confirmed, patients were managed conservatively (most patients maintained acceptable biochemistry) and if their symptoms or biochemistry warranted, patients were re-listed for transplantation. In this matched cohort, no patients required re-listing and one patient with IC developed biliary sepsis and died suddenly as a result.

Our propensity match used CIT as a confounding variable, hence the mean times were similar between the groups (means of 7.3 hours for DCD and 7.4 hours for DBD recipients, standardised difference 0.094). The mean FWIT for DCD grafts was 20 minutes, which lies just within the "marginal" range for FWIT according to BTS guidelines. When using standard procurement and preservation techniques, limiting the FWIT in DCD retrievals is crucial in reducing the development of IC. When compared to other determinants of marginality, it is likely FWIT has the greatest impact on graft function after transplantation. It has been calculated that one minute of additional warm ischemia can increase the risk of IC or hepatic necrosis by up to 16%(39). Normothermic machine liver perfusion (NMLP) has shown promise in terms of *in situ* normothermic regional perfusion(40), preservation(41), viability testing(42) and reconditioning of liver grafts. Our centre performed the first transplantation of a discarded liver graft after viability testing using NMLP(43). In the future, cellular therapy may also offer some benefit in terms of reducing the immunological insults triggered by warm ischemia(44, 45).

The donor risk index introduced by Feng *et al* focuses on donor factors, as well as CIT and retrieval location (which itself is closely linked to CIT) and has been reported to be predictive of graft survival(46). The mean DRI for our DCD recipient cohort of 2.82 would ordinarily predict a 1-year graft survival of 71.4%. In this cohort, 1-year graft survival was 87.6% (the predicted graft survival rate for a DRI score of <1). After removing DCD as a determining factor of graft survival, the mean DRI reduced to 1.87 (vs. DBD 1.80 p=0.077). The BAR score was devised in 2011 and based upon 37,255 patients in the UNOS (United Network for Organ Sharing) database. (47). Given that neither warm ischemia nor donor type are taken into account in BAR scoring, the mean BAR score for DCD recipients was 4.88 and 4.40 for DBD recipients. The survival rates of our matched cohorts (1-year 87.6% DCD and 88.8% DBD) are in keeping with published data that suggests a score of 4-5 predicts 1-year patient survival to be 89-92%. Our mean BAR score is low because no patients underwent re-transplant, or were on pre-operative life support, and our mean MELD score was 16 (+-5.7). This is lower than 20, the average MELD of patients in the USA prior to transplant. An explanation of why the MELD across the cohort is low is because of the exclusion of acute liver failure and re-transplant patients from our analysis. Patients with HCC also generally have a lower MELD score than those with end stage chronic liver disease – 27.3% of the whole cohort had HCC with a mean MELD of 14. The mean MELD scores within the matched groups are even lower (14 DCD and 13.7 DBD). DCD recipients are generally chosen as they have a lower MELD than the typical chronic liver disease patient and DBD recipients were matched to them. Due to the size of the DCD cohort, it was not possible to perform any meaningful matched analysis on a higher MELD subset. With the introduction of machine perfusion, in the future it may be possible to safely transplant marginal donors into higher risk recipients and compare outcomes in such a cohort.

There are several reasons why we the authors believe we can achieve such results. Other than being simply a high-volume centre, we employ a number of strategies. At our hospital, the decision to choose a recipient for a DCD graft is made between the transplant surgeon and hepatology consultant and low risk recipients are chosen for DCD grafts on the basis that they can better cope with a reperfusion insult that can occur when using marginal grafts. They are also usually easier to explant which helps keep CIT to a minimum. Low risk in terms of aetiology usually means patients with low MELD scores and/or those with HCC (hence the 37.6% incidence of HCC in DCD recipients compared to 24.1% in DBD recipients in the whole cohort [Table 2.1]). In addition, when transplanting marginal grafts our consultant surgeons are acutely aware of the importance of keeping the second period of warm ischemia at implantation to a minimum. Recipient's older than 50 years are only chosen if they do not have diabetes or cardiovascular disease and DCD grafts are rejected if they are moderately steatotic or stiff following preservation. CIT is kept strictly under 8 hours and we do accept livers that have been exposed to a FWIT of up to 40 minutes (but only if other criteria were within normal range). To extend into the category of marginal donors, donor age is the boundary that we invariably push, frequently accepting DCD grafts from donors over the age of 50.

A number of the consultants have started to employ the technique of hepatic artery-first (HA-first) reperfusion when utilising marginal grafts, as they believe it reduces the risk of post-reperfusion cardiovascular instability. A matched study of 40 DCD transplants performed at our centre showed that HA first reperfusion increased intra-operative stability and reduced the incidence of post-reperfusion syndrome and peak post-transplant bilirubin(48). A much larger study is required to investigate the benefits of HA-first reperfusion further. In addition to what has already been discussed in the methods in terms of procurement, DCD donors are ordinarily

withdrawn on ITU, as long as it is not situated too far from the operating room, in which case they are withdrawn in the adjacent anaesthetic room. Following asystole, there is a 5-minute stand-down prior to bringing the patient to the operating room. Thirty-eight point five (38.5%) of DCD retrievals were performed by teams from other centres (compared to 14.4% of DBD retrievals) – another indication of our willingness to accept and transplant marginal donors that have been rejected by other centres. We are happy to do so because of the understanding that all UK retrieval teams follow the same rigorous procurement guidelines laid out by the BTS. We do not utilise thrombolytics or other specific techniques to target the microcirculation. Vendrell *et al* demonstrated there was no role for the use of exogenous fibrinolysis(49). A study by Simon *et al* demonstrated no formation of microthrombi in DCD biopsies at different stages of cold storage, which they felt made it less likely that microthrombi are involved in the pathophysiology of non-anastomotic strictures after liver transplantation(50). Time from extubation to arrest (even if oxygen saturations or blood pressure remain stable) is generally limited to 60 minutes, after which a liver would not normally be procured even if a patient arrested following a subsequently acceptable FWIT whilst waiting the remaining 2-3 hours for kidney procurement. However, this group could be a target for the viability testing of livers using NMLP(43).

In conclusion, this propensity-matched single-centre cohort study supports the notion that with appropriate recipient selection and other techniques, the use of DCD's, including those deemed marginal as per national guidelines, can be used safely and produce outcomes comparable to those seen when using DBD grafts in similar recipients. In spite of accepted risks such as acute kidney injury and ischemic cholangiopathy, they remain a crucial source of donors at a time when the demand for liver transplantation is increasing.

2.2.6 Acknowledgements

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2.2.7 Disclosure

The authors have nothing to disclose

2.2.8 Supplementary Material

2.2.8.1 Propensity Score Matching

A multivariable binary logistic regression model is first produced, in order to predict the probability of each patient receiving a DCD liver, based on a range of factors of clinical relevance. This probability is referred to as the propensity score, and patients with similar scores are then paired together. Whilst individual pairs of patients are not necessarily well matched on all of the clinically relevant factors being considered, the two groups of paired patients (DCD and DBD) as a whole will be balanced with respect to these factors. Once the score had been produced for each patient, the "case-control matching" dialogue in IBM SPSS Statistics 22 (IBM Corp. Armonk, NY) was used to pair the DCD and DBD patients. Patients were matched 1:1 without replacement, meaning that each DCD patient could only be matched to one DBD patient, and that each DBD patient could only be included in one pair. A caliper of 0.05 was used when matching, meaning that DCD patients could only be matched to DBD

patients if the difference between the propensity scores was within ±0.05.

Supplementary Table S2.1 Logistic regression model used to generate the propensity score

Donor Factors	***Odds Ratio (95% CI)***	***p-Value***
Age		0.020
<= 27	1	-
28 - 38	0.39 (0.18 - 0.82)	0.013
39 - 43	0.22 (0.09 - 0.51)	<0.001
44 - 47	0.45 (0.22 - 0.95)	0.035
48 - 52	0.65 (0.33 - 1.29)	0.218
53 - 55	0.27 (0.11 - 0.70)	0.007
56 - 59	0.67 (0.33 - 1.36)	0.268
60 - 64	0.50 (0.24 - 1.04)	0.062
65 - 69	0.68 (0.33 - 1.39)	0.290
70+	0.49 (0.22 - 1.10)	0.083
Sex		0.182
Female	1	-
Male	1.31 (0.88 - 1.93)	0.182
BMI	0.94 (0.90 - 0.98)	0.004
Days on ventilator	0.98 (0.92 - 1.04)	0.450
Cold ischaemic time (hours)	0.67 (0.61 - 0.73)	<0.001
Recipient Factors		
Age		0.595
<= 39	1	-
40 – 45	1.13 (0.48 - 2.70)	0.776
46 – 49	1.06 (0.44 - 2.58)	0.894
50 – 52	1.64 (0.69 - 3.87)	0.260
53 – 55	1.45 (0.61 - 3.44)	0.397
56 – 58	0.89 (0.37 - 2.15)	0.801
59 – 60	2.13 (0.90 - 5.07)	0.087
61 – 62	1.30 (0.54 - 3.12)	0.563
63 – 65	1.51 (0.65 - 3.52)	0.338
66+	1.51 (0.64 - 3.53)	0.347
Sex		0.865
Female	1	-
Male	0.96 (0.61 - 1.51)	0.865
Diagnosis		0.029
Alcoholic Cirrhosis	1	-
Hepatitis B Cirrhosis	0.66 (0.26 - 1.66)	0.379
Hepatitis C Cirrhosis	1.18 (0.70 - 1.97)	0.539
Non-alcoholic Steatohepatitis	1.41 (0.66 - 3.02)	0.381
Other	0.45 (0.25 - 0.82)	0.009
Primary Biliary Cirrhosis	1.29 (0.69 - 2.42)	0.419
Primary Sclerosing Cholangitis	0.81 (0.41 - 1.59)	0.539
BMI	0.99 (0.95 - 1.03)	0.521
MELD	0.91 (0.88 - 0.94)	<0.001

From a binary logistic regression model, with the type of organ as a dependent variable, with DBD being the reference category

Supplementary Table S2.2 Causes of re-graft and death in matched recipients and unmatched DCDs

	DCD (n=187)	*DBD (n=187)*	*u-DCD (n=47)*
<30 days			
Re-graft	PNF (4 [1 patient died 8 days post re-graft]), HAT (4), Rejection (1)	PNF (2), HAT (1)	HAT (1) – survived
Died	PNF (1 - not re-grafted), sepsis (3), cardiac complications (3), pulmonary complications (2), haemorrhage (2)	Haemorrhage (1), cerebrovascular accident (2), pulmonary complications (3), sepsis (1)	PNF (1) – not re-grafted
>30 days – 1 year			
Re-graft	Hepatic artery stenosis resulting in liver biliary abscesses (1), recurrent rejection (1)	HAT (3) – all died	
Died	HAT (3), sepsis (4), suicide (1), recurrence of HCC (1), chronic rejection (1), cardiac complications (1)	Cardiac complications (1), gastrointestinal bleed (2), sepsis (4), recurrent HCC (3), recurrent cholangiocarcinoma (incidental histological finding post-transplant) (1)	Hepatic biliary abscess following full embolization for HA pseudoaneurysm (1)
>1 year			
Re-graft	HAT (1) – died intra-operatively due to thrombotic event	HAT (1) – died due to post-op acute respiratory distress syndrome	
Died	Ischaemic cholangiopathy (1), cardiac complications (2), sepsis (1), pulmonary complications (1), GI bleed (1), recurrent HCC (7)	Sepsis (3), tuberculosis (2), pulmonary complications (6), GI bleed (1), cardiac complications (2), lymphoma (1), chronic rejection (1), recurrence of HCC (10), HCV recurrence (3), HAT (2)	

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PART II – VIABILITY TESTING USING NORMOTHERMIC MACHINE PERFUSION

CHAPTER 3 - THE USE OF NORMOTHERMIC MACHINE PERFUSION TO TEST DONOR LIVER VIABILITY

3.1 DEVELOPMENT OF CLINICAL CRITERIA FOR FUNCTIONAL ASSESSMENT TO PREDICT PRIMARY NONFUNCTION OF HIGH-RISK LIVERS USING NORMOTHERMIC MACHINE PERFUSION

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Authors: Mergental H*[1,2], Stephenson BTF*[1], Laing RW*[1,2], Kirkham AJ*[3], Neil DAH[2], Wallace LL[1], Boteon YL[1,2], Widmer J[1], Bhogal RH [1,2], Perera MTPR[2], Smith A[2], Reynolds GM[1], Yap C[3], Hübscher SG[1], Mirza DF[1,2], Afford SC[1]

*Authors contributed equally

Affiliations:

[1] National Institute for Health Research Birmingham, Liver Biomedical Research Centre, College of Medical and Dental Sciences, University of Birmingham.

[2] Liver Unit, Queen Elizabeth Hospital Birmingham, University Hospitals Birmingham National Health Service Foundation Trust.

[3] Cancer Research UK Clinical Trials Unit, Institute of Cancer and Genomics Sciences, University of Birmingham, Birmingham, United Kingdom.

3.3.1 Abstract

Increased utilisation of high-risk allografts is critical to meet the demand for liver transplantation. We aimed to identify criteria predicting viability of organs, currently declined for clinical transplantation, using functional assessment during normothermic machine perfusion.

Twelve discarded human livers were subjected to normothermic machine perfusion following static cold storage. Livers were perfused with a packed red cell-based fluid at 37ºC for 6 hours. Multilevel statistical models for repeated measures were employed to investigate the trend of perfusate blood gas profiles and vascular flow characteristics over time and the effect of lactate clearing and non-clearing ability of the livers. The relationship of lactate clearance capability with bile production and histological and molecular findings were also examined.

After 2 hours of perfusion, median lactate concentrations were 3.0mmol/L and 14.6mmol/L in the lactate clearing and non-clearing groups respectively. Lactate clearing livers produced more bile and maintained a stable perfusate pH and vascular flow greater than 150mL/min and 500mL/min through the hepatic artery and portal vein respectively. Histology revealed discrepancies between subjectively discarded livers compared to objective findings. There were minimal morphological changes in the lactate clearing group whereas non-lactate clearing livers often showed hepatocellular injury and reduced glycogen deposition. ATP levels in the lactate clearing group increased compared to the non-lactate clearing livers. We propose composite viability criteria consisting of lactate clearance, pH maintenance, bile production, vascular flow patterns and liver macroscopic appearance. These have been tested successfully in clinical transplantation.

Normothermic machine perfusion allows an objective assessment of liver function that may reduce the risk and permit utilisation of currently unused high-risk livers.

3.3.2 Introduction

The demand for donor organs in liver transplantation greatly exceeds supply, whilst the global incidence of end-stage liver disease continues to rise, further increasing demand(1). In the UK during 2016 to 2017, 19% of patients listed for liver transplantation were either removed from the waiting list (15%) or died (4%) within one year of listing(2). Despite the increasing utilisation of grafts from donors after circulatory death (DCD) and high-risk donors after brain death (DBD), together known as extended criteria donors, waiting list mortality has not decreased(3). Their use is associated with a higher incidence of early post-transplant complications such as primary non-function, early allograft dysfunction and/or renal failure(4-6). Utilisation of high-risk organs remains low, with 159 out of 1041 livers procured in the UK during 2016-17 being discarded. Only 35% of all potential DCD livers were transplanted, due to DCD donation failing to proceed, inconsistencies in interpreting donor history and laboratory results, macroscopic or histological assessment, surgeon experience and the transplanting centre's expertise in marginal organ utilisation(7-10). These largely subjective factors impact upon the selection process and can compromise patient safety by resulting in the acceptance of high-risk marginal grafts that fail to function, or conversely potentially usable organs being discarded due to a perceived risk of post-transplant complications.

Normothermic machine perfusion (NMP) of the liver is a novel technology developed to reduce ischaemic damage and provide superior organ preservation compared to static cold storage. The purported advantages of NMP include: A) attenuation of ischaemia reperfusion injury, B) assessment of liver function prior to transplantation, C) improvement of transplant logistics and D) the potential to deliver therapeutics to recondition currently unusable livers, enabling subsequent transplantation(11-14).

The aim of this study was to develop a standardised protocol for NMP allowing functional assessment of donor livers rejected for transplantation, and to subsequently propose real-time criteria that predict liver viability. Outcomes of functional assessment were then correlated with histopathological assessment, currently the gold-standard to assess transplantability of extended criteria donor livers.

3.3.3 Materials and Methods

3.3.3.1 Source of discarded human livers

The study included 12 consecutively perfused livers offered to our team for research, regardless of cause, between May 2013 and June 2015. All organs were procured by the UK National Organ Retrieval Service, using standardised surgical protocols(15), with the primary intention of clinical transplantation and were subsequently declined by all UK centres. Ethical approval for the study was granted by the National Research Ethics Service committee in London-Surrey Borders (reference number 13/LO/1928). Consent to use donor tissues for research was obtained by specialist nurses in organ donation from the donor's next of kin during consent for organ donation. All livers were preserved in University of Wisconsin preservation fluid and exposed to a variable period of static cold storage.

3.3.3.2 Normothermic machine perfusion of the liver

The liver preparation for NMP was analogous to clinical transplantation. Whilst bathed in slushed ice, any redundant tissues were removed. The portal vein was cleaned to its bifurcation and hepatic artery dissected to the gastroduodenal artery. Straight cannulae were used for the artery (size varied according to size of vessel) and curved 20 French Medos cannulae were inserted into the portal vein. Prior to commencing NMP, livers were flushed with 2 litres of 10% dextrose solution at 37°C as per our unit's transplant protocol. The liver was then placed into the machine's reservoir, and the cannulae primed with perfusion fluid and connected to the perfusion circuits. Where required, a wider artery, from the same donor and surplus to transplant requirements, was anastomosed to the existing hepatic artery to permit cannulation. NMP was performed using the Liver Assist device (Organ Assist, Groningen, The Netherlands) which provides dual perfusion of the hepatic arterial and portal venous systems, in a semi-

closed circuit, using two rotatory pumps that produce pulsatile and non-pulsatile flows respectively.

The initial pressure settings of 30mmHg for the artery and 8mmHg for the portal vein were increased to 50mmHg and 10mmHg respectively within 30 minutes of commencing NMP. The pressure was set with the aim to maintain stable flows with adequate liver perfusion, however, in situations the flows (in particular in the arterial circuit) were decreasing arterial flows the perfusion pressures were raised in attempt to maintain adequate flows. The temperature was initially set to 25^{o}C and increased incrementally to 37^{o}C within 30 minutes. Oxygen was supplied via a Sechrist air/oxygen blender (S3500CP-G, Inspiration Healthcare Ltd., Leicester, UK). The fraction of inspired oxygen was set at 0.21 with 1L of flow per minute across each oxygenator, in accordance with the manufacturer's instructions. The perfusion fluid was based on 3 units of liver donor-specific blood group, Rhesus-negative, packed red cells obtained from the UK National Health Service Blood and Transplant. The constitution of the perfusion fluid is detailed in Table 3.1.

Table 3.1 Perfusion fluid constitution

	Amount *(Initial bulk fluid administrated into reservoir)*
Oxygen carrier	
Packed red blood cells	3 units
Drug	
Human albumin solution 5%	1000 ml
Heparin*	10,000 IU
Sodium bicarbonate 8.4%†	30 ml
Calcium gluconate 10%	10 ml
Vancomycin	500 mg
Gentamicin	60 mg
Continuous infusions	
Epoprostenol	2 μg/ml, commenced at 4 ml/hour and titrated as necessary
Intermittent drug administration	
Aminoplasmal 10%‡	50 ml bolus every 6 hours
Dextrose 10%	Infusion as necessary according to perfusate glucose concentration

Note: * bolus repeated every 3 hours; †bolus 10-30ml administrated if perfusate pH<7.00 to maintain pH>7.20; ‡ Cernevit 2ml and phytomenadione 1mg (0.1ml) added to Aminoplasmal 500ml bottle.

3.3.3.3 Data and sample collection protocol

Flow rates, pressures and resistances in the hepatic arterial and portal venous circuits were recorded every 30 minutes. Concurrently, 2mL of perfusate from the arterial and venous circuits were collected for immediate blood gas analysis using the Cobas b 221 blood gas analyser (Roche Diagnostics, Indianapolis, USA). If produced, bile was collected cumulatively and weighed at the end of the procedure. Liver biopsies were taken immediately prior to starting NMP, at 3 hours and either after 6 hours or the end of NMP, whichever was earlier. The tissue sample was divided and fixed in formalin as well as snap frozen in liquid nitrogen. The summary of the sampling protocol is shown in Figure 3.1A.

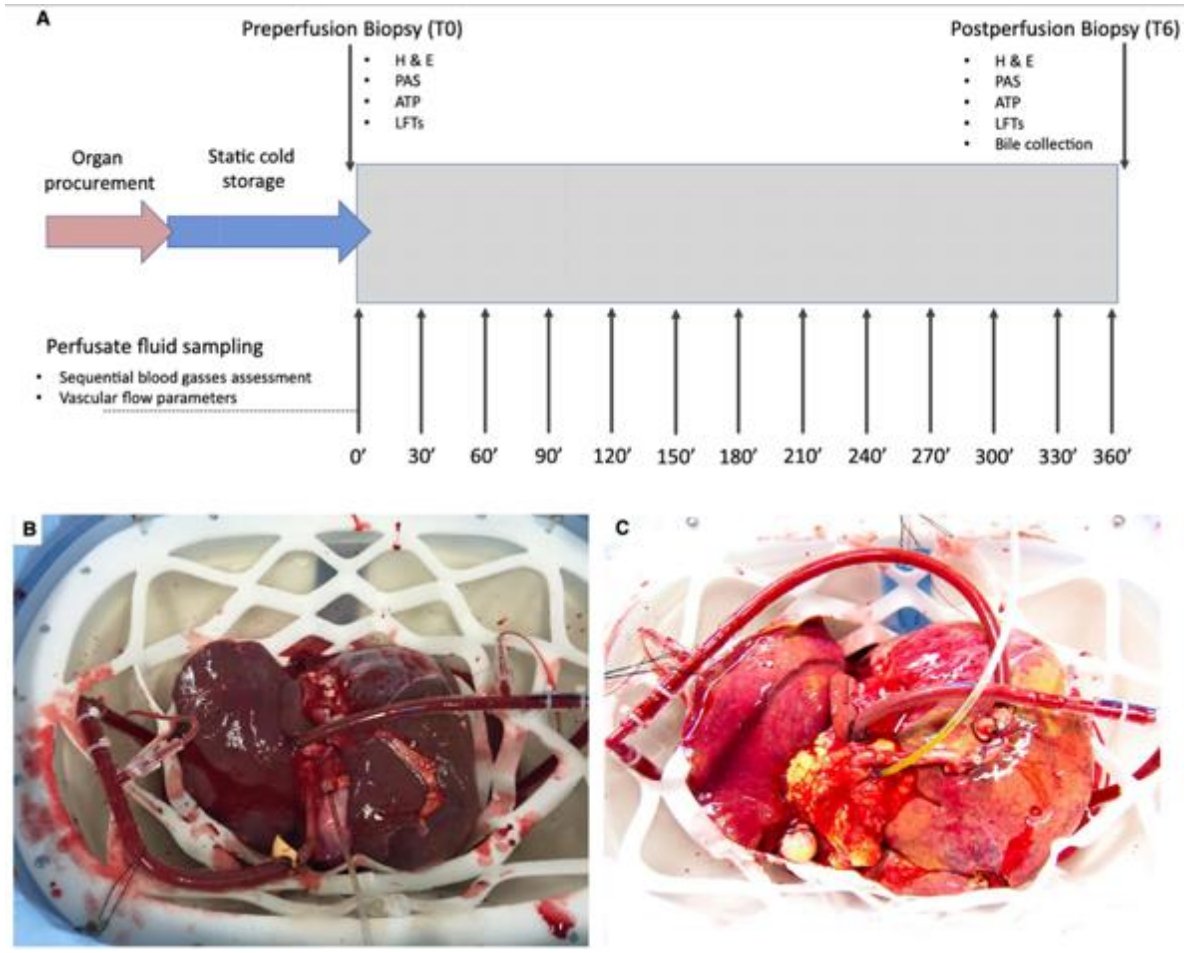


Figure 3.1 Study design and macroscopic appearance of viable and non-viable liver

Panel 3.1A details the study design, and the perfusate fluid and biopsy sampling protocol. Panel 3.1B shows a well-perfused liver with optimal macroscopic appearance. The organ was rejected for transplantation due to the incidental discovery of a malignant melanoma. The liver began to function shortly after commencing the perfusion, and the vascular flows and blood gas profile patterns were used to help define criteria for liver graft viability (perfusion number 8). Panel 3.1C is a steatotic liver with suboptimal macroscopic appearance; this organ did not meet the viability criteria (perfusion number 2).

3.3.3.4 Assessment of physiology

The perfusate from the arterial and venous outflow was analysed to measure partial pressures of O_2, and CO_2, pH, base excess, bicarbonate, O_2 saturation, haemoglobin, haematocrit, sodium, potassium, chloride, calcium, glucose and lactate concentrations. A perfusate pH less than 7.00 was corrected using 20mL boluses of 8.4% sodium bicarbonate. Oxygen consumption per gram of liver tissue was calculated based on oxygen delivery and oxygen extraction from the arterial and hepatic venous elements of the circuit respectively. Oxygen extraction ratio was calculated as the ratio of oxygen consumption to oxygen delivery.

3.3.3.5 Histopathological assessment

After paraffin embedding and processing, liver biopsies were stained with haematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). Biopsies were assessed for pre-existing acute or chronic liver injury, large and small droplet macrovesicular steatosis, coagulative necrosis, intrahepatic bile duct injury (apoptosis, vacuolation and lifting of epithelium from basement membrane), hepatocyte plate injury (hepatocyte loss of cohesion, detachment of hepatocyte plates from the sinusoidal lining) and glycogen depletion, that were recorded as percentages of cells affected(16). Histological assessment was conducted by independent experienced liver transplant pathologists, blinded to the designated viability.

For ultrastructural examination by transmission electron microscopy, 2mm biopsy pieces were fixed in 2.5% glutaraldehyde and processed to a resin block, and photomicrographs taken at x13.000 magnification of mitochondria within random hepatocytes and examined for signs of injury(17).

3.3.3.6 Assessment of Adenosine Trisphosphate

Measurements of Adenosine Triphosphate (ATP) were performed from snap frozen tissue by immediate homogenisation in SONOP buffer (0.372g EDTA in 130mL H_2O and NaOH (pH 10.9) + 370mL 96% ethanol) using the GentleMacs system. Protein concentration was determined using Pierce BCA Protein Assay kit (Thermo Scientific Inc. Rockford, USA). An ATP Bioluminescent Assay kit (FLAA, Sigma-Aldrich Inc, St Louis, USA) was used to determine concentrations from a calibration curve on the same plate, corrected for amount of protein, and expressed as nmol/g protein.

3.3.3.7 Assessment of liver cellular damage by microRNA analysis

The extent of the liver damage was estimated by microRNA122 qPCR analysis. RNA was isolated using Qiagen RNeasy kits (Eqicon, Vedbaek, Denmark) with the inclusion of Exiqon synthetic Spike-in templates as controls(18). On column DNase digestion eliminated genomic DNA. RNA samples were assessed on a TapeStation (Agilent Technologies Inc. Santa Clara, USA) using 10ng RNA per cDNA synthesis reaction with Exiqon cDNA synthesis reagents (miRCURY LNA™ Universal microRNA PCR kit) on a Labcycler (SensoQuest, Gottingen, Germany). Real-time PCR was performed on a Roche LC480 using the miRCURY LNA Universal RT microRNA PCR kit following reagent and protocol guidelines. Ct values were generated via the Absolute Quantitation and 2nd derivative method and relative quantities calculated.

3.3.3.8 Statistical methods

Twenty-seven perfusion parameters were recorded over a 6-hour period at approximately 30 minute intervals (details shown in supplementary Table S3.1). These were plotted against time, giving each liver its own observable trajectory, enabling trends to be visualised. Due to small

sample size the mean, standard deviation (SD), minimum and maximum values have been presented at initiation of NMP and then after two, four and six hours of perfusion (Table 3.3). The effect of lactate clearing and non-lactate clearing liver status on the change in liver function parameters (lactate and glucose metabolism, pH, arterial and portal flow rates, haematocrit, oxygen extraction ratio and oxygen consumption) were explored through multilevel linear models for repeated measures. Random intercept and slope effects were assigned at the liver level. Where linear relationships were not observed data were transformed as appropriate. Explanatory variables bicarbonate, carbon dioxide and base excess were adjusted for in the pH model, hepatic artery pressure and hepatic artery resistance were adjusted for in the hepatic artery flow rate model, and portal vein pressure and portal vein resistance were adjusted for in the portal vein flow rate model. An indicator variable based on lactate clearing trajectories, denoted as "Lactate Clearing" (LC) and "Non-Lactate Clearing" (non-LC), and its interaction with time were included in each model and included if found to be significant. For these exploratory analyses, as the sample size is small, any potential interactions between lactate clearance and time with p-value <0.2 would be presented. Models were estimated using the method of maximum likelihood estimation and selected using likelihood ratio tests.

Missing data were recorded as: lactate 7.7%, glucose, arterial and portal flow rates 8.3%, pH 11.5%; haematocrit 18.6%, oxygen extraction ratio and oxygen consumption 20.5%. The multilevel models approach used is tolerant of missing data under a missing at random assumption. Multilevel modelling was performed using Stata version 14.2 (StataCorp LLC, Texas, USA).

The bile production, ATP and microRNA levels were compared with Mann–Whitney U-test, with the statistical level of significance set at $p<0.05$, using GraphPad Prism (GraphPad Software, La Jolla, California USA) software.

3.3.4 Results

3.3.4.1 Donor demographics, chronology and reasons for discarding livers

Eight livers included in the study were from DCD donors. The median donor age was 56 (range 30–76) years and the body mass index 30 (23-47) kg/m^2. The median cold ischaemic time (CIT) was 483 (range 380–797) minutes. Three livers were discarded due to steatosis, 2 for extrahepatic primary donor malignancy, 2 for excessive CIT and 2 for excessive donor warm ischaemic time (WIT). The detailed characteristics of the included livers are provided in Table 3.2.

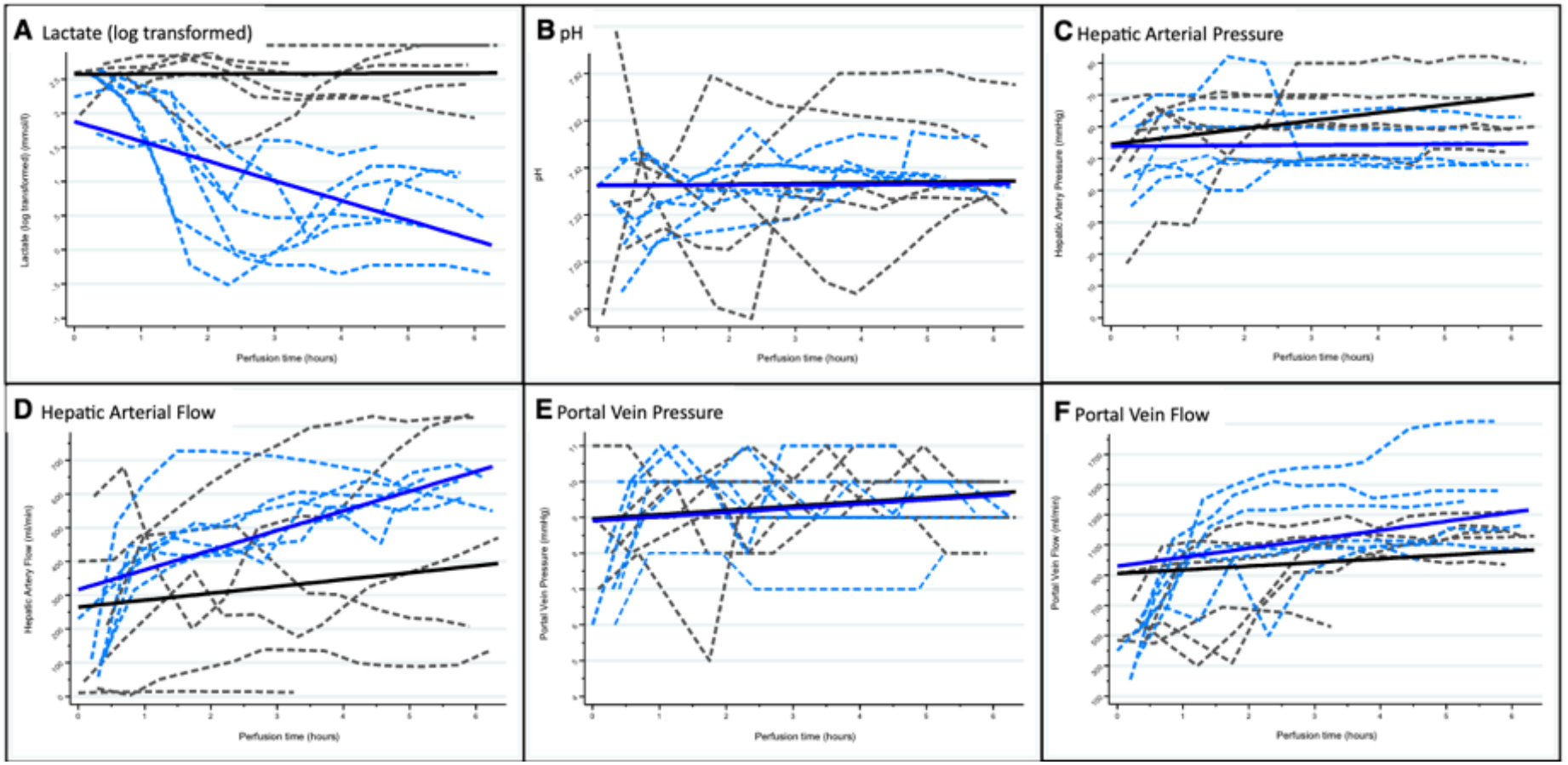
Table 3.2 Donor demographics and chronology

	Non-LC*						LC*					
Perfusion number	1	2	3	4	5	6	1†	2	3	4	5	6†
Donor age (years)	55	55	76	60	46	71	30	69	55	57	70	50
Donor sex	Female	Male	Female	Female	Male	Male	Male	Male	Male	Male	Female	Female
BMI (kg/m^2)	47	33	28	36	23	30	25	31	24	25	34	45
Blood group	B+	A+	O+	A+	O+	O-	A+	O+	O+	A+	O+	O+
Cause of death	Meningitis	ICH	ICH	HBI	ICH	HBI	HBI	HBI	Meningitis	ICH	HBI	HBI
Donor type	DBD	DCD	DCD	DCD	DBD	DCD	DCD	DBD	DBD	DCD	DCD	DCD
Agonal period (mins)	NA	14	8	17	NA	31	100	NA	NA	14	16	29
Primary WIT (mins)	NA	12	17	15	NA	12	12	NA	NA	14	18	11
Liver weight (grams)	2420	2130	1775	1712	1961	2310	1997	2400	2300	1752	1650	1943
Steatosis assessment††	Mod.	Mod.	Nil	Mod.	Mild	Mod.	Nil	Mild	Nil	Mild	Mild	Nil
Cold ischemic time (mins)	792	797	554	491	380	467	445	496	454	532	583	408
Donor risk index	1.85	2.64	3.23	2.77	1.41	3.22	1.77	1.78	1.61	2.36	3.05	2.39
Reason for discard	Steatosis.	Steatosis.	CIT§	Steatosis.	ITU	Perfusion	WIT¶	Steatosis	Cancer	Cancer	CIT§	WIT¶

NOTE: Agonal period in DCD procurement was defined as the period between withdrawal of treatment to circulatory arrest. Primary WIT in DCD procurement designs time from circulatory arrest to in situ organ perfusion.
*The livers are grouped according to the lactate metabolism (viability criteria) rather than the chronological order of the perfusion.
†Designates livers that were transplanted
††Subjective assessment by the retrieval and/or transplant surgeon.
§Prolonged CIT.
||Poor quality liver graft perfusion.
¶Extensive WIT and CIT.

3.3.4.2 Liver functional assessment

Initial graphical data explorations were performed with the aim of observing any trends over time. Individual livers' response data were recorded for lactate, glucose, arterial and portal flows, pH, oxygen extraction ratio, oxygen consumption and haematocrit (Figure 3.2 and Table 3.3). The results for lactate measurements showed two distinct groups; one had a sharp fall in lactate levels which subsequently stabilised at lower levels, designated as the lactate clearing group, whereas the other showed fluctuations and rises in the lactate level over time, known as the non-lactate clearing group. No other response variable measured showed a similar performance demarcation, although the lactate clearing livers did appear to show similarities of behaviours when plotted.


A Lactate (log transformed)
Lactate (log transformed) (mmol/l)
Perfusion time (hours)
B pH
pH
Perfusion time (hours)
C Hepatic Arterial Pressure
Hepatic Artery Pressure (mmHg)
Perfusion time (hours)
D Hepatic Arterial Flow
Hepatic Artery Flow (ml/min)
Perfusion time (hours)
E Portal Vein Pressure
Portal Vein Pressure (mmHg)
Perfusion time (hours)
F Portal Vein Flow
Portal Vein Flow (ml/min)
Perfusion time (hours)

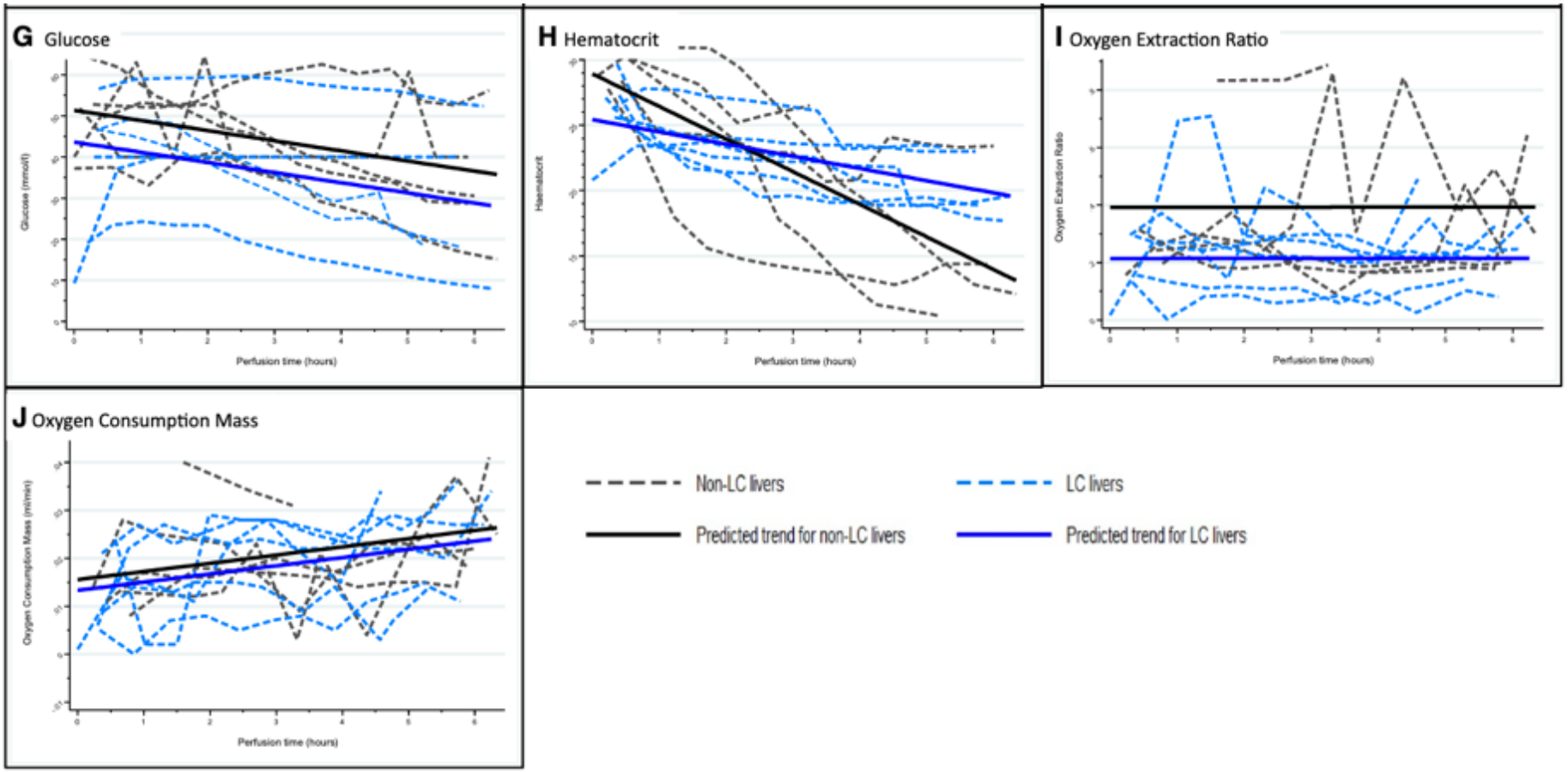

G Glucose
Glucose (mmol/l)
Perfusion time (hours)
H Hematocrit
Haematocrit
Perfusion time (hours)
I Oxygen Extraction Ratio
Oxygen Extraction Ratio
Perfusion time (hours)
J Oxygen Consumption Mass
Oxygen Consumption Mass (ml/min)
Perfusion time (hours)
Non-LC livers
LC livers
Predicted trend for non-LC livers
Predicted trend for LC livers

Figure 3.2 Multilevel random intercept and slope model findings

Panel 3.2A: Lactate levels (mmol/L) measured during perfusions for individual livers with linear predicted estimate trajectories for LC and non-LC groups – lactate levels were found to be significantly lower in the LC group ($p<0.001$). Panel 3.2B: Hepatic artery flow rates (mL/min/g) measured during perfusions for individual livers with linear predicted estimate trajectories for LC and non-LC groups – no evidence of a difference was observed between LC and non-LC groups ($p=0.824$). Panel 3.2C: Portal vein flow rates (mL/min/g) measured during perfusions for individual livers with linear predicted estimate trajectories for LC and non-LC groups - no evidence of a difference was observed between LC and non-LC groups ($p=0.500$). Panel 3.2D: Glucose levels (mmol/L) measured during perfusions for individual livers with linear predicted estimate trajectories for LC and non-LC groups – no evidence of a difference was observed between LC and non-LC groups ($p=0.148$). Panel 3.2E: pH measured during perfusions for individual livers with linear predicted estimate trajectories for LC and non-LC groups – on average pH was significantly lower in the LC group ($p<0.001$). Panel 3.2F: Haematocrit measured during perfusions for individual livers with linear predicted estimate trajectories for LC and non-LC groups - no evidence of a difference was observed between LC and non-LC groups ($p=0.818$). Panel 3.2G: Oxygen extraction ratio measured during perfusions for individual livers with linear predicted estimate trajectories for LC and non-LC groups – no explanatory variables were found to be significant. Panel 3.2H: Oxygen consumption (mL/min/g) measured during perfusions for individual livers with linear predicted estimate trajectories for LC and non-LC groups - no evidence of a difference was observed between LC and non-LC groups ($p=0.579$).

Table 3.3 Liver functional assessment parameters

	Non-LC				LC			
Time (hours)	0	2	4	6	0	2	4	6
Lactate (mmol/L)	13.7 (4.1) [7.2-20.0]	14.6 (5.7) [4.4-20.0]	13.7 (4.5) [9.2-20.0]	14.6 (5.7) [6.9-20.0]	10.5 (3.3) [5.5-13.9]	3.0 (1.7) [0.6-5.5]	2.1 (1.1) [0.7-4.0]	2.1 (1.1) [0.7-3.1]
Glucose (mmol/L)	49.3 (9.7) [37.2-64.1]	50.5 (9.7) [39.5-64.5]	40.3 (12.4) [26.2-60.3]	34.1 (15.2) [15.1-56.2]	36.4 (18.3) [9.3-56.6]	41.3 (12.9) [23.3-59.3]	34.1 (14.8) [14.2-56.7]	29.6 (20.2) [8.0-52.4]
pH	7.3 (0.5) [6.8-8.0]	7.3 (0.3) [6.8-7.8]	7.4 (0.4) [6.9-7.8]	7.4 (0.2) [7.2-7.8]	7.2 (0.2) [6.9-7.5]	7.3 (0.1) [7.2-7.4]	7.4 (0.1) [7.3-7.6]	7.4 (0.1) [7.3-7.6]
Arterial Flow (mL/min)	213.8 (238.5) [11.0-593.0]	316.3 (224.4) [103.0-631.0]	412.6 (269.7) [98.0-810.0]	495.2 (330.8) [136.0-835.0]	155.0 (96.2) [58.0-313.0]	524.2 (118.8) [426.0-727.0]	575.2 (43.1) [527.0-638.0]	621.2 (52.1) [550.0-682.0]
Arterial Flow Rate (mL/min/g)	0.1 (0.1) [0.01-0.5]	0.2 (0.1) [0.1-0.3]	0.2 (0.1) [0.1-0.4]	0.3 (0.2) [0.1-0.5]	0.1 (0.05) [0.03-0.2]	0.3 (0.1) [0.2-0.4]	0.3 (0.1) [0.2-0.4]	0.3 (0.1) [0.3-0.4]
Portal Flow (mL/min)	613.3 (177.7) [470.0-910.0]	962.5 (261.5) [690.0-1250.0]	1120.0 (70.7) [1030.0-1210.0]	1158.0 (125.2) [970.0-1320.0]	458.3 (166.8) [210.0-630.0]	1176.0 (192.3) [1000.0-1430.0]	1330.0 (225.1) [1100.0-1650.0]	1418.0 (320.3) [1070.0-1920.0]
Portal Flow Rate (mL/min/g)	0.3 (0.1) [0.2-0.4]	0.5 (0.1) [0.4-0.7]	0.6 (0.1) [0.4-0.7]	0.6 (0.1) [0.4-0.7]	0.2 (0.1) [0.1-0.4]	0.6 (0.2) [0.4-0.9]	0.7 (0.1) [0.5-0.9]	0.7 (0.3) [0.5-0.9]
Haematocrit (%)	29.0 (1.4) [27.8-31.1]	23.3 (5.4) [14.8-29.5]	16.0 (4.4) [11.3-20.8]	16.6 (6.0) [12.1-23.4]	26.2 (3.0) [20.8-29.7]	22.6 (1.3) [21.3-24.0]	21.3 (2.0) [18.9-23.5]	19.8 (2.3) [17.7-23.0]
Oxygen Consumption (mL/min)	24.2 (1.4) [23.2-25.2]	34.1 (7.8) [25.1-39.4]	23.8 (13.7) [7.3-39.6]	46.8 (11.1) [31.0-57.0]	15.0 (13.3) [1.4-37.0]	34.2 (17.4) [15.8-58.5]	32.3 (15.7) [18.5-83.9]	54.2 (28.1) [18.5-83.9]
Oxygen Consumption, Mass	0.013 (0.002) [0.011-0.014]	0.017 (0.005) [0.013-0.023]	0.013 (0.006) [0.004-0.017]	0.027 (0.010) [0.018-0.041]	0.008 (0.008) [0.001-0.021]	0.018 (0.009) [0.008-0.029]	0.016 (0.008) [0.005-0.024]	0.027 (0.011) [0.011-0.036]
Oxygen Extraction Ratio	0.2 (0.1) [0.2-0.3]	0.2 (0.04) [0.2-0.3]	0.3 (0.3) [0.2-0.8]	0.3 (0.2) [0.2-0.6]	0.2 (0.1) [0.02-0.3]	0.2 (0.1) [0.1-0.3]	0.2 (0.1) [0.1-0.2]	0.3 (0.1) [0.1-0.4]

NOTE: Data are given as mean (SD) [range].

3.3.4.3 Multilevel Random Intercept and Slope Models

Results from multilevel modelling found that lactate levels demonstrated a significant difference in trend over time ($p<0.001$), with LC livers being lower in comparison to non-LC livers. After adjusting for bicarbonate ($p<0.001$), carbon dioxide ($p<0.001$) and excess base ($p<0.001$), pH levels increased over time ($p=0.003$) although LC livers appear to have a gentler increasing trend compared to non-LC livers ($p=0.10$). There was a difference in the trend of hepatic arterial pressure over time ($p=0.08$) with a much steeper increasing trend in the non-LC livers compared to the LC livers ($p=0.08$). Changes in arterial flow, after adjusting for arterial resistance ($p=0.007$) and arterial pressure ($p=0.14$) and their subsequent interaction ($p=0.01$), showed a slightly higher increasing trend in LC livers over time ($p=0.13$). Portal vein pressure showed an increasing trend over time ($p=0.07$) however there was no significant difference between LC and non-LC livers ($p=0.90$). Portal vein flow increased over time ($p=0.13$), with LC livers having a higher increment in flow rate ($p=0.12$) after adjusting for portal vein pressure ($p<0.001$), portal vein resistance ($p=0.25$) and their interactions ($p=0.001$). Glucose levels decreased significantly over time ($p=0.006$), with LC livers being 7.8mmol/L lower on average compared to non-LC livers ($p=0.15$). Haematocrit demonstrated a significant reduction over time ($p<0.001$) with LC livers showing a gentler decreasing trend ($p=0.01$). Oxygen extraction ratio was found not to change significantly over time, but on average for LC livers was 0.2 lower than non-LC livers ($p=0.07$). A significant increase in oxygen consumption over time was observed ($p<0.001$) however there appears to be no difference between LC and non-LC livers ($p=0.85$). The multilevel model parameters are provided in Table 3.4.

Table 3.4 Multilevel random effects model parameters examining liver response variables during perfusion

Response Variables	Explanatory Variables	Estimate (95% CI)	*P* Value
Lactate (log) (mmol/L)	Time (hours)	0.003 (–0.1 to 0.1)	0.96
	LC indicator	–0.7 (–1.2 to –0.2)	0.005
	Interaction: LC indicator × time	–0.3 (–0.4 to –0.2)	<0.001
pH	Time (hours)	0.003 (0.001 to 0.006)	0.003
	LC indicator	0.002 (–0.02 to 0.02)	0.85
	Interaction: LC indicator × time	–0.002 (–0.005 to 0.0004)	0.1
	CHCO3	–0.05 (–0.06 to –0.05)	<0.001
	pCO2	–0.006 (–0.008 to –0.05)	<0.001
	Base excess	0.06 (0.06 to 0.06)	<0.001
Hepatic artery pressure (mm Hg)	Time (hours)	2.5 (0.7 to 4.3)	0.008
	LC indicator	–0.6 (–14.0 to 12.8)	0.93
	Interaction: LC indicator × time	–2.3 (–4.9 to 0.2)	0.08
Hepatic artery flow (mL/minute)	Time (hours)	20.4 (–15.2 to 55.9)	0.26
	LC indicator	51.7 (–91.6 to 195.0)	0.48
	Interaction: LC indicator × time	38.0 (–10.6 to 86.6)	0.13
	Hepatic artery pressure	–2.3 (–5.4 to 0.8)	0.14
	Hepatic artery resistance	–224.9 (–387.1 to –62.7)	0.007
	Interaction: pressure × resistance	3.3 (0.8 to 5.7)	0.01
Portal vein pressure (mm Hg)	Time (hours)	0.1 (–0.01 to 0.2)	0.07
	LC indicator	–0.06 (–1.0 to 0.9)	0.9
Portal vein flow (mL/minute)	Time (hours)	24.5 (–6.8 to 55.8)	0.13
	LC indicator	48.6 (–36.9 to 134.1)	0.27
	Interaction: LC indicator × time	34.9 (–8.6 to 78.5)	0.12
	Portal vein pressure	163.4 (108.4 to 218.4)	<0.001
	Portal vein resistance	21,183.4 (–14,933.6 to 57,300.4)	0.25
	Interaction: pressure × resistance	–6972.7 (–11,140.1 to –2805.3)	0.001
Glucose (mmol/L)	Time (hours)	–2.5 (–4.2 to –0.7)	0.006
	LC indicator	–7.8 (–18.3 to 2.8)	0.15
Haematocrit (%)	Time (hours)	–2.5 (–3.4 to –1.6)	<0.001
	LC indicator	–3.5 (–7.4 to 0.4)	0.08
	Interaction: LC indicator × time	1.6 (0.3 to 2.8)	0.01
Oxygen extraction ratio	Time (hours)	0.0002 (–0.02 to 0.02)	0.98
	LC indicator	–0.2 (–0.4 to 0.01)	0.07
Oxygen consumption (mL/minute)	Time (hours)	3.8 (1.7 to 5.8)	<0.001
	LC indicator	–1.4 (–16.5 to 13.6)	0.85

3.3.4.4 Bile production

There were significant differences in cumulative bile production between LC and non-LC groups. There was more sustained bile production in the LC group, although this only occurred in 4 livers. In the non-LC group, only one liver produced bile at the end of the NMP (2.6g at 6 hours). After 6 hours, the median bile production for LC and non-LC groups was 6.5g vs. 0.0g (p=0.03) respectively.

3.3.4.5 Histological findings

There was a significant discrepancy between the subjective assessment of liver quality performed by the organ retrieval or transplant surgeon and the subsequent histological findings. Microscopic evaluation confirmed only mild large droplet macrovesicular steatosis in livers declined for steatosis. Histology did not reveal any fibrosis in the liver declined for this presumed diagnosis.

None of the livers displayed significant large droplet steatosis, and at most showed only a mild degree (maximum of 15%; Figure 3.3A). Small droplet macrovesicular steatosis was greater in the non-LC livers (Table 3.5 and Figure 3.3B). Ischaemic-type coagulative necrosis was minimal across both groups (Figure 3.3C). Lost cohesion of hepatocytes, predominantly in zone 3, was observed in the non-LC group (Figure 3.3K) with all post-NMP livers showing variable amounts of hepatocyte detachment (LC 1.5%, 0-10% vs non-LC 15%, 1-40%).

There was no difference in amount of glycogen depletion pre-NMP between the groups (Figure 3.3D and 3.3H; LC 80% depletion, 5-95% vs. non-viable 75% depletion, 5-99%). At the end of the perfusion, the LC group displayed increased PAS staining (Figure 3.3E v Figure 3.3I; LC 22.5% glycogen depletion, 5-80% vs. non-LC 80% depletion, 10-90%), indicating that

viable livers were able to uptake glucose and store this as glycogen (Figure 3.3F) or maintain glycogen stores if initially high.

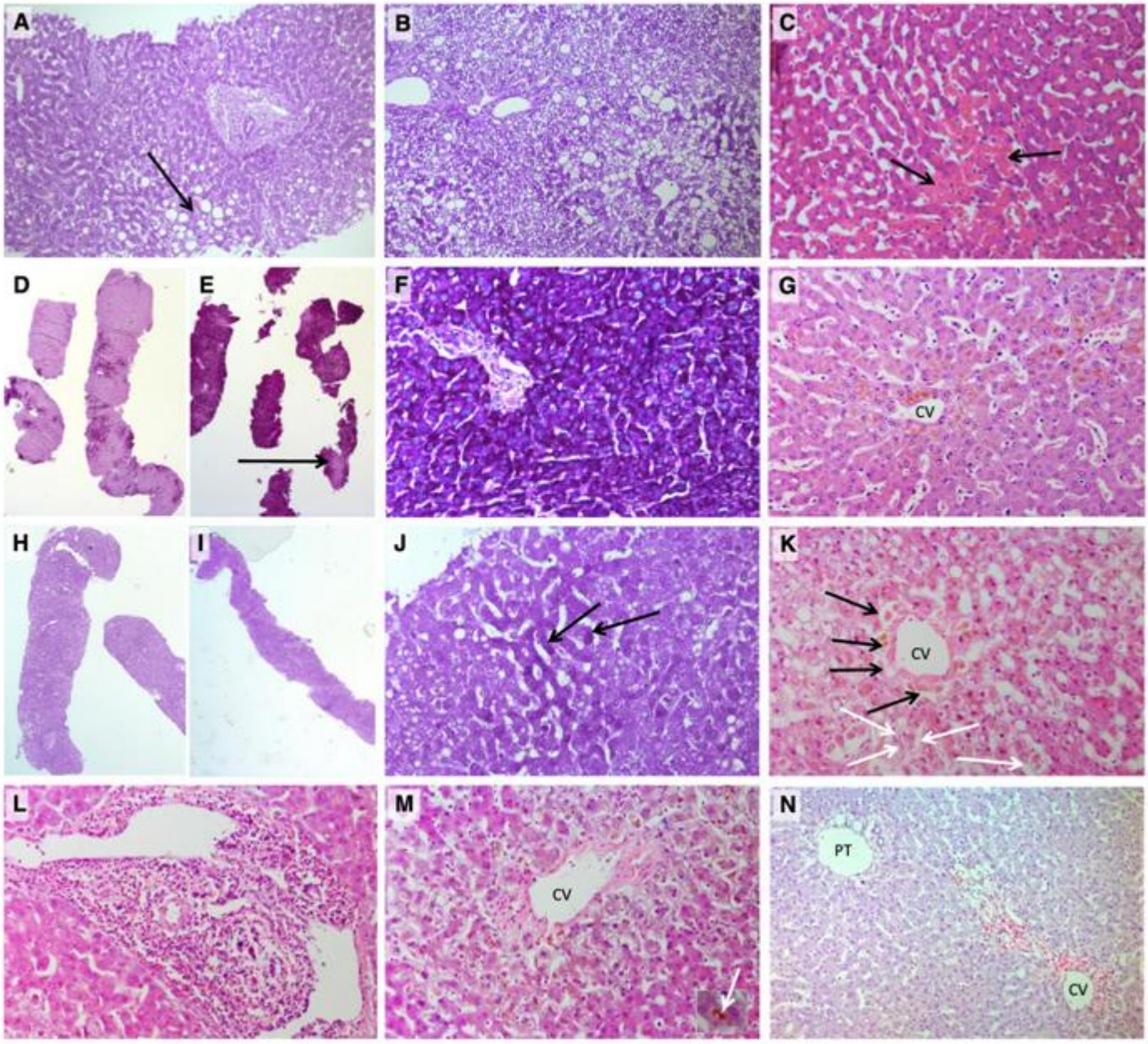


Figure 3.3 Histological findings

Panel 3.3A shows Periodic acid-Schiff (PAS) stained section of liver 4 which had the most severe large droplet macrovesicular steatosis (arrow), the type of fat considered in evaluating suitability for transplantation. This was mild involving up to 15% of hepatocytes. The liver was turned down on macroscopic assessment of steatosis [original objective x10]. Panel 3.3B captures a PAS stained section of liver 1 pre-NMP with extensive small droplet microvesicular steatosis, where hepatocyte cytoplasm contains often numerous small droplets of fat which do not displace the hepatocyte nuclei. Several large fat droplets are also present. This liver was turned down due to the macroscopic appearance of steatosis; large droplet steatosis was mild involving only 5% of hepatocytes in the whole biopsy. It is likely that the small droplet steatosis

was also seen macroscopically. This is not traditionally considered in assessing a liver for transplantation and indicates the requirement of a liver biopsy to accurately assess the type and amount of both types of fat droplets [original objective x10]. Panel 3.3C shows Haematoxylin & Eosin (H&E) stained sections of LC liver 12, 6 hours post NMP, showing a small area of coagulative necrosis where the cells become hypereosinophilic (arrows). This was seen to an equal extent in both viable and non-viable livers pre- and post-NMP and was very mild in this series of livers. Panels 3.3D-F show PAS stain from LC liver 6, and Panels 3.3H-J non-LC liver 4. Both livers demonstrated marked glycogen depletion pre-NMP (3.3D and 3.3H); whilst post-NMP the viable liver has restored its glycogen stores (3.3E, F), the non-viable liver (3.3I, J) remains significantly glycogen depleted. Bright magenta staining of the cytoplasm indicates glycogen, and pale pink staining indicates no glycogen (arrow, 3.3E). The few darker staining hepatocytes containing some glycogen are indicated (3.3J). [D, E, H, I original objective x2; F, J original objective x20]. Panel 3.3G demonstrates LC, viable liver 8 after 6 hours of NMP, revealing normal hepatocyte plate morphology and attachment of hepatocyte plates to the central vein (CV). Panel 3.3K shows non-LC, non-viable liver number 3.3, 6 hours after NMP showing loss of cohesion of hepatocytes from each other and from the sinusoidal lining (arrows) and from the central vein.

Panels 3.3L and M show H&E stained sections of liver 5 which was turned down for transplantation based on its macroscopic appearance. This liver had a portal hepatitis (3.3L) and severe zone 3 cholestasis (3.3M, inset – high power of bile plug, arrow) [original objective x20 for both]. Panel 3.3N shows H&E stained section of liver 7 discarded because macroscopically thought to have fibrosis. There is no fibrosis present. There is a normal portal tract (PT) showing no fibrous expansion. The abnormality present is centred around the central vein consisting of confluent areas of hepatocyte loss in which there is variable haemorrhage/congestion (red colour of red blood cells seen) and pigment laden macrophages [original objective x10].

The intrahepatic bile ducts displayed greater injury, in particular apoptosis of biliary epithelial cells, in the non-LC group (median of 1 vs 0) compared to LC group. Detailed histological findings are shown in Table 3.5. The ultrastructural assessment by transmission electron micrograph demonstrated the mitochondria were not swollen in either liver group, however flocculent densities, a sign of irreversible cell injury, were present in many mitochondria in the non-LC livers, but were not present in the LC livers (Figure 3.4).

Table 3.5 Histological features on liver biopsies

	Non-LC						LC					
Perfusion number	**1**	**2**	**3**	**4**	**5**	**6**	**1 ***	**2**	**3**	**4**	**5**	**6 *, §**
Large-droplet steatosis, % †	5	5	<5	15	0	0	0	0	0	0	0	<1
Small-droplet steatosis, % ††	90	30	<5	40	0	0	5	10	0	0	0	10
Glycogen depletion, % § (pre/post NMP)	30/90	75/90	99/80	90/80	—	05/10	80/15	—	5/5	85/75	40/30	95/10
Detached hepatocytes, % ‖ (pre-NMP/post-NMP)	0/1	4/30	0/40	20/15	—	0/5	0/1	—/0	0/0	10/10	0/5	1/2
Bile duct injury ¶ (pre-NMP/post-NMP)	0/2	0/2	0/1	0/0	—	0/1	0/1	—/0	0/0	0/1	0/0	0/0
Coagulative necrosis, % # (pre-NMP/post-NMP)	0/1	0/0	0/0	0/5	—	0/10	0/0	—/0	0/0	0/2	0/10	0/5
Other findings	Micro-thrombi	Mild portal hepatitis		Patchy congestion	Hepatitis with severe cholestasis	Mild portal oedema with eosinophils		l-2 week-old lytic zone 3 necrosis				
Time of 2nd biopsy (hours)	6	3.2	6	6	6	6	4.5	6	6		6	5

NOTE: Values designated with "—" are missing.

* Designates livers that were transplanted.

† Large-droplet macrovesicular steatosis is defined as a single large fat droplet within the hepatocyte cytoplasm displacing the nucleus; values are % of hepatocytes containing fat.

†† Small-droplet macrovesicular steatosis is defined as fat droplets, usually multiple, within the cytoplasm of the hepatocyte that do not displace the nucleus; values are % of hepatocytes containing fat.

§ Glycogen depletion is graded as the % of hepatocytes that do not contain glycogen.

‖ Detached hepatocytes is the % of hepatocytes that have lost cohesion from each other and from the sinusoidal lining.

¶ Bile duct injury is defined as apoptotic debris within the wall or lumen or loss of cohesion between the epithelium and basement membrane; it is graded as 0 (nil), 1 (minimal), and 2 (present).

Necrosis is depicted as the percent of total hepatocytes in the biopsy that shows classical ischemic-type coagulative necrosis.

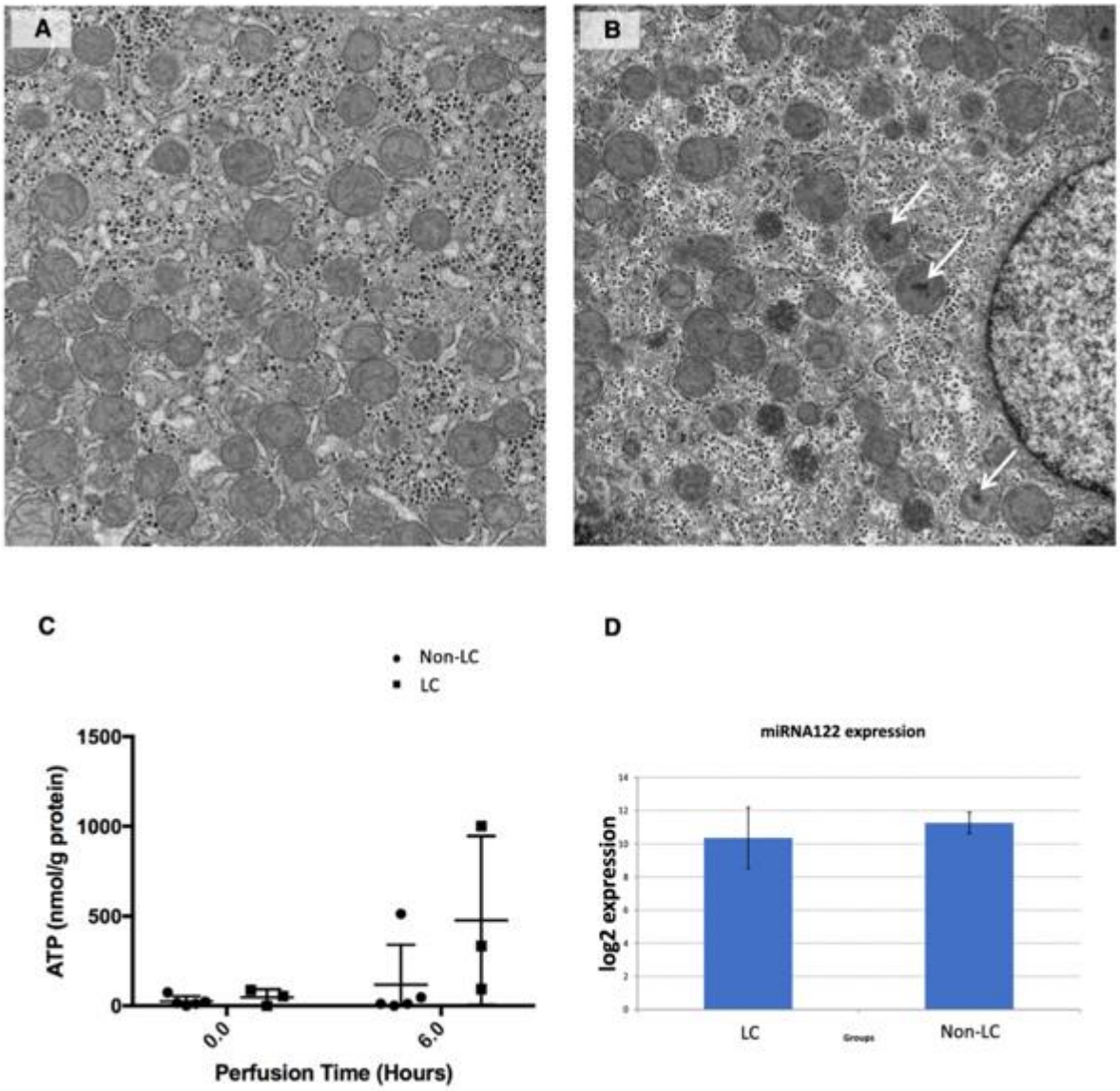


Figure 3.4 Transmission electron micrographs and adenosine triphosphate and microRNA analyses

Panel 3.4A shows a lactate clearing, viable liver number 9, and Panel 3.4B a non-clearing, non-viable liver number 10. Both microphotographs were taken from post-perfusion (T6) biopsy samples. In the non-viable liver, flocculent densities can be seen within several of the mitochondria (white arrows), which indicate irreversible cell injury. Christae are still apparent within other mitochondria and within the viable liver (3.4A) in which no flocculent densities were observed. The mitochondria of both livers are not swollen. (original magnification x13.000). Panel 3.4C illustrates pre-perfusion and post-perfusion ATP levels, showing increase

in the LC livers contrasting with minimal change observed in non-LC livers. Panel 3.4D shows microRNA assays to assess the extent of cellular damage. This analysis did not reveal any difference between LC and non-LC groups.

3.3.4.6 Adenosine triphosphate findings

The ATP analysis was performed from 8 livers, showing non-significant differences between median pre-perfusion levels (54.6 vs 15.8, p=0.42), followed by a trend for increasement in the LC livers at 6 hours, contrasting with reduced ATP levels in the non-LC group (334.6 vs 11.9, p=0.18). Details are shown in Table 3.6.

Table 3.6 Liver Perfusion Parameters and Proposed Viability Criteria

	Non-LC						**LC**					
Liver number	**1**	**2**	**3**	**4**	**5**	**6**	1 *	**2**	**3**	**4**	**5**	6 *
Perfusion time (minutes)	541	192	501	1102	738	394	393	277	378	403	388	316
Lactate T0 (mmol/L)	>20.0	13.4	13	13.3	7.2	15.2	7.6	9.4	12.9	13.9	13.9	5.5
Lactate T2 (mmol/L)	19.2	16.4	20	12.5	4.4	15.1	1.2	4.6	0.6	5.5	3.2	3
Trough lactate (mmol/L)	12.8	13.4	13	8.8	4.4	6.9	0.7	2.1	0.6	1.2	0.8	1.4
Bile production T6 (grams)	0	0	0	0	2.6	0	23	6.1	10.4	0	6.9	0
ATP T0 (nmol/g protein) †	15.8	—	12.1	0	24.6	74.7	—	—	54.6	88.1	0	—
ATP T6 (nmol/g protein) †	46.6	—	0.6	11.5	11.9	512.8	—	—	334.6	1001.9	93.5	—
ALT (IU/L)† T0	—	—	4055	—	—	2888	—	—	574	—	2603	3673
ALT (IU/L)† peak value			—			5017			1498	10,772	3803	6851

Major criteria: Trough lactate level of <2.5 mmol/L Presence of bile production

Minor criteria: Perfusate pH of >7.30; Stable arterial flow of more than 150 mL/minute and portal flow more than 500 mL/minute; homogeneous liver perfusion with soft consistency of the parenchyma

NOTE: A viable liver graft has to meet ≥1 major and ≥2 of the minor criteria. All parameters are assessed 120 minutes after commencing the perfusion. To ensure recipient safety and to minimize risks of presence of a pre-existing liver disease or irreparable liver damage, only organs meeting the following criteria were considered for the pilot clinical transplant series: maximum donor age of 70 years, CITs of <16 hours for livers from donors after brain death, or <10 hours from DCD, donor WIT (systolic blood pressure <50 mm Hg to aortic perfusion) in DCD organs <60 minutes, absence of hepatitis B, hepatitis C, or human immunodeficiency virus infection, and healthy macroscopic appearance without signs of fibrosis or cirrhosis (Mergental et al.12).

*Designates livers that were transplanted.

†Values designated with "—" are missing.

3.3.4.7 Assessment of liver cellular damage by microRNA analysis

For the purpose of the assay Sp6 was used as the inter-plate calibrator, Sp4 as the internal amplification control. Outliers with Ct values >37 were excluded. The samples were normalised to the reference gene miRNA-23b, converted to relative quantities and a log scale. Pre-processed normalised data did not reveal any difference between LC and non-LC livers (U-value of 39, p=0.25)

3.3.4.8 Viability assessment criteria

Two livers were declined for unexpected malignancy confirmed in other organs after the retrieval. These two livers had a favourable macroscopic appearance (Figure 3.1B) and donor characteristics, and during NMP demonstrated properties expected of livers post-transplant, enabling us to propose perfusion parameters associated with functioning livers. The ability of livers to clear lactate appeared to be a substantial marker to divide the livers into two groups. Bile production was closely related to lactate clearance, however its negative predictive value was low.

In defining clinically usable viability criteria to assess function of high-risk and/or discarded livers, our main objective was to ensure transplant recipient safety. We designed a composite viability measure consisting of lactate clearance and/or bile production (major criteria), in combination with additional minor criteria of stable arterial and portal flows, perfusate pH and favourable macroscopic assessment by the transplant surgeon (Table 3.6, Figure 3.2).

3.3.5 Discussion

NMP has been developed to overcome shortcomings and organ damage occurring during static cold storage. Preserving the liver in near-physiological conditions at normothermia, with oxygen and nutrients, allows for *ex-vivo* functional assessment. Our key objective when commencing the NMP programme was to develop a protocol to evaluate liver function and define criteria characteristic of a viable liver with a view to preventing primary non-function whilst utilising high-risk extended criteria organs. This research, performed on discarded donor livers that had been exposed to a variable period of static cold storage, assumed that during NMP potentially transplantable livers would behave similarly to an allograft following its implantation. Two livers in the study had, barring incidental donor malignancy, otherwise favourable donor characteristics and macroscopic appearances, with NMP commencing after a short duration of cold ischaemia. Provided favourable perfusion characteristics had been observed, if post-procurement biopsies from the suspicious donor tissues had not shown malignancy these organs could have been transplanted. The demonstrated perfusion characteristics and metabolic activity in these two livers were similarly observed in four other livers. The most striking functional indicator in these six livers was their ability to metabolise lactate to near physiological levels within 2 hours of NMP, a quality not seen in the other six high-risk livers. This LC group was expected to consist of viable, transplantable livers. The remaining six non-LC livers were deemed non-viable. A more detailed analysis of the liver perfusion characteristics showed livers that metabolised lactate were more likely to maintain a physiological pH without intervention, establish physiological flow rates in both the hepatic artery and portal vein, and have a less declining haematocrit. We also added evidence of bile production as this is generally accepted as a favourable indicator of graft function, although its absence is not proof of non-function. Using a composite of these parameters aimed to maximise patient safety.

The present study reveals unique data and novel observations. These are the first criteria to be successfully tested in clinical practice and subsequently adopted within a clinical study of viability testing and transplantation of discarded human livers(12). The criteria are easy to measure and consist of familiar parameters. Lactate concentration is one of a number of indicators of graft function in the peri-transplantation period and as such, its inclusion facilitated clinical adoption of the protocol(19). This is the first report that includes marginal organs that were so severely damaged we were unable to maintain the perfusion for 6 hours, which has enabled us to assess the full spectrum of liver function. The proposed criteria appear to correlate closely with the current gold-standard assessment of liver transplantability, histopathological assessment. We have demonstrated the quite marked variability in the assessment of steatosis by the retrieving or transplanting surgeon and the histology of the liver. In this era of progressive organ shortage, such inconsistency may contribute to the wastage of potentially usable livers, further highlighting the urgent need to develop objective assessment methods to improve the relatively low utilisation of high-risk organs.

The Groningen group was the first to demonstrate the feasibility of NMP on 4 discarded human livers. The livers were subjected to 6 hours of NMP following a median CIT of 6h 55m, with all organs showing recovery of function and being deemed viable(20). The inferior outcomes of some livers from our series may be explained in part by the CIT being on average 2 hours longer. A subsequent study from the Groningen group reported an NMP series on 12 discarded livers, proposing six hours of cumulative bile production greater than 20 grams as a marker of a good liver function(21). We were unable to define a cut-off volume because some viable organs in our series did not produce bile. We concur with the observation reported by Sutton et al. of significantly lower lactate levels in the livers with a high bile output.

The Cambridge group advocated assessment based on perfusate transaminases and bile pH(14). The authors observed a significant correlation between the alanine transaminase (ALT) in the perfusate measured after 2 hours' perfusion and the peak ALT post-transplant levels within the first week(14). They also hypothesised that the liver's capacity to produce an alkaline bile (pH>7.4) might be a good marker of cholangiocyte function, possibly identifying a selection of organs with a low risk of developing ischaemic-type biliary lesions. If validated, this observation might revolutionise DCD liver utilisation. However, issues with bile collection, such as technical problems with bile duct cannulation, could lead to discarding usable livers. We agree with findings from the Cleveland group that the importance of bile production in the context of NMP is possibly overestimated(22).

NMP provides the opportunity to explore multiple parameters and it is still to be determined which can best predict post-transplant outcomes. We anticipate that future assessment methods will include more sophisticated techniques, including perfusate proteomic and metabolomic profiling to identify sensitive biomarkers, which could be used in conjunction with the proposed viability criteria to provide further objective measurement of liver functional integrity(23-26). In this study, we also present the outcome of microRNA122 quantitation, frequently used as a marker of tissue injury. The assay system we developed was technically robust and well validated. We identified and utilised an appropriate control microRNA and included positive (spiked) controls. We were unable to show a difference in microRNA 122 levels between the livers defined viable (LC) or non-viable (non-LC). This suggests that whilst microRNA122 may correlate with the degree of tissue damage, it would not appear to be of value in the determination of liver function according to our proposed criteria.

Lactate is the intermediate metabolite of pyruvate within the glycolysis metabolic pathway. In NMP, hyperlactataemia is predominantly due to relative tissue hypoxia resulting from impaired liver blood flow and decreased gluconeogenesis. In this setting, lactate production may exceed its clearance and may be an indicator for real-time liver function monitoring. Viability assessment based principally on lactate clearance offers several advantages compared to other proposed markers: lactate clearance can be measured 30-90 minutes earlier than bile production, providing a particular advantage when using machines designed for relatively short perfusions; lactate can be measured sequentially, providing a trend, and the rate of decline in lactate concentration adjusted for mass of liver tissue (Δlactate/g) may be an even better parameter for characterising the metabolic capacity of the liver compared to simple cut-off levels. This aspect is under active investigation by our group.

The comprehensive histopathological assessment reflected differences between the livers that were consistent with the grouping based on lactate clearance. The development of subtle zone 3 changes to hepatocytes/hepatocyte plates with loss of cell adhesion between them and loss of contact with the sinusoidal lining, features reminiscent of autolytic changes seen at post-mortem, suggest that this is an ischaemic injury modified by lack of tissue response. Hepatocyte glycogen stained by PAS was maintained at higher levels or increased in the LC compared with the non-LC livers over the course of the perfusion, suggesting increased glycogen replenishment. Small droplet microvesicular steatosis has been seen to develop during cold storage and subsequently following reperfusion, suggesting that this may also be a response to ischaemia reperfusion injury(16). Taken together, these results support our hypothesis and suggest that grouping these discarded livers into viable and non-viable groups according to objective functional parameters has merit.

In implementing this novel strategy into our organ selection pathway, patient safety was the highest priority. We set an initial target of meeting criteria within two hours of starting NMP. We appreciate that some "non-viable" organs according to the proposed criteria may still be salvaged by delaying the cut-off for viability assessment or by increasing the required lactate value. Whether livers from a "grey zone" of organs achieving lactate levels of 2.5–4mmol/L later can be used, or if supplementary therapeutic interventions might allow safe transplantation of these organs is an important area of ongoing research(27).

A limitation of our findings is incomplete perfusate transaminases and their correlation with the lactate measurements. Transaminase concentrations have often been used as a surrogate marker of hepatic injury related to the machine perfusion procedure and transplantation(11, 28). Due to progressive perfusate haemolysis during NMP, we were only able to obtain complete sets of perfusate transaminases from four perfusions(8, 10, 11 and 12). In each, there was a steady increase in ALT over the course of the 6-hour perfusion (6851 IU in the liver that was successfully transplanted). Currently, we are unable to comment whether transaminase levels might be used as a reliable indicator of liver function or if they represent a snapshot of the extent of cellular injury that occurred prior to commencing NMP. Another limitation of the proposed criteria is that the primary focus is on function during the early post-transplant period, aiming to prevent early allograft dysfunction and primary non-function, but they do not provide any information concerning the likely long-term post-transplant outcome. We were unable to provide robust data regarding bile duct condition that could be compared in the context of the Groningen and Cambridge groups' dedicated research on ischaemic cholangiopathy(29, 30).
The utilisation of high-risk organs remains globally low, with the principal reasons for rejecting livers being steatosis, poor organ flushing and prolonged cold ischaemic times. Whilst this proportion may differ between countries, these indications clearly imply clinicians' fear of

primary non-function. Ischaemic cholangiopathy as a rationale for liver discard would be pertinent only to DCD donors, particularly for those with prolonged warm ischaemic times. For DBD livers however, the risk of developing cholangiopathy is insignificantly low(9). We believe the proposed criteria, focused primarily on the risk of primary non-function, might globally increase the utilisation of currently wasted livers.

The proposed criteria are, to our knowledge, the first to be used successfully to select and transplant viable livers from the current pool of unutilised organs(12, 31). Having used these for over 3 years, we gained experience and developed confidence in the viability criteria, allowing us to progress to transplanting a subset of these originally discarded livers successfully.

These criteria were tested in a clinical pilot published previously and all patients included in that series are well, with normal liver function and to date three years or longer of follow-up. With our increased experience, we now believe the proposed criteria, used as a starting point for our subsequent work, including the VITTAL study, are conservative and can be further refined(32).

In summary, this study introduces a composite of viability criteria including lactate concentration, bile production and vascular flow patterns. The introduction of objective, real-time methods of assessment are urgently required to address the under-utilisation of high-risk livers. NMP may lead to considerable expansion of the donor pool available for transplantation. Whilst an assessment of viability is important to prevent early post-transplant graft failure, the effects on long-term transplant outcomes are yet to be determined.

3.3.6 Acknowledgements

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3.3.7 Disclosure

Hynek Mergental and Simon C. Afford initiated the study and were responsible for the management of the research project. Barnaby T. F. Stephenson, Jeannette Widmer, Richard W. Laing, Amanda Smith, and Hynek Mergental performed the machine perfusions. Barnaby T. F. Stephenson, Jeannette Widmer, Richard W. Laing, and Gary M. Reynolds collected the samples and data. Desley A. H. Neil, Simon C. Afford, and Stefan G. Hübscher analysed the histology samples. Darius F. Mirza, M. Thamara P. R. Perera, and Hynek Mergental provided the surgical expertise and prepared livers for the perfusions. Lorraine L. Wallace, Yuri L. Boteon, and Richard W. Laing analysed the tissue and perfusate samples. Amanda J. Kirkham and Christina Yap performed the statistical modelling and interpretation. Barnaby T. F. Stephenson, Richard W. Laing, and Yuri L. Boteon carried out other statistical analyses. Barnaby T. F. Stephenson, Hynek Mergental, Richard W. Laing, Desley A. H. Neil, and Simon

C. Afford were responsible for the data interpretation and preparation of the manuscript draft. Amanda Smith and Hynek Mergental edited the text and were responsible for the manuscript submission. All co-authors actively contributed to the project and approved to the final manuscript version. The machine perfusion research was funded by the Queen Elizabeth Hospital Birmingham Charity's Liver Foundation UK and supported by the Medical Research Council and collaboration with the Organ Assist company. Barnaby T. F. Stephenson's salary was funded from the Medical Research Council research fellowship; Richard W. Laing and Yuri L. Boteon are research fellows funded by the National Institute for Health Research (NIHR) Wellcome Trust via the VITTAL clinical trial. The authors are employees of the University Hospitals Birmingham National Health Service (NHS) Foundation Trust or University of Birmingham, and none received any payment or have any conflict of interest related to this manuscript. The normothermic liver perfusion machine was on loan, and the perfusion kits were purchased from Organ Assist, Groningen, the Netherlands. This article includes independent research work supported by the NIHR Birmingham Liver Biomedical Research Unit and the Liver Unit at the Queen Elizabeth Hospital, University Hospitals Birmingham NHS Foundation Trust, and the views expressed are those of the authors and not necessarily those of the NHS, the NIHR, or the Department of Health. Barnaby T. F. Stephenson received grants/contracts of more than $10,000 per year from the Medical Research Council (Clinical Research Training).

3.3.8 Supplementary data

Table S3.1 List of all measured perfusion parameters

	Non-Lactate Clearing (N=6) **Mean** (SD); [min, max]				**Lactate Clearing (N=6)** **Mean** (SD);[min, max]			
	Perfusion time = 0	**Perfusion time = 2hrs**	**Perfusion time = 4hrs**	**Perfusion time = 6hrs**	**Perfusion time = 0**	**Perfusion time = 2hrs**	**Perfusion time = 4hrs**	**Perfusion time = 6hrs**
Lactate (mmol/L)	**13.7** (4.1); [7.2, 20.0]	**14.6** (5.7); [4.4, 20.0]	**13.7** (4.5); [9.2, 20.0]	**14.6** (5.7); [6.9, 20.0]	**10.5** (3.3); [5.5, 13.9]	**3.0** (1.7); [0.6, 5.5]	**2.1** (1.1); [0.7, 4.0]	**2.1** (1.1); [0.7, 3.1]
Glucose	**49.3** (9.7); [37.2, 64.1]	**50.5** (9.7); [39.5, 64.5]	**40.3** (12.4); [26.2, 60.3]	**34.1** (15.2); [15.1, 56.2]	**36.4** (18.3 [9.3, 56.6]	**41.3** (12.9); [23.3, 59.3]	**34.1** (14.8); [14.2, 56.7]	**29.6** (20.2); [8.0, 52.4]
Arterial flow (mL/min)	**213.8** (238.5); [11.0, 593.0]	**316.3** (224.4); [103.0, 631.0]	**412.6** (269.7); [98.0, 810.0]	**495.2** (330.8); [136.0, 835.0]	**155.0** (96.2); [58.0, 313.0]	**524.2** (118.8); [426.0, 727.0]	**575.2** (43.1); [527.0, 638.0]	**621.2** (52.1); [550.0, 682.0]
Arterial flow rate (mL/min/g)	**0.1** (0.1); [0.01, 0.5]	**0.2** (0.1); [0.1, 0.3]	**0.2** (0.1); [0.1, 0.4]	**0.3** (0.2); [0.1, 0.5]	**0.1** (0.05); [0.03, 0.2]	**0.3** (0.1); [0.2, 0.4]	**0.3** (0.1); [0.2, 0.4]	**0.3** (0.1); [0.3, 0.4]
Arterial resistance	**1.3** (1.6); [0.03, 4.2]	**0.3** (0.2); [0.1, 0.6]	**0.3** (0.3); [0.1, 0.8]	**0.2** (0.2); [0.1, 0.6]	**0.4** (0.1); [0.2, 0.6]	**0.1]** (0.04); [0.1, 0.2]	**0.1** (0.01); [0.1, 0.1]	**0.09** (0.01); [0.08, 0.10]
Arterial pressure (mm/Hg)	**48.0** (17.3); [17.0, 68.0]	**57.5** (5.7); [49.0, 61.0]	**64.4** (11.7); [51.0, 82.0]	**64.0** (10.8); [52.0, 80.0]	**47.3** (10.4); [35.0, 60.0]	**59.0** (16.2); [40.0, 82.0]	**54.6** (7.7); [48.0, 66.0]	**54.6** (6.4); [48.0, 63.0]
Portal flow(mL/min)	**613.3** (177.7); [470.0, 910.0]	**962.5** (261.5); [690.0, 1250.0]	**1120.0** (70.7); [1030.0, 1210.0]	**1158.0** (125.2); [970.0, 1320.0]	**458.3** (166.8); [210.0, 630.0]	**1176.0** (192.3); [1000.0, 1430.0]	**1330.0** (225.1); [1100.0, 1650.0]	**1418.0** (320.3); [1070.0, 1920.0]
Portal flow rate (mL/min/g)	**0.3** (0.1); [0.2, 0.4]	**0.5** (0.1); [0.4, 0.7]	**0.6** (0.1); [0.4, 0.7]	**0.6** (0.1); [0.4, 0.7]	**0.2** (0.1); [0.1, 0.4]	**0.6** (0.2); [0.4, 0.9]	**0.7** (0.1); [0.5, 0.9]	**0.7** (0.3); [0.5, 0.9]
Portal resistance	**0.015** (0.003); [0.012, 0.019]	**0.010** (0.003); [0.007, 0.013]	**0.009** (0.001); [0.008, 0.011]	**0.008** (0.001); [0.007, 0.010]	**0.02** (0.0001); [0.01, 0.04]	**0.008** (0.002); [0.006, 0.010]	**0.007** (0.002); [0.005, 0.01]	**0.007** (0.001); [0.005, 0.008]
Portal pressure (mm/Hg)	**8.8** (1.3); [7.0, 11.0]	**8.8** (1.0); [8.0, 10.0]	**9.8** (0.8); [9.0, 11.0]	**9.4** (0.9); [8.0, 10.0]	**7.3** (1.0); [6.0, 10.0)	**9.6** (0.9); [8.0, 10.0]	**9.4** (1.5); [7.0, 11.0]	**9.0** (0.7); [8.0, 10.0]
pH	**7.3** (0.5); [6.8, 8.0]	**7.3** (0.3); [6.8, 7.8]	**7.4** (0.4); [6.9, 7.8]	**7.4** (0.2); [7.2, 7.8]	**7.2** (0.2); [6.9, 7.5]	**7.3** (0.1); [7.2, 7.4]	**7.4** (0.1); [7.3, 7.6]	**7.4** (0.1); [7.3, 7.6]
HCO3 (mmol/L)	**10.7** (7.7); [2.3, 17.4]	**9.8** (7.2); [1.7, 20.1]	**14.2** (8.6); [5.7, 26.8]	**26.4** (10.5); [14.5, 40.7]	**7.2** (0.2); [1.4, 10.0]	**10.3** (3.5); [6.0, 15.7]	**12.1** (4.7); [6.2, 18.7]	**16.4** (6.0); [9.2, 22.9]
pCO2 (kPa)	**2.4** (1.9); [1.1, 3.7]	**2.3** (0.8); [1.6, 3.5]	**3.1** (1.5); [1.4, 4.9]	**5.4** (1.8); [3.8, 8.5]	**1.5** (0.7); [0.6, 2.0]	**2.8** (1.8); [1.6, 6.0]	**2.5** (1.1); [1.4, 4.3]	**3.8** (1.8); [2.3, 6.3]
pO2 (kPa)	**33.1**(21.3); [14.5, 62.8]	**55.5** (29.4); [23.6, 89.0]	**56.7** (27.6); [26.4, 82.1]	**32.2** (26.6); [10.2, 72.6]	**24.3** (12.2); [4.2, 42.6]	**15.4** (8.2); [6.6, 27.3]	**23.0** (8.3); [7.0, 31.2]	**13.7** (4.8); [8.1, 13.4]
Base Excess (mmol/L)	**-18.6** (6.2); [-25.5, -13.5]	**-15.7** (11.9); [-30.6, 1.0]	**-9.8** (12.6); [-24.2, 5.5]	**1.8** (12.7); [-12.0, 20.2]	**-20.8** (6.6); [-28.4, -12.3]	**-13.9** (2.5); [-18.0, 11.8]	**-11.0** (4.4); [-17.6, -5.7]	**-7.8** (6.1); [-15.0, -2.3]
Na (mmol/L)	**106.8** (6.5); [100.9, 114.3]	**132.4** (25.7); [93.7, 172.8]	**151.3** (16.9); [130.8, 173.1]	**158.4** (24.5); [136.6, 196.5]	**124.3** (12.0); [113.5, 147.9]	**129.5** (6.1); [121.0, 135.8]	**131.8** (5.7); [125.0, 138.7]	**137.6** (9.4); [126.8, 149.5]
K (mmol/L)	**15.3** (1.9); [13.6, 17.4]	**15.0** (3.1); [10.4, 17.2]	**12.8** (5.1); [6.0, 18.9]	**11.7** (4.6); [6.0, 17.1]	**14.7** (4.0); [8.4, 18.4]	**14.4** (3.6); [10.4, 19.6]	**13.1** (3.4); [7.5, 17.6]	**13.6** (6.0); [5.3, 19.4]
Cl (mmol/L)	**85.3** (4.5); [79.4, 89.7]	**88.9** (8.2); [76.4, 99.0]	**93.8** (5.2); [86.2, 100.3]	**91.4** (5.9); [83.9, 99.4]	**102.4** (10.2); [93.8, 118.8]	**101.1** (11.7); [89.8, 120.9]	**101.1** (11.9); [93.8, 124.7]	**98.1** (3.9); [94.4, 102.7]
Ca (mmol/L)	**0.6** (.); [0.6, 0.6] n=1	**0.7** (0.5); [0.3, 1.5]	**0.9** (0.7); [0.3, 1.7]	**0.8** (0.6); [0.3, 1.7]	**0.6** (0.2); [0.3, 0.8]	**0.4** (0.2); [0.3, 0.6]	**0.4** (0.1); [0.3, 0.5]	**0.29** (0.05); [0.25, 0.34]
tHb (g/L)	**77.6** (1.6); [75.8, 79.6]	**71.7** (6.3); [64.2, 81.7]	**65.6** (6.1); [58.6, 73.0]	**60.4** (15.1); [46.7, 84.8]	**86.6** (11.0); [74.4, 102.7]	**79.4** (3.7); [74.0, 84.0]	**76.7** (2.2); [73.7, 79.0]	**77.0** (2.7); [73.6, 80.1]
O2Hb (%)	**96.9** (1.7); [94.4, 98.3]	**91.8** (7.8); [78.5, 98.2]	**93.9** (6.0); [84.4, 98.2]	**91.4** (6.9); [81.1, 97.8]	**97.6** (0.8); [96.9, 99.1]	**96.8** (1.7); [95.2, 99.2]	**97.5** (0.9); [96.4, 99.1]	**94.6** (3.5); [89.6, 97.0]
COHb (%)	**1.1** (0.1); [1.0, 1.2]	**1.2** (0.5); [0.7, 2.1]	**1.0** (0.2); [0.8, 1.4]	**1.2** (0.1); [1.1, 1.4]	**1.4** (0.5); [1.0, 2.4]	**1.2** (0.1); [1.0, 1.3]	**1.4** (0.5);[1.0, 2.3]	**1.6** (0.6); [1.1, 2.5]
H.Hb (%)	**1.5** (1.6); [0.3, 3.9]	**0.5** (0.2); [0.4, 0.7]	**0.5** (0.1); [0.4, 0.7]	**1.6** (1.3); [0.4, 3.1]	**0.8** (0.3); [0.3, 1.3]	**1.8** (1.3); [0.7, 3.3]	**0.8** (0.1); [0.6, 0.9]	**3.2** (2.9); [1.0, 7.3]
MetHb (%)	**0.5** (0.05); [0.4, 0.5]	**6.7** (7.8); [0.5, 20.4]	**4.6** (6.1); [0.4, 14.4]	**5.8** (7.6); [0.3, 16.7]	**0.5** (0.1); [0.4, 0.6]	**0.6** (0.2); [0.5, 0.9]	**0.6** (0.1); [0.5, 0.7]	**0.6** (0.1); [0.4, 0.7]
SO2 (%)	**98.5** (1.6); [96.1, 99.7]	**99.5** (0.2); [99.2, 99.6]	**99.5** (0.2); [99.3, 99.6]	**98.3** (1.4); [96.8, 99.6]	**99.1** (0.3); [98.7, 99.7]	**98.2** (1.3); [96.7, 99.3]	**99.2** (0.1); [99.1, 99.4]	**96.7** (2.9); [92.5, 98.9]
Haematocrit (%)	**29.0** (1.4); [27.8, 31.1]	**23.3** (5.4); [14.8, 29.5]	**16.0** (4.4); [11.3, 20.8]	**16.6** (6.0); [12.1, 23.4]	**26.2** (3.0); [20.8, 29.7]	**22.6** (1.3); [21.3, 24.0]	**21.3** (2.0); [18.9, 23.5]	**19.8** (2.3); *[*17.7, 23.0]
O2 consumption	**24.2** (1.4); [23.2, 25.2]	**34.1** (7.8); [25.1, 39.4]	**23.8** (13.7); [7.3, 39.6]	**46.8** (11.1); [31.0, 57.0]	**15.0** (13.3); [1.4, 37.0]	**34.2** (17.4); [15.8, 58.5]	**32.3** (15.7); [18.5, 83.9]	**54.2** (28.1); [18.5, 83.9]
O2 consumption mass	**0.013** (0.002); [0.011, 0.014]	**0.017** (0.005); [0.013, 0.023]	**0.013** (0.006); [0.004, 0.017]	**0.027** (0.010); [0.018, 0.041]	**0.008** (0.008); [0.001, 0.021]	**0.018** (0.009); [0.008, 0.029]	**0.016** (0.008); [0.005, 0.024]	**0.027** (0.011); [0.011, 0.036]
Oxygen extraction ratio	**0.2** (0.1); [0.2, 0.3]	**0.2** (0.04); [0.2, 0.3]	**0.3** (0.3); [0.2, 0.8]	**0.3** (0.2); [0.2, 0.6]	**0.2** (0.1); [0.02, 0.3]	**0.2** (0.1); [0.1, 0.3]	**0.2** (0.1); [0.1, 0.2]	**0.3** (0.1); [0.1, 0.4]

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CHAPTER 4 - THE USE OF DISCARDED DONOR LIVERS FOLLOWING VIABILITY TESTING USING NMP-L

4.1 TRANSPLANTATION OF DECLINED LIVER ALLOGRAFTS FOLLOWING NORMOTHERMIC EX-SITU EVALUATION

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Authors: Mergental H[1,2*], Perera MTPR[1*], Laing RW[2], Muiesan P[1], Isaac JR[1], Smith A[1], Stephenson BTF[2], Cilliers H[1], Neil DAH[1], Hübscher SG[1], Afford SC[2], Mirza DF[1,2]

Affiliations:

[1] Liver Unit, Queen Elizabeth Hospital Birmingham, University Hospitals Birmingham NHS Foundation Trust, United Kingdom

[2] National Institute for Health Research (NIHR) Birmingham Liver Biomedical Research Unit and Centre for Liver Research, Institute of Immunology and Immunotherapy, Institute for Biomedical Research, College of Medical and Dental Sciences, University of Birmingham, United Kingdom

4.4.1 Abstract

The demand for liver transplantation exceeds supply with rising waiting list mortality. Utilisation of high-risk organs is low and a substantial number of procured livers are discarded. We report the first series of five transplants with rejected livers following viability assessment by normothermic machine perfusion of the liver (NMP-L). The evaluation protocol consisted of perfusate lactate, bile production, vascular flows and liver appearance. All livers were exposed to a variable period of static cold storage prior to commencing NMP-L. Four organs were recovered from donors after circulatory death and rejected due to prolonged donor warm ischaemic times; one liver from a brain death donor was declined for high liver function tests. The median (range) total graft preservation time was 798 (724-951) minutes. The transplant procedure was uneventful in every recipient with immediate function in all grafts. The median in-hospital stay was 10 (6-14) days. At present, all recipients are well, with normalised liver function tests at median follow-up of 7 (6-19) months. Viability assessment of high-risk grafts using NMP-L provides specific information on liver function and can permit their transplantation while minimising the recipient risk of primary graft non-function. This novel approach may increase organ availability for liver transplantation.

4.4.2 Introduction

Deaths from liver disease have soared by 40% in the last decade, killing 11,000 a year in England at an average age of 59 years(1). Liver transplantation (LT) is highly successful in treating end-stage disease, but access is restricted by the number of available organs and approximately 20% of patients die whilst awaiting transplantation(2-5). To address this, more transplants are performed using high-risk organs, from donors with co-morbidities or relative contraindications(6-9). These organs, termed "marginal" or "extended criteria" grafts, are more susceptible to cold ischaemia, and have an increased risk of graft failure, recipient morbidity and mortality(7, 10). The devastating consequences of graft failure following LT preclude greater utilisation of high-risk livers. For example, in 2014-15, of 1,282 identified UK donors, only 924 (72%) livers were deemed suitable for retrieval and 812 (63%) were subsequently transplanted(2). Data from the United States are similar and the latest report of the Organ Procurement and Transplant Network showed that only 6,312 out of 8,144 (73%) potential donor livers were transplanted(3). Over the same period, in these two countries combined more than 3,200 patients died or were removed from the transplant waiting list for being too sick for transplantation(3, 11).

Normothermic machine perfusion of the liver (NMP-L) is a novel technique, substituting the detrimental effect of static cold storage (SCS) by preserving the organs in near-physiological conditions, with oxygen and nutrients at 37°C. The preserved metabolic activity at normothermia not only prevents further graft damage caused by ischaemia, but allows *ex-situ* monitoring of liver function by permitting objective assessment of liver biochemistry, blood flow and bile production. The complexity of dual - arterial and portal - liver inflow has proved technically challenging. The first machine introduced into clinical practice was recently developed by the Oxford group, and was used for the pilot liver transplant series using standard

criteria organs preserved by NMP-L, completely avoiding SCS(12). Our pre-clinical studies on discarded livers showed that perfusate lactate clearance in combination with bile production and stable blood flow rates are sensitive parameters predictive of graft viability, and in August 2014 our group carried out the first-in-man transplant of such a liver graft(13, 14). Here, we present the first five recipients of NMP-L treated rejected liver allografts.

4.4.3 Methods

4.4.3.1 Study design

This series evolved from a research project of viability testing of rejected human livers where NMP-L based viability criteria were established and a perfusion fluid was developed to facilitate resuscitation of high-risk organs. After defining viability criteria, we obtained approval from the hospital ethics and novel therapeutic committees in June 2014 to perform a pilot series of five clinical transplants. Here we present the results of six consecutive NMP-Ls, commenced with an intention to perform clinical transplantation in carefully selected and consented adults with grafts that met viability criteria.

4.4.3.2 Source of rejected human livers

Based on donor history and laboratory results, the livers (except donor four with progressively rising liver function tests) were initially accepted and procured by one of the teams from the UK National Organ Retrieval Service, using a nationally agreed surgical protocol, with the intention of transplantation(15). All grafts were initially preserved in University of Wisconsin preservation fluid at 4^{o}C.

On arrival at the transplanting centre, each liver was assessed and deemed unsuitable by the consultant surgeon. The liver was then offered to and turned down by all UK liver transplant centres and then offered for use in our pilot study by the NHSBT co-ordinating office. Ethical approval for the study was granted by the University Hospital Birmingham NHS Foundation Trust Novel Therapeutics and NHSBT Ethics Committees.

To ensure safety, risks were minimised by excluding livers with a significant pre-existing disease, and all grafts in this study met the following inclusion criteria: cold ischaemic times

(CIT) less than 16 hours for livers from donors after brain death (DBD), or less than 10 hours from donors after circulatory death (DCD), donor warm ischaemic time (dWIT; defined as the interval between systolic blood pressure less than 50mmHg or oxygen saturation less than 70% to aortic perfusion) in DCD organs less than 60 minutes, absence of hepatitis B, hepatitis C, or human immunodeficiency virus infection, and a macroscopic appearance without fibrosis or cirrhosis. Following a review of the protocol after the unsuccessful perfusion 2, an additional criteria of maximum donor age of 65 years was added.

We were offered about 15 livers for machine perfusion research over the study period but utilised only a proportion of these due to the limited availability of personnel to perform the perfusions.

4.4.3.3 Clinical protocol for liver viability testing

Graft preparation was analogous to the standard back-table procedure, and the portal vein was dissected and cannulated. The celiac trunk branches were ligated and the hepatic artery was dissected to the gastroduodenal artery. We routinely attached an iliac artery interposition graft to the aortic patch to facilitate the insertion of the arterial perfusion cannula.

The perfusion fluid was based on 3 units of the donor liver specific blood group, Rhesus-negative, packed red cells, supplemented with 1000ml human albumin solution 5%, 30ml sodium bicarbonate 8.4% and 10ml calcium gluconate 10%. The circuit was loaded with 10,000 IU heparin, 500mg vancomycin and 60mg gentamicin prior to connecting the liver, with the continuous infusion of epoprostenol (8μg/hour).

NMP-L was then commenced, using two different devices. Livers from donors 1 to 5 were

perfused with Liver Assist (Organ Assist, the Netherlands). This device provides a pulsatile arterial and continuous non-pulsatile portal flow via two independent rotary pump circuits. The liver from donor 6 was perfused with the OrganOx Metra device (OrganOx, UK) delivering continuous non-pulsatile arterial and portal flows powered by one rotary pump. Organ viability was assessed within three hours of perfusion. In a viable liver the perfusate lactate level had to be less than 2.5mmol/L or the liver had to produce bile, in combination with at least two of the following three criteria: 1) perfusate pH greater than 7.30, 2) stable arterial flow of more than 150ml and portal venous flow more than 500ml per minute, and 3) homogeneous graft perfusion with soft consistency of the parenchyma.

4.4.3.4 Histology

Menghini liver biopsies were obtained at three time points: 1) pre-NMP-L, 2) at the end of NMP-L, and 3) following reperfusion of the implanted liver. The cut end of the common bile duct was obtained post-NMP-L. All biopsies were placed in 10% formalin and processed by standard procedures to a paraffin block. Sections stained with Haematoxylin and Eosin (H&E) and Periodic Acid Schiff (PAS) were examined for the percentage of large droplet (ld) and small droplet (sd) macrovesicular steatosis (MS), hepatocyte necrosis and glycogen depletion. Preservation-reperfusion injury in post-reperfusion biopsies was graded based on these features together with neutrophil infiltration. The grading system used has been developed in-house over many years by correlation with peak post-operative transaminases (unpublished data) and evolved from examination of sequential findings prior to retrieval, during cold storage and following reperfusion(16). Bile duct biopsies were assessed for loss of the lining epithelium, epithelial damage in superficial and deep peribiliary glands, stromal necrosis, arteriolar necrosis and thrombosis according to previously published criteria(17). Histological assessments were all performed after graft implantation and did not therefore impact on

decisions concerning viability assessment.

4.4.3.5 Transplant recipients

The recipients were patients listed for transplantation at Queen Elizabeth Hospital Birmingham, UK. All patients received an explanation about the principles of NMP-L during consenting for LT. When a recovered viable liver graft became available, the consultant surgeon familiar with the project re-explained the procedure in detail and obtained patients' additional consent to accept the graft. Recipients considered for this study had low surgical perioperative risk as assessed by the multi-disciplinary team during the listing process. Patients with hepatocellular carcinoma, with a high risk of waiting list dropout due to tumour progression, were regarded as favourable recipients.

4.4.3.6 Liver transplant procedure and patient follow up

The grafts were implanted with the vena cava preserving technique. After completing the native liver hepatectomy, the NMP-L was stopped and the graft was flushed with 2 litres of cold Histidine-Tryptophan-Ketoglutarate solution, vascular and bile duct cannulas were removed and bile duct and liver biopsies were taken. The graft was immediately implanted and reperfused in the standard manner. The perioperative data, post-transplant laboratory results and details of the patient's recovery course were collected. Following discharge from the hospital, patients were reviewed in the outpatient clinic with weekly (1^{st} month) and then fortnightly (2^{nd} to 3^{rd} month) frequency.

4.4.3.7 Funding source

The project was funded by QEHB Charities (Liver Foundation) and supported by the National Institute for Health Research (NIHR) Birmingham Liver Biomedical Research Unit. The Organ

Assist (n=5 livers) and OrganOx Metra (n=1) devices used were on loan and neither of the two manufacturers had any role in the study design, data collection, analysis, interpretation or the manuscript preparation. The authors are employees of the University Hospital Birmingham NHS Foundation Trust or University of Birmingham and none of them received any payment or have any conflict of interest related to this manuscript.

4.4.4 Results

The median donor age was 49 (range 29-54) years. Four livers were recovered from DCD and two from DBD donors. There was an even split between the liver offers initially accepted and retrieved by our team versus other teams. The median CIT was 422 (387-474) minutes. Five out of six livers met the viability criteria and were used for transplantation. The detailed demographics and graft characteristics are provided in Tables 4.1 and 4.2.

Table 4.1 Donor demographics and liver characteristics

	Donor 1 (Transplant 1)	**Donor 2 (Discarded)**	**Donor 3 (Transplant 2)**	**Donor 4 (Transplant 3)**	**Donor 5 (Transplant 4)**	**Donor 6 (Transplant 5)**
Donor information						
Age	29	69	49	49	46	51
Donor type	DCD	DBD	DCD	DBD	DCD	DCD
Sex	Male	Male	Female	Female	Male	Female
Height (cm)	173	174	169	161	179	165
Body weight (kg)	75	94	130	52	90	90
Body mass index (kg/m^2)	25	31	45	20	28	33
Premorbid cardiac arrest (downtime minutes)	Yes (58)	Yes (multiple)	Yes (35)	No	Yes (40)	No
Peak ALT (IU/L)	137	2264[a]	52	997[b]	1297[c]	49
Days on ventilator	8	27	2	7	6	2
Comorbidities and history	Diabetes mellitus (type 1)	Bladder cancer (recent surgery) hypertension	Paracetamol overdoses, DVT hypertension	Suprasellar meningioma (recent surgery)	Alcohol misuse	Diabetes mellitus (type 2) hypertension
Cause of death	Hypoxic brain injury	Hypoxic brain injury	Hypoxic brain injury	Intracranial haemorrhage	Hypoxic brain injury	Intracranial haemorrhage
Liver characteristics						
Liver weight (g)	1997	2400	1943	1382	2486	2522
Donor warm ischemic time (min)	109	NA	36	NA	31	19
Cold ischemic time (min)	422	518	406	387	453	474
Donor risk index	2.31	1.97	2.36	1.83	2.25	3.03
Graft offering[d]	Fast-track	Full offer	Full offer	Fast-track	Fast-track	Fast-track
Retrieval team and location[e]	Regional[f]	Regional	Regional	Extra-zonal	Extra-zonal	Extra-zonal
Reason for initial rejection	Long dWIT, poor liver flush	High LFTs, biopsy findings	Long dWIT, donor history, BMI	High LFTs, macroscopic appearance	Long dWIT, macroscopic appearance	Macroscopic appearance

Table 4.2 Machine perfusion characteristics of perfused donor livers

	Donor 1 (Transplant 1)	**Donor 2 (Discarded)**	**Donor 3 (Transplant 2)**	**Donor 4 (Transplant 3)**	**Donor 5 (Transplant 4)**	**Donor 6 (Transplant 5)**
Machine perfusion parameters						
Perfusion device	Organ Assist	Organ Assist	Organ Assist	Organ Assist	Organ Assist	OrganOx Metra
Lactate (mmol/L)						
Highest	13.3	11.4	5.5	13.1	12.4	13.9
Lowest	0.7	2.1	1.4	2.2	1.2	0.9
Last	0.7	4.5	1.4	2.4	1.2	2.8
Total bile production (g)	23.2	6.1	0	18.5	11.3	0
Mean arterial flow (mL/min)	558	491	476	623	654	360
Mean portal vein flow (L/min)	0.8	0.7	1.2	1.5	1.3	1.1
Mean liver mass perfusion (mL/g/min)	0.68	0.49	0.88	1.54	0.78	0.36
Perfusion time (min)	416	255	318	564	345	305
Total preservation time (min)	838	773	724	951	798	779
Transplanted	Yes	No	Yes	Yes	Yes	Yes
Operation lactate levels (mmol/L)						
Lactate peak/end of surgery	7.0/4.5	NA	4.3/3.0	4.0/2.9	5.0/3.3	3.6/1.4

ALT, alanine transaminase; DBD, donor after brain death; DCD, donor after circulatory death; DVT, deep vein thrombosis; dWIT, donor warm ischemic time; LFTs, liver function tests; NA, not applicable; UHB, University Hospitals Birmingham.

[a] ALT 2264 IU/L post cardiac arrest, reducing to 883 IU/L at time of retrieval.

[b] ALT progressively rising to 997 IU/L at the time of retrieval.

[c] ALT 1297 IU/L post cardiac arrest, reducing to 257 IU/L at time of retrieval.

[d] Fast-track offers denotes the liver was offered following refusal by other teams, often after it was procured and inspected by the retrieval team

[e] Regional liver procurements were performed by the UHB team, with the expected travel time back to the hospital less than 3 h; extra-zonal procurements were performed by other teams, with the expected shipment time greater than 3 h.

[f] Expected travel time greater than 4 h.

4.4.5 Donor history details and reasons for initial graft rejection

Donor one (DCD) was a 29-year-old diabetic male admitted with cardiac arrest, having elevated liver function tests (LFTs). The dWIT was 109 minutes and the graft appearance patchy. The liver was rejected due to prolonged dWIT and poor perfusion.

Donor two (DBD) was a 69-year-old male ventilated for 27 days following surgery for ascending aorta dissection, with a peak ALT of 2,264 IU/L and multiple cardiac arrests. The liver was rejected based on history and LFTs.

Donor three (DCD) was a 49-year-old female with body mass index (BMI) 45kg/m^2 with a history of hypertension, depression with two suicide attempts with paracetamol overdose, and deep vein thrombosis with an infected chronic leg ulcer. The liver was rejected due to the prolonged dWIT (36 minutes) in combination with high BMI.

Donor four (DBD) was a 54-year-old female with an intracranial bleed post-resection of a suprasellar meningioma. Because of rising LFTs (ALT 997 IU/L on day of donation), the liver was not accepted.

Donor five (DCD) was a 46-year-old male who collapsed with a cardiac arrest of 40 minutes duration. He was a known heavy drinker and the admission ALT was 1,297 IU/L. The graft was rejected due to its large size (2,486g) and abnormal LFTs.

Donor six (DCD) was a 51-year-old male with an intracranial haemorrhage, diabetes on metformin and BMI 33kg/m^2. The liver was rejected due to large size (2,522g) and steatotic appearance on macroscopic assessment.

4.4.6 Viability evaluation

All but one liver met defined criteria for viability and showed signs of function as assessed by the perfusate lactate clearance and bile production. The median starting lactate level was 9.9mmol/L that decreased in two hours to the median level 1.5mmol/L. The median NMP-L time was 332 (318-564) minutes. The total preservation time of the transplanted livers was 798 (724-951) minutes.

The donor two liver did not meet viability criteria, despite initially showing a rapid lactate clearance with levels decreasing from 11.4mmol/L to 2.1mmol/L within the first two hours of perfusion. The liver had aberrant arterial anatomy, with an accessory right hepatic artery rising from the superior mesenteric artery. Despite a presence of back-flow bleeding from the artery stump after graft connection to the device, there was noticeable colour difference on the liver surface after 90 minutes of perfusion, prompting arterial reconstruction. Following re-established inflow via the accessory artery, lactate levels rose and did not normalise within the three hour time frame, and the liver was discarded. This event suggests that any vascular reconstruction for aberrant arterial anatomy should be performed prior to commencing the perfusion.

The donor six graft function recovery occurred soon after starting NMP-L with fluctuations and increase of lactate levels during the later perfusion course. In terms of the decision-making we consider the key lactate reading as being the one taken at the time of graft viability assessment (the point when lactate drops below 2.5mmol/L, or the reading taken at 3 hours after commencing NMP-L). Although we continued to measure the parameters every 30 minutes thereafter, these values do not have any impact on the transplantation procedure, as

the recipient operation is already in progress. Details of the NMP-L parameters, graft function, and transplantation procedure are provided in Table 4.1 and Figure 4.1.

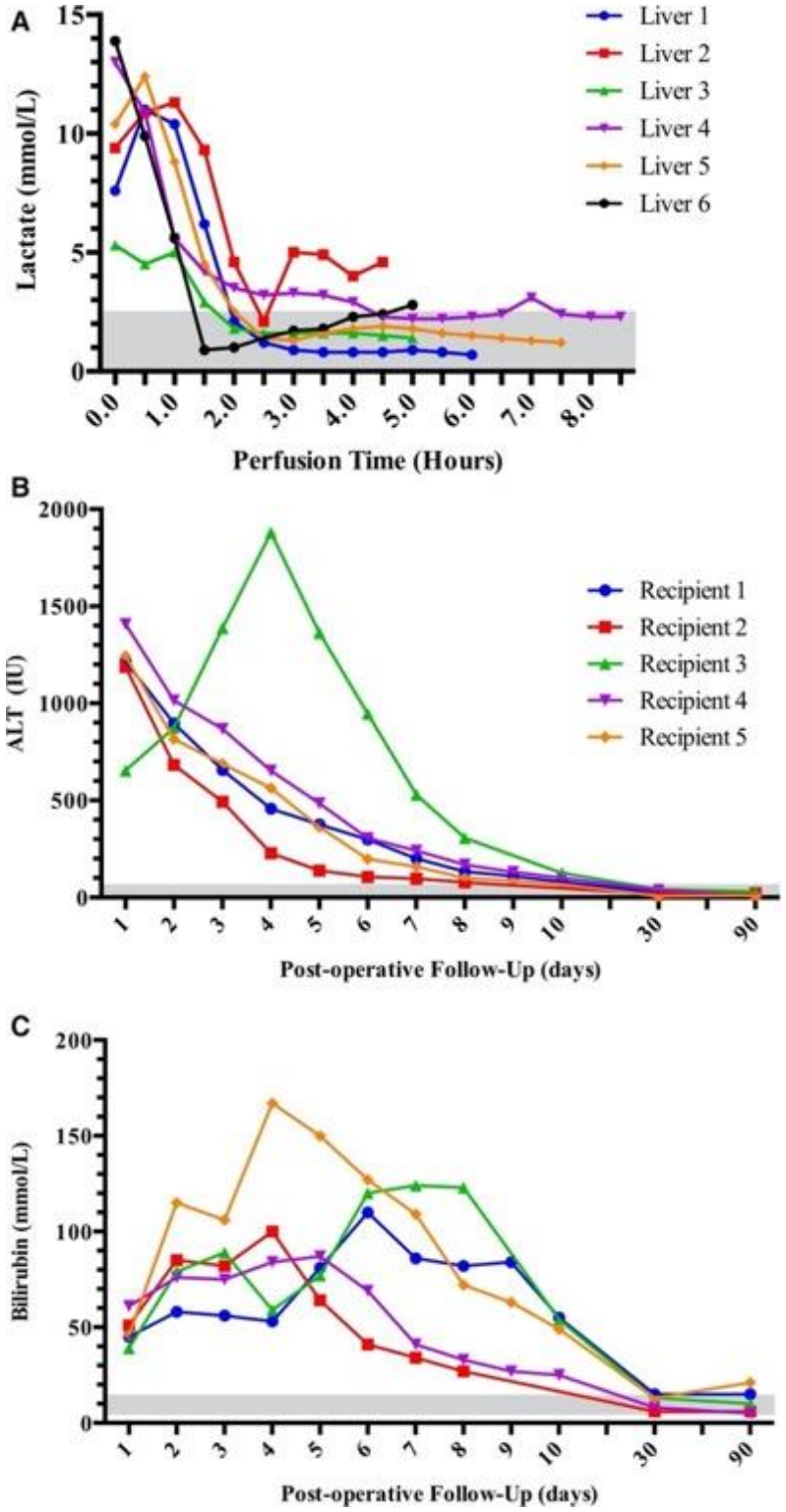

Figure 4.1 Viability assessment by the perfusate lactate clearance and the posttransplant liver function tests.

Panel (A) shows the lactate clearance during the normothermic perfusion. All livers demonstrated metabolic activity, and perfusate lactate levels dropped below 3.0 mmol/L. In liver number 2 the lactate levels fell to 2.1 mmol/L, but started to rise again after 150 min. The organ failed to meet the viability criteria and was not used for transplantation. Panel (B) shows the posttransplant changes in the ALT levels; the enzyme is often used as a surrogate marker for preservation-related liver injury. The initial posttransplant levels were similar in all livers, with progressive improvement within the first posttransplant week. In all recipients the ALT levels were normal within the first month after transplantation. Panel (C) demonstrates a similar improvement pattern with bilirubin levels. In recipient number 1, bilirubin levels slightly increased later during follow-up and the magnetic cholangiography performed at 6 months posttransplant revealed a mild anastomotic biliary stricture. The bilirubin level normalized with conservative management of ursodeoxycholic acid medication. ALT, alanine transaminase.

4.4.7 Histological findings

No significant large droplet steatosis was seen in these livers, with the majority (4/6) also having negligible sdMS and two having mild (<33%) sdMS (Figure 4.2A, B). Hepatocyte necrosis (Figure 4.2C, D) of more than just a few cells was present in one liver which was transplanted (30% increasing to 50% post-transplant), and in the one which did not reach transplant criteria (15% hepatocyte loss from necrosis at an earlier time point). In 4/5 of the transplanted livers glycogen stores appeared to be replenished during NMP-L (Figure 4.2E, F). The injury post-transplant varied from mild to severe.

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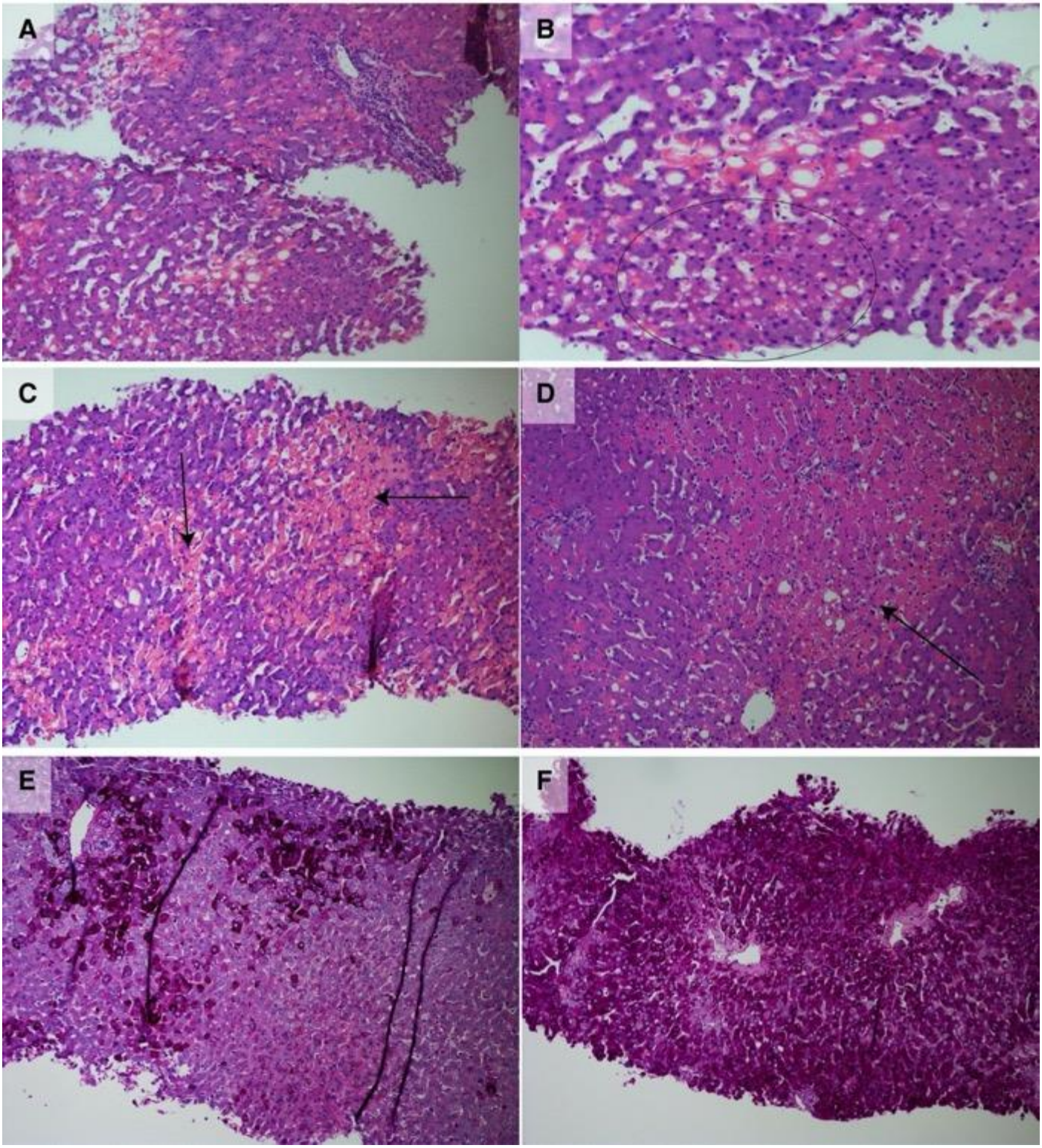

Figure 4.2 Histological findings in liver biopsies.

Panels (A) and (B) show pre-NMP-L H&E-stained biopsies from liver number 5. Panel (A) shows negligible large droplet macrovesicular steatosis (10× objective). Panel (B) is a higher magnification showing small droplet macrovesicular steatosis involving roughly 20–30% of the hepatocytes. This is seen within the circled area as tiny white holes in the hepatocytes. This type of steatosis, often referred to as microvesicular steatosis, is not considered to be important

in determining the amount of fat in an assessment for transplantation. None of the livers had more than 5% large droplet steatosis, the type that determines suitability for transplantation (20× objective). Panels (C) and (D) demonstrate areas of necrosis seen as the pale pink hepatocytes (arrows) in post-NMP-L biopsies from liver number 5. Panel (C) shows approximately 30% necrosis in the preimplantation biopsy. Panel (D) shows an increase in the number of necrotic hepatocytes in the postreperfusion biopsy, approximating to 50% of the liver parenchyma. This liver showed the most necrosis in this presented series; this degree of necrosis is considered unfavourable by currently used assessment standards. The additional information provided by the functional assessment using the normothermic perfusion confirmed the liver viability and the graft was successfully transplanted with immediate intraoperative recovery of the function and good patient recovery (both sections H&E, 10× objective). Panels (E) and (F) are PAS-stained sections of biopsies from liver number 1 in which glycogen in hepatocytes stains dark pink. Panel (E) shows the pre-NMP-L biopsy with moderate glycogen depletion. Panel (F) shows the post-NMP-L biopsy with increased glycogen content, now amounting to only mild depletion (both 10× objective). H&E, haematoxylin and eosin; NMP-L, normothermic machine perfusion of the liver; PAS, periodic acid–Schiff.

Bile duct injury (Figure 4.3) was generally mild: there were only mild epithelial changes in deep peribiliary glands in the livers transplanted. One post-NMP-L bile duct biopsy showed mild, two moderate and three severe stromal nuclear loss. Mild arteriolar necrosis was seen in three of the post-NMP-L biopsies. Thrombosis was not seen. The detailed findings are provided in Table 4.3

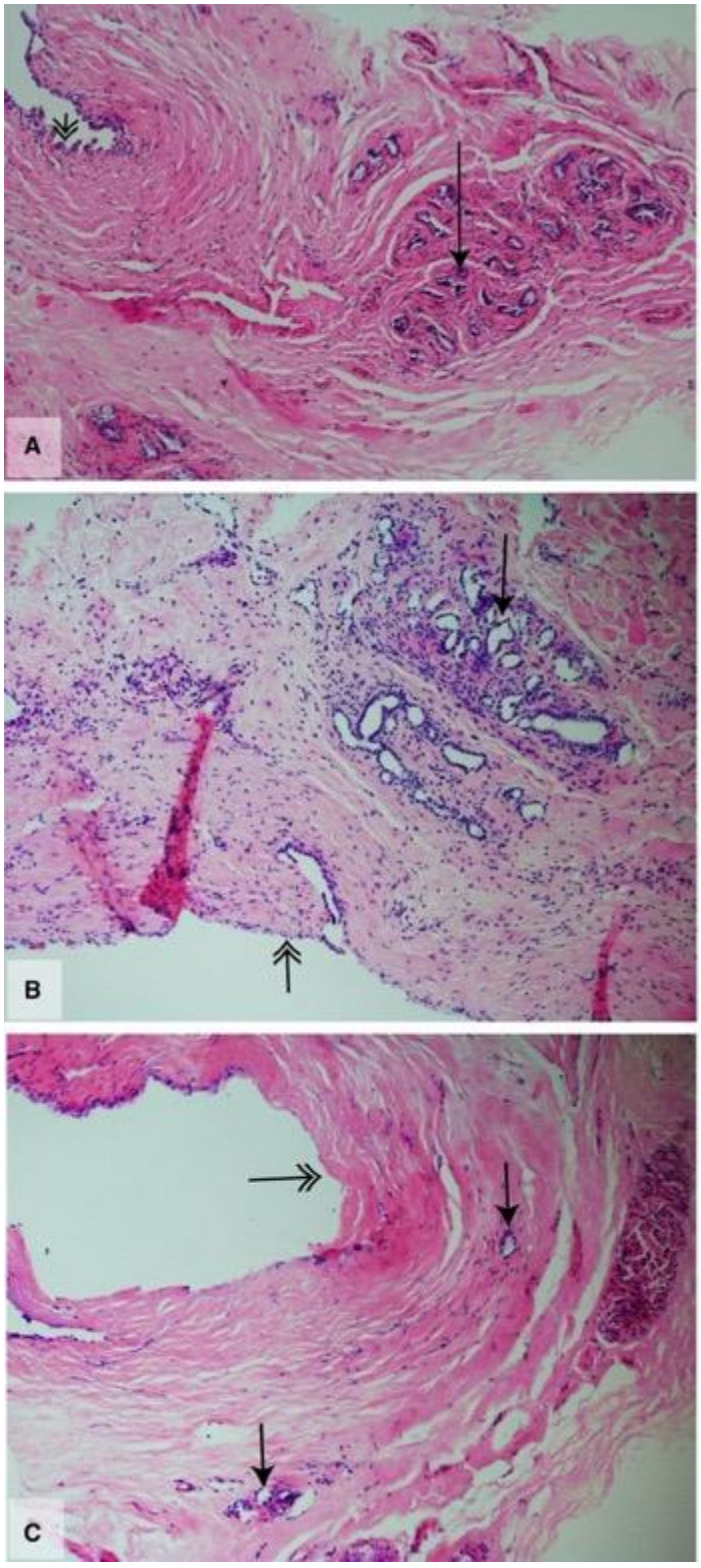

Figure 4.3 Bile duct histology.

This figure demonstrates H&E-stained sections of bile duct. The double arrowhead shows the surface epithelial lining and the single arrowhead points to a deep peribiliary plexus. Panel (A) shows the surface epithelium is intact in this part of the bile duct with relatively mild changes

to the deep peribiliary glands in liver number 6. Panel (B) displays partial surface epithelial loss with well-preserved peribiliary glands in liver 4. Panel (C) shows another fragment of bile duct from liver 6 in which there is moderately extensive loss of surface epithelium, with stromal nuclear loss deep to the double arrowhead; the deep peribiliary glands in this area look moderately injured (all 10× objective). H&E, haematoxylin and eosin.

Table 4.3 Histological features on liver biopsies

	Donor 1 (Transplant 1)	**Donor 2 (Discarded)**	**Donor 3 (Transplant 2)**	**Donor 4 (Transplant 3)**	**Donor 5 (Transplant 4)**	**Donor 6 (Transplant 5)**
Large droplet macrovesicular steatosis[a]						
Pre-NMP-L	None	NA	NA	None	<5%	None
Post-NMP-L	None	None	None	<5%	<5%	None
Post-reperfusion	None	NA	None	<5%	<5%	None
Small droplet macrovesicular steatosis[b]						
Pre-NMP-L	<5%	NA	NA	20%	20%	<5%
Post-NMP-L	<5%	30%	<5%	<5%	20%	None
Post-reperfusion	None	NA	10%	<5%	25%	10%
Necrosis[c]						
Pre-NMP-L	None	NA	NA	5%	None	None
Post-NMP-L	1%	15% (old)	5%	None	30%	None
Post-reperfusion	1%	NA	10%	1%	50%	5%
Glycogen depletion[d]						
Pre-NMP-L	Moderate–severe		Moderate	Minimal	Severe	Mild–moderate
Post-NMP-L	Mild	Severe	Mild–moderate	Moderate–severe	Mild	None
Post-reperfusion	Moderate	NA	Moderate–severe	Moderate	Moderate–severe	Moderate–severe
Post-reperfusion injury	Mild	NA	Moderate	Moderate	Severe	Moderate–severe
Bile duct biopsies[e]						
Superficial epithelium	>50%	>50%	>50%	<50%	<50%	<50%
Superficial PBG	>50%	>50%	>50%	<50%	<50%	<50%
Deep PBG	<50%	>50%	<50%	<50%	<50%	<50%
Stromal nuclear loss	Severe	Severe	Severe	Mild	Moderate	Moderate
Arterial medial loss	Mild	Mild	None	None	Mild	None
Thrombi	None	None	None	None	None	None
Haemorrhage	None	None	None	None	None	None

NA, not applicable/available; NMP-L, normothermic machine perfusion of the liver; PAS, periodic acid–Schiff; PBG, peribiliary gland.

[a] Large droplet macrovesicular steatosis is defined as a single large fat droplet within the hepatocyte cytoplasm displacing the nucleus. Mild <1/3, moderate 1/3–2/3, and severe >2/3 of hepatocytes contain large droplet macrovesicular fat.

[b] Small droplet macrovesicular steatosis is defined as fat droplets, usually multiple within the cytoplasm of the hepatocyte, which do not displace the nucleus. Mild <1/3, moderate 1/3–2/3, and severe >2/3 of hepatocytes contain small droplet macrovesicular fat.

[c] Necrosis is depicted as the percent of total hepatocytes in the biopsy that are necrotic.
[d] Glycogen depletion is graded as mild—up to 20% of nonnecrotic hepatocytes do not contain PAS-positive glycogen, moderate 20–95% of hepatocytes do not contain glycogen, and severe >95% of hepatocytes do not contain glycogen.
[e] Classification grading as follows: loss of surface and peribiliary glands none – no loss, mild ≤50%, and severe >50% loss of cells; stromal nuclear loss none – no loss, mild ≤25%, moderate 25–50% loss, severe >50% loss; arterial medial loss none – no loss of nuclei from media, mild – incomplete nuclear loss in ≤50% of arteries/arterioles, moderate >50% incomplete nuclear loss, severe – complete necrosis of wall in >50% of arteries/arterioles.

4.4.8 Patient outcomes

The median recipient age was 56 (47-66) years. The transplantation procedure was uneventful for every recipient with immediate function recovery in all grafts. The median intensive therapy unit (ITU) stay was 3 (2-6) days, with one early ITU readmission in a patient who developed acute coronary syndrome 8 days following surgery, requiring percutaneous coronary intervention with stent insertion. The median in-hospital stay was 10 (6-15) days. To date, all patients are well, with normalised liver tests at a median follow-up of 7 (6-19) months. Recipient three (donor 4 liver) showed a different post-transplant ALT profile compared to the other recipients and this may be related to the severe pre-retrieval injury as documented by the progressively rising ALT (peak 997 IU/L) within 24 hours prior to donation. This might also explain the different pattern of its lactate clearance and in this particular case the viability criteria were met by bile production rather than lactate level at 3 hours. The recipient demographics and outcome details are provided in Table 4.4.

Table 4.4 Recipient demographics and outcomes

	Recipient 1 (donor 1)	**Recipient 2 (donor 3)**	**Recipient 3 (donor 4)**	**Recipient 4 (donor 5)**	**Recipient 5 (donor 6)**
Age at transplant (years)	46	56	66	65	56
Sex	Male	Male	Male	Male	Female
Primary aetiology	Alcohol	NAFLD	Alcohol and NAFLD	Hemochromatosis	Alcohol
Indication for transplant	Encephalopathy	Refractory ascites	HCC	HCC	Refractory ascites
MELD at LT	17	9	7	7	8
UKELD at LT	55	49	51	47	51
Waiting list time (months)	2	6	7	1	3
ITU stay (days)	5	2	3	6	3
Early allograft dysfunction[a]	No	No	No	No	No
Renal replacement therapy	No	No	No	Yes (10 days)	No
In hospital stay (days)	12	7	6	15	10
Post-transplant complications[b]	None	None	None	Grade IVb (MI, PCI, RRT)	None
Liver function tests					
Peak ALT (IU/L)	1215	1188	1879	1408	1242
Peak bilirubin	110	100	124	87	167
At 1 month					
ALT (IU/L)	24	17	43	38	6
Bili (μmol/L)	15	6	13	8	13
ALP (IU/L)	73	113	114	178	64
At 3 months					
ALT (IU/L)	16	21	29	8	10
Bili (μmol/L)	15	6	10	5	21
ALP (IU/L)	135	103	79	63	81
Creatinine (μmol/L)					
At 1 month	90	67	78	168	62
At 3 months	82	77	98	147	92

ALP, alkaline phosphatase; ALT, alanine transferase; AST, aspartate transferase; Bili, bilirubin; HCC, hepatocellular carcinoma; ITU, intensive therapy unit; LT, liver transplantation; MELD, Mayo end-stage liver disease score; MI, myocardial infarction; NAFLD, non-alcoholic fatty liver disease; PCI, percutaneous coronary intervention; RRT, renal replacement therapy; UKELD, UK model for end-stage liver disease score.

[a] Early allograft dysfunction consists of the presence of one or more of the following variables: (1) bilirubin 10 mg/dL on postoperative day 7; (2) INR 1.6 on postoperative day 7; (3) aminotransferase level (ALT or AST) >2000 IU/L within the first 7 postoperative days (Olthoff Kulik et al, 2010)

[b] According to Clavien-Dindo classification (Clavien Barkun et al, 2009)

4.4.9 Discussion

The consequences of transplanting a liver which fails to function are potentially dire. NMP-L offers the opportunity to assess and improve the quality of high-risk livers deemed unsuitable for transplantation. To our knowledge this report describes the first patient series of "rejected" liver allografts transplanted following successful assessment and resuscitation by NMP-L. This pilot study shows that a proportion of high-risk donor livers might be transplanted by subjecting them to viability testing during NMP-L, without compromising patient safety in a cohort of low-risk recipients.

Since transplantation was established as a highly successful treatment almost half a century ago, scarcity of suitable donors has become a worldwide factor limiting access to this treatment. Ongoing advancements, ranging from the improved management of intracranial vascular malformations to the vast improvements in road traffic safety, have had an impact on decreasing the availability of DBD organ donors. National and international regulatory bodies have proposed strategies and identified funding to overcome the shortage, but these are largely based on increasing the number of extended criteria organs, known to be associated with a higher risk for the recipient(18).

Machine perfusion technology has shown promising results in preserving cardiothoracic and abdominal organs(12, 19-23). Although most of the reported series showed its feasibility in organs acceptable for transplantation, the technology has already demonstrated the potential to expand the donor pool. For example, the team at St Vincent's Hospital in Sydney recently reported a series of heart transplants using allografts recovered from donors after circulatory death that were previously deemed unfeasible(20).

Normothermic perfusion replicating near-physiological conditions *ex-vivo* has for a long time been regarded as the optimal machine perfusion strategy, but has required advanced technology that was previously not available. Several groups have successfully pursued simpler hypothermic machine perfusion (HMP)(21, 24, 25). The early adoption of HMP was also facilitated by the negligible risk of graft loss related to potential device malfunction. Clinical trials of hypothermic machine perfusion of kidneys have demonstrated improved results in renal transplantation(23, 26). Numerous teams have reported encouraging outcomes following HMP of the liver, however the first reported high-risk graft series demonstrated a high incidence of biliary complications and also primary non-function was observed(21, 27).

The devastating consequences of primary graft non-function in cardiothoracic and liver transplantation preclude further extension of organ acceptance criteria. The utilisation of high-risk hearts or lungs is only 30-40%, which might relate to the use of ventricular assist devices and extracorporeal membrane oxygenation as a bridge to transplantation until a lower risk donor becomes available. In contrast, the constant growth in demand for liver transplants has extended utilisation of marginal livers to 70-80%, often compromising post-transplant outcomes and patients' safety(7, 10).

The limits in the utilisation of high-risk livers have been explored in countries such as the UK, where these organs can be allocated to lower risk recipients(28, 29). The protocol presented here may transform use of high-risk livers. Diminishing the risk of primary non-function or severe dysfunction, with their often fatal consequences, might allow further evolution of this novel approach and permit safe allocation of high-risk organs to the sickest recipients, benefiting the patients with the highest waiting list mortality(30).

In this series, the livers were declined by all the UK transplant units and NMP-L was commenced following a variable period of static cold storage, with no differences observed between the use of the two available devices, and five out of six tested grafts were viable. During the period of this pilot study, there were 149 (81 DCD, 68 DBD) livers meeting the study inclusion criteria discarded in the UK. Recovering 70% of these organs would allow over 100 additional liver transplantations in the UK, increasing the number of available organs by 15% (unpublished data, courtesy of Sally Rushton, National Health Service Blood and Transplant). We envisage that viability testing will transform the organ selection and acceptance process and this case series represents a promising start. The technique showed favourable outcomes in a pre-defined subgroup of high-risk organs. Nevertheless, the presented results must be taken cautiously and seen as a feasibility report. One limitation is that this small group of livers did not include any organs with moderate or severe large droplet fatty change (macrosteatosis), a key risk factor for initial graft dysfunction / primary non-function. Other potential limitations could be the additional costs and challenges of wider implementation of NMP-L technology and expertise, but this may be justified by the increases in transplant activity and improved organ utilisation. In addition, our study shows the feasibility to perform NMP-L following SCS and inspection at the transplant centre, with logistical and financial advantages, and may allow targeting of livers that would benefit most from NMP-L.

This report demonstrates that a proportion of currently rejected livers might be salvaged by subjecting them to NMP-L and viability testing. Use of this technology may transform the utilisation of high-risk organs and improve access for patients to transplantation.

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4.4.11 Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the American Journal of Transplantation.

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CHAPTER 5 - VITTAL: THE PROTOCOL

5.1 VIABILITY TESTING AND TRANSPLANTATION OF MARGINAL LIVERS (VITTAL) USING NORMOTHERMIC MACHINE PERFUSION: STUDY PROTOCOL FOR AN OPEN-LABEL, NON-RANDOMISED, PROSPECTIVE, SINGLE-ARM TRIAL.

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Authors: Laing RW[1,2*], Mergental H[1,2*], Yap C[3], Kirkham A[3], Whilku M[3], Barton D[3], Curbishley S[2], Boteon YL[1,2], Neil DA[1], Hübscher SG[1,2], Perera MTPR[1,2], Muiesan P[1,2], Isaac J[1], Roberts KJ[1], Cilliers H[1], Afford SC[2], Mirza DF[1,2].

Affiliations:

[1] Liver Unit, Queen Elizabeth Hospital, University Hospitals Birmingham NHS Foundation Trust, UK

[2] National Institute for Health Research (NIHR) Birmingham Liver Biomedical Research Unit, Institute of Immunology and Immunotherapy, University of Birmingham, UK

[3] Cancer Research UK Clinical Trials Unit, Institute of Cancer and Genomic Sciences, University of Birmingham, UK

5.5.1 Abstract

The use of marginal, or extended criteria donor livers is increasing. These organs carry a greater risk of initial dysfunction and early failure, as well as inferior long-term outcomes. As such, many are rejected due to a perceived risk of use and utilisation varies widely between centres. Ex-situ normothermic machine perfusion of the liver (NMP-L) may enable the safe transplantation of organs that meet defined objective criteria denoting their high-risk status and are currently being declined for use by all the UK transplant centres.

VITTAL (Viability testing and transplantation of marginal livers) is an open label, non-randomised, prospective, single arm trial designed to determine whether currently unused donor livers can be salvaged and safely transplanted with equivalent outcomes in terms of patient survival. The procured rejected livers must meet pre-defined criteria that objectively denote their marginal condition. The liver is subjected to NMP-L following a period of static cold storage. Organs metabolising lactate to ≤2.5mmol/L within 4 hours of the perfusion commencing in combination with two or more of the following parameters – bile production, metabolism of glucose, a hepatic arterial flow rate ≥150ml/min and a portal venous flow rate ≥500ml/min, a pH ≥7.30 and/or maintain a homogenous perfusion – will be considered viable and transplanted into a suitable consented recipient. The co-primary outcome measures are the success rate of NMP-L to produce a transplantable organ and 90-day patient post-transplant survival.

The protocol was approved by the National Research Ethics Service (London – Dulwich Research Ethics Committee, 16/LO/1056), the Medicines and Healthcare Products Regulatory Agency and is endorsed by the National Health Service Blood and Transplant Research, Innovation and Novel Technologies Advisory Group. The findings of this trial will be disseminated through national and international presentations and peer-reviewed publications.

Registration

Clinicaltrials.gov NCT02740608

5.5.2 Strengths and Limitations of the Study

- The study will answer the question: "Can ex-situ end-ischemic normothermic machine perfusion safely increase the number of transplantable livers?"
- The study aims to establish objective liver viability criteria and biomarkers that may enable point-of-care assessment of liver quality
- The study has clearly defined criteria characterising the discarded organs
- Incorporation of an adaptive three-stage trial design provides opportunities to assess patients' safety, allowing for early trial termination if necessary
- The trial includes low and moderate risk recipients only – the suitability for high-risk recipients will require further testing

5.5.3 Introduction

5.5.3.1 Liver transplantation

Liver transplantation is a highly successful treatment for end stage liver disease, fulminant hepatic failure and early stage primary liver cancer. Deaths from liver disease have soared by 40 per cent in a decade and continue to rise. Liver disease kills 11,000 a year in England and the average age of death from liver disease (59 years), continues to decrease(1). Over the past 50 years, transplant techniques and outcomes have greatly improved and 5-year survival rates of 70-80% mean that transplantation has become the mainstay of treatment for an increasing number of patients with chronic liver disease, metabolic disorders, acute liver failure and malignancy. As such, the demand for donor livers greatly exceeds supply and approximately 20% of patients die whilst awaiting transplantation(2). In Europe, the most common indications for liver transplantation are cirrhosis (68%), malignancy (14%), and acute hepatic failure (8%). The main causes for cirrhosis in Europe are the hepatotropic viruses and alcohol related liver disease(3). Non-alcoholic fatty liver disease is an emergent cause and despite health campaigns, the incidence continues to rise. In the UK, it is predicted that the incidences of end stage liver disease and hepatocellular carcinoma will increase substantially during the next decade, exacerbating the existing shortage of donor livers.

5.5.3.2 The UK Liver Transplant Programme

Between March 2015 and April 2016, there were 1161 new waiting list registrations in the UK, and 878 transplants were carried out. Of the 621 patients on the list as of April 2015, 22% died or were removed from the list (n=135) due to deteriorating health(4). This is reflected across other countries to the extent that a patient is now more likely to die within the first 12 months of being listed than the first 12 months' post-transplant(5). Over the past decade there has been a very modest increase in the use of standard or 'ideal' organ donors (those retrieved from

young donors following a diagnosis of brain-stem death, DBD). In response, centres have utilised donors following circulatory death (DCD) and sub-optimal "marginal" or “extended criteria” donors (those of older age, livers with a presence of steatosis etc.).

5.5.3.3 Responding to the shortage

There are several ways to respond to the shortage. Organ donation policies are undergoing changes however there is a lack of well-controlled scientific evidence on which to base decisions regarding policy-making and opinions are strong and divided. Spain has the highest organ donation rates and operates an opt-out system, however the rise in rates only started approximately 10 years after the system’s introduction. Wales is the most recent country to go down this route, however unlike in Spain, next of kin consent is still required before patients can become organ donors. More likely, the increased Spanish donation rates are due to a combination of factors – the creation of a transplant coordination network that operates at hospital, regional and national levels, the placement of transplant coordinators at each procurement hospital and the improvement in the quality of information received by the public. Living donation is one potential means to increase the number of liver transplants, using surgical techniques developed for liver resection and ‘liver splitting’ (which uses a single liver for transplantation into two recipients). The major limitations are most patients do not have a willing or suitable living donor and there are concerns about the risks to the healthy donor. The reported risk of donor death is estimated at 0.2% but the risk of serious complications is much higher(6, 7). Although programmes have had some success in countries without deceased donor programmes, living donor transplantation will be unlikely to have a significant impact on the shortage of donor livers in most countries.

5.5.3.4 The use of "marginal" or "extended criteria" donors

As discussed, a rising proportion of transplants are carried out using "marginal" or "extended criteria" grafts, procured from obese or elderly donors with multiple co-morbidities(8). These livers are significantly more susceptible to cold storage-related ischaemic injury, which increases the risk of graft failure, and recipient morbidity and mortality. Reflecting the issues with these sub-optimal grafts, in 2014/15, of 1282 solid organ donors, only 924 (72.1%) livers were deemed suitable for retrieval and only 812 (63.3%) were subsequently transplanted(9). The duration of the functional warm ischemic time (FWIT) is an important determinant of outcome. The recent document 'Donation After Circulatory Death' published by a steering group on behalf of the British Transplantation Society and Intensive Care Society suggested that the stand-down time from the onset of functional warm ischaemia for DCD liver transplantation was 30 minutes (although 20 minutes is ideal), and that age was an important factor. Because of this, a number of livers will be retrieved from DCDs that fall into the "marginal donor" category and may not go on to be transplanted(10).

Several donor parameters have been identified as relative risk factors for poor outcome including age; steatosis; DCD donation; split livers; prolonged cold ischaemia time (>12 hours). These were all developed using North American data and formulated into an algorithm known as the Donor Risk Index (DRI), and later validated using European data(11, 12). The British Transplantation Society have published their own guidelines on the utilisation of donor organs and use criteria in Table 5.1 to distinguish between grafts of varying quality.

Table 5.1 Criteria for donor quality as per British Transplantation Society UK Guidelines for Donors after Circulatory death

Good livers – *All should be used* (DBDs and DCDs)	**Ideal Livers** – *All should be used* (DCDs)	**Marginal Donors** – *Use selectively* (DCDs)	**Absolute contraindication**s to using liver as donor organ
Age <50	Age <50 years	Age >50 years	DCD with macrovesicular steatosis >30%
Normal LFTs	Weight <100kg	Weight >100kg	ESLD
<5 days on ICU	FWIT <20 mins	FWIT 20-30mins	Acute liver failure
Low levels of inotropic support	CIT <8 hours	CIT 8-12 hrs	Acute liver injury that's not improving
<30% Steatosis	<15% Steatosis	>15% Steatosis	
No active sepsis	ICU stay <5 days	ICU stay >5 days	

CIT, cold ischemic time; DBD, donor following brain death; DCD, donor following circulatory death; ESLD, end-stage liver disease; FWIT, functional warm ischemic time; ICU, intensive care stay; LFTs, liver function tests

5.5.3.5 Organ Preservation

The current standard of donor liver preservation is based on static cold storage (SCS)(13). During SCS, organs are flushed and cooled with specific chilled preservation solutions (University of Wisconsin [UW] solution is used most commonly although Histidine-Tryptophan-Ketoglutarate (HTK) solution is also used less widely) and ice is added to the abdominal cavity. After retrieval, the organ is placed in fluid-filled sterile plastic bags for transportation and stored in preservation solution within an ice-box until transplantation. Although the available preservation solutions differ in chemical composition, their function is essentially the same. The hypothermia aims to reduce the liver's metabolic activity and the solution aims to reduce the cellular swelling. This is a consequence of anaerobic metabolism resulting in depletion of adenosine triphosphate stores leading to influx of free calcium and activation of phospholipases(14). Cooling the organ slows metabolism approximately 12-fold but cannot prevent its dysfunction and the eventual destruction of cellular integrity. Ischaemia-reperfusion is an important factor influencing graft outcome(15). The ischaemic phase starts

early in the procurement process (swings in blood pressure following brain-death or due to the functional warm ischaemic time in non-heart beating donors) and triggers a complex cascade of cellular and molecular events including the release of pro-inflammatory mediators and chemotaxis of cell types that initiate progressive immunological processes. During the reperfusion phase, “the reflow paradox” causes infiltration of the tissues by leucocytes and cellular injury occurs through a series of pathways that include lipid peroxidation and the creation of reactive oxygen species(16). The most common manifestation of the ischaemia-reperfusion process is delayed graft function, which is the inability of the organ to fulfil the physiological needs of the recipient and is associated with graft failure, re-transplantation and death(17). Static cold storage therefore is unable to reverse the injury sustained during donor death and procurement, causes injury due to the cooling process, limits the preservation time and prevents physiological assessment prior to transplantation.

5.5.3.6 In-situ organ reconditioning

To reverse or diminish the injury, many cytoprotective strategies have been tested in experimental models of transplantation and several have been shown to have therapeutic potential, including gene therapy(18, 19), cytokine or growth factor administration(20-22), vasodilating agents and ischemic pre-conditioning(23, 24). Treatment of the organ during preservation has major logistic and ethical advantages over any attempt to achieve the same effects by treating the donor (therapeutic interventions before declaration of death are not currently permitted unless they are of potential benefit to the donor). Recently there has been published early experience with normothermic regional perfusion of DCD donors, nevertheless the feasibility and benefit of this experimental approach is yet to be shown(25).

5.5.4 Normothermic Machine Perfusion of the Liver (NMP-L)

Bretschneider and Starzl first attempted machine perfusion of the liver in the late 1960's. Although hypothermic machine perfusion (HMP) has been shown some promise in clinical studies, NMP-L combats the limitations of SCS previously described by aiming to maintain the organ at the body's natural temperature while providing oxygen, nutrition and the essential substrates necessary for adequate cellular metabolism. Providing a homeostatic environment theoretically enables us to extend our storage period and test the organs physiological parameters. To date only one clinical trial of 20 adult recipients of livers maintained by HMP has been published showing a reduction in early graft dysfunction (5% vs 25% $p<0.08$) as well as a significant reduction in serum injury markers in the HMP group. A joint pilot trial between Oxford University, King's College Hospital London and University Hospitals Birmingham Foundation Trust (UHBFT) recruited 20 patients into an NMP-L phase 1 study and concluded the procedure was feasible and safe when used on current conventional donor acceptance criteria(26). Following this, a 220-patient phase III international clinical trial entitled "COPE WP2" has completed recruitment and the results are eagerly awaited. The Liver Unit at University Hospital Birmingham NHS Foundation Trust contributed to this multi-centre international trial by randomising 50% of the study patients.

Our group believes NMP-L enables the donor organ to be functionally assessed, thereby increasing transplant safety. It can also extend organ preservation times to improve transplant logistics and donor organ utilisation. There are several devices available on the market, but only the OrganOx *metra*™ has been widely used in the clinical transplant setting(26). Our team has performed over 70 liver transplants with grafts preserved on this machine and has gained broad experience by using this device. The OrganOx *metra*™ is the leading device in terms of the number of clinical transplants undertaken, with more than 100 machine-perfused livers

transplanted in the Phase III randomised European trial, together with 20 livers in the Phase I safety study and further on-going trials in North America. For these reasons, we have decided to use the OrganOx *metra*™ device for the proposed study.

The device consists of a unit that cradles the liver, a perfusate reservoir, oxygenators, pumps operating at physiological pressures and a closed tubing system that connects the unit to the portal vein, hepatic artery and vena cava. The constituents of the perfusate can vary but generally consist of whole blood for oxygen carriage, sources of nutrition (glucose, insulin, amino acids), anti-thrombotic agents (heparin, epoprostenol), antibiotics and acid-base agents which help reduce cellular oedema, cholestasis, microvascular injury and the effects of free-radicals.

5.5.4.1 Benefits of NMP-L

NMP-L does not simply benefit marginal DCD organs that have been exposed to a damaging FWIT. Brain-stem death is a catastrophic physiological event associated with profound hypotension (parasympathetic response) followed by hypertension, tachycardia and high levels of circulating catecholamines (sympathetic surge) followed by another reduction in the sympathetic outflow. These dramatic swings can cause significant graft ischaemia prior to retrieval. Diabetes insipidus occurs in 70-80% of brain dead patients causing severe hypernatremia (associated with primary liver graft non-function), hypokalaemia, hypocalcaemia, hypophosphatemia and hypomagnesaemia(27, 28). Pirenne *et* al described seven cases when livers from DBD's between 70 and 80 years old were used with "favourable outcomes"(29). More recently, groups from Italy have reported excellent outcomes using grafts from octogenarian donors(30, 31). NMP-L could however play an important role in preconditioning and assessing such organs prior to transplantation.

Hellinger et al were unable to identify a benefit using NMP-L in 1997; however, it was the first study of its kind(32). In 2001, Schon used NMP-L to preserve and re-condition livers that had been exposed to 1 hour of warm ischaemia. These livers were then transplanted into pigs which all survived longer than 7 days. The group that received livers preserved using SCS had no survivors(33). Several studies have been published by the Oxford group, responsible for OrganOx *metra*™. Imber *et al* published results from a study on a porcine model comparing NMP-L with SCS controls. They showed livers preserved using NMP-L were significantly superior ($P<0.05$) to SCS livers "in terms of bile production, factor V production, glucose metabolism, and galactose clearance", whilst SCS livers had higher perfusate levels of hepatocellular enzymes and more cellular damage(34). The same year they successfully perfused and maintained 5 porcine livers for 72 hours, managing to maintain normal physiological parameters, pH, protein synthesis and histological architecture(35). In 2009, Brockman et al simulated DBD and DCD scenarios in a porcine model. After five hours of preservation (NMP-L vs SCS) there was no difference seen in preservation method in either the DCD or DBD graft recipients. After 20 hours of preservation however, both DCD and DBD grafts that had been preserved using NMP-L were superior to their SCS counterparts with respect to enzyme release, histological changes and recipient survival. Of note, there was no difference in survival between DCD and DBD NMP-L-preserved graft recipients (83% and 86% respectively)(36).

5.5.4.2 Pre-clinical research and pilot study

Our team's pre-clinical research on rejected human livers has demonstrated that metabolism of lactate, in combination with bile production, maintenance of physiological pH, and stable blood flow rates, are sensitive parameters predictive of organ viability. In April 2014, the UHBFT

Novel Therapeutics Committee approved a pilot clinical project for transplantation of five reconditioned liver grafts, initially deemed unusable for transplantation. In this series, livers were declined by all the UK transplant units, after which NMP-L commenced following a variable period of SCS. Still, five out of six tested livers met the viability criteria and were successfully transplanted(37). Although this pilot project showed that viability testing has the potential to transform the organ selection and acceptance process of high-risk livers, our observation primarily provided the feasibility and short-term outcome data. In addition, this cohort also demonstrated the feasibility of performing NMP-L within a "back-to-base" model, i.e. following SCS and inspection at the transplant centre. This offers logistical and financial advantages over using NMP-L in place of SCS and may target livers that would benefit the most from NMP-L. More research in this area is required and this was recognised by the Health Innovation Challenge Committee of the Wellcome Trust who awarded our study group a research grant to fund this trial. We have demonstrated so far, that a proportion of currently rejected liver allografts might be salvaged by subjecting them to NMP-L and viability testing. Use of this technology could transform the utilisation of high-risk organs and may improve access to treatment for thousands of patients awaiting liver transplantation globally.

5.5.5 Methods

5.5.5.1 Study Design Overview

VITTAL is an open label, non-randomised, prospective, single arm trial, using normothermic machine liver perfusion (NMP-L) testing viability and transplantation of marginal livers. It is being conducted at a single site (UHBFT). The design utilises two linked components assessing: (A) - the feasibility of NMP-L as a technique to increase the number of transplantable livers; and (B) - achievement of successful transplantation of the NMP-L treated marginal livers. (A) utilises a two-stage adaptive design(38), requiring up to 53 marginal livers

to be perfused. (B) utilises a three-stage adaptive design(39) and requires 22 NMP-L treated marginal livers to be transplanted. Success is measured by 90-day patient survival – a nationally accepted, monitored and continually audited outcome following liver transplantation.

5.5.5.2 Ethical and regulatory approval

The National Research Ethics Service (NRES) London-Dulwich (REC reference 16/LO/1056, Protocol number RG 15-240) and the Medicines and Healthcare Products Regulatory Agency (MHRA) approved all versions of the study protocol. This trial will use the OrganOx *metra* ™ device following a variable period of SCS to evaluate organ viability pre-transplant procedure. The OrganOx *metra* ™ device currently has a CE mark for liver organ transport and not organ evaluation. The use of the device within this clinical trial is therefore off registration and UK Competent authority (MHRA) clinical trial Investigation: No Objection was obtained (MHRA ref: CI/2016/0031. In addition, approval from the Research and Development (R&D) department at UHBFT and from NHSBT's RINTAG was obtained prior to the start of screening.

5.5.5.3 Graft entry into study and subsequent preparation

The patient and donor liver pathways can be seen in Figure 5.1. All livers will be retrieved with the intention and standardised technique to use them for transplantation. Following the retrieval procedure at the donor hospital the liver will be placed in ice-cold preservation solution on the back-table and transported (according to local protocol). If the liver is allocated to UHBFT, if it is then considered not suitable for use it must be rejected by the on-call transplanting surgeon. For the liver to be considered un-transplantable, the liver will be inspected by the on-call transplant surgeon and another transplant surgeon in the department. The liver will then be

offered as a Fast Track graft to the other centres around the UK. If rejected by all centres and if consent for research was taken, it will be considered for use in VITTAL. Livers offered to our unit as Fast Track offers from other centres will undergo the same 2-consultant rejection process. An appropriate consented potential recipient will be selected by the transplant surgeon and contacted by the coordinator and will come into hospital for admission. The co-ordinator will request 3 units of packed red blood cells, matched to the intended recipient, for use in the OrganOx *metra*™ device. The liver will be prepared according to the procedure for preparing the device for use and placing the organ on the device (described in detail in the OrganOx *metra*™ Instructions for Use (IFU) document (version 13.0, 12-Mar-2016). The liver will be weighed prior to being connected to the device. If cannulation proves impossible, the liver will be rejected as previously intended. If the liver meets the criteria for transplantation, the recipient explantation will commence and the procedure for removing the liver from the device is also described in the IFU. Implantation and reperfusion of the liver will proceed as per the usual practice of the implanting centre. The patient will be clerked as if they were being admitted for a standard liver transplant.

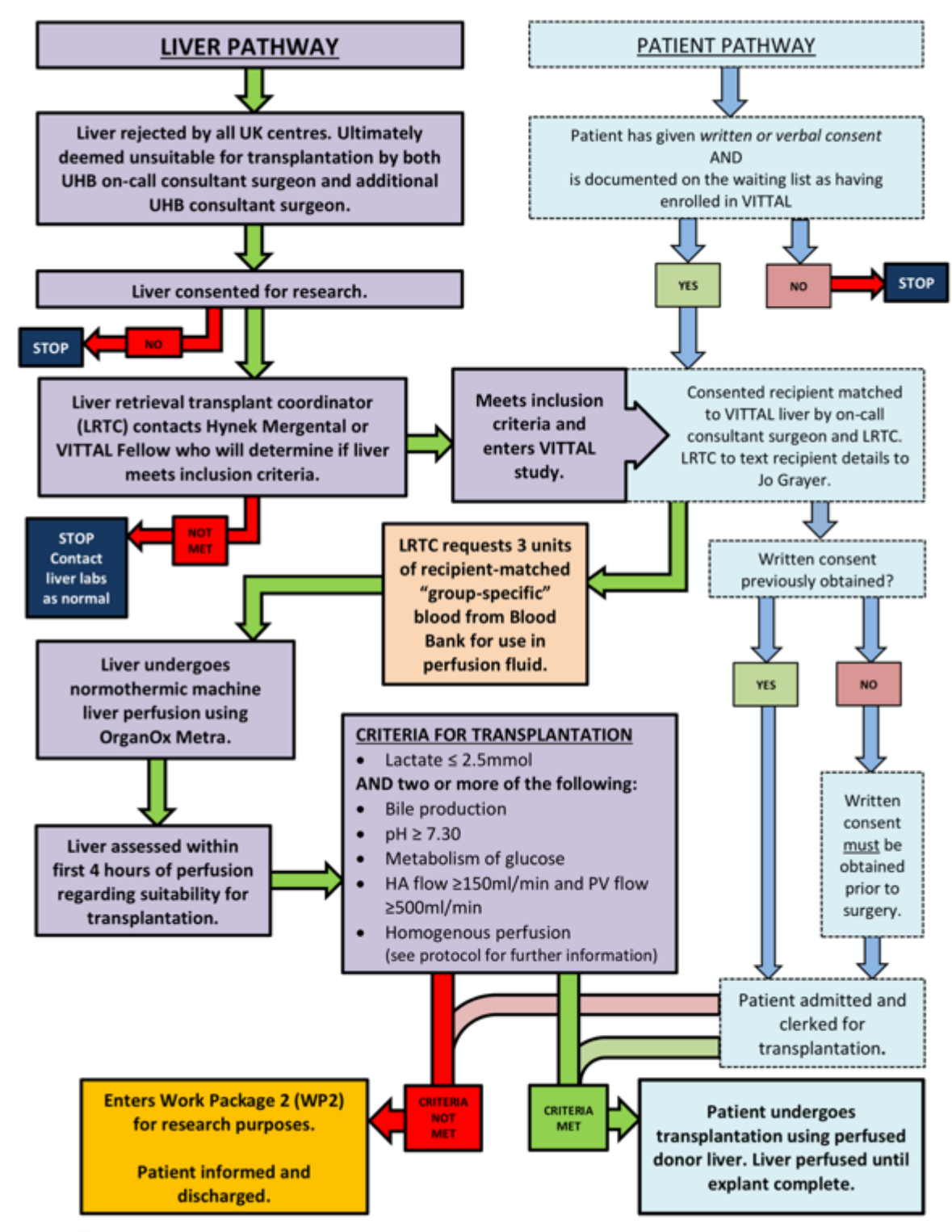

Figure 5.1 Patient and donor liver pathways.

HA, hepatic artery; PV, portal vein; UHB, University Hospitals Birmingham; VITTAL, Viability testing and transplantation of marginal livers.

5.5.5.4 Perfusion of the graft

The machine will be primed with a perfusate suitable for NMP-L and will use packed red cells as the oxygen carrier. During the perfusion, biochemical analysis of the blood-based perfusate will be performed using a Cobas biochemical point-of-care analyser (Roche Diagnostics) which will give results for pH, pO2, pCO2, Bicarb, Base excess, Calcium, Chloride, Sodium, Potassium, Haemoglobin, Haematocrit, Lactate and Glucose. Arterial and portal venous flows, resistances and pressures will also be recorded. Samples to be collected are detailed in Table 5.2.

Table 5.2 Trial sample collection schedule**.**

Perfusate samples	Hepatic arterial and hepatic venous biochemistry (point-of-care)	Pre-perfusion Every 30 minutes during perfusion	Cobas point-of-care desktop analyser
	Perfusate supernatant	Pre-perfusion Every 15 mins for first hour Every hour thereafter	5x1ml aliquots Stored at -80°C
Liver samples	Liver biopsy	L1 Pre-perfusion L2 After 4 hours L3 at end of perfusion* L4 Post-reperfusion	16G core needle biopsy Divided into segment for formalin, segment for frozen and piece for electron microscopy.
	Common bile duct	CBD1 Pre-perfusion CBD2 Post-reperfusion	Formalin
Bile samples	(if produced)	B2 sample at 2 hours B4 sample at 4 hours B6 sample at 6 hours	Total volume recorded and 2ml samples snap frozen at these time points
Patient Samples	Biochemistry Haematology Clotting	Visits 1, 2, 3, 4, Extended follow-up	Standard of care
	Serum, Plasma, mononuclear cells (PBMC)	Visit 1 (pre-operative [post-induction of anaesthesia], post-reperfusion Day 4 post-op) Visits 2, 3, 4,	Additional research samples
	Urine	Visit 1 (pre-operative [post-induction], post-reperfusion Day 4 post-op)	Additional research samples

*if lasting longer than 6 hours

The duration of machine perfusion will be dictated by logistics and the recipient's explant, but should not be less than 4 hours or more than 24 hours. For a graft to be considered for transplantation it *must* –

- Metabolise lactate to less than or equal to 2.5mmol/L within 4 hours of the start of the perfusion

And meet at least 2 of the following criteria within 4 hours of the start of perfusion:

- Demonstrate evidence of bile production
- Maintain a pH greater than 7.30
- Show evidence of glucose metabolism
- Maintain stable hepatic arterial flow of more than or equal to 150 mL/ minute and portal flow more than or equal to 500 mL/minute
- Achieve homogeneous graft perfusion with soft consistency of the parenchyma

Once the transplanting surgeon is content that the liver has met the criteria required for transplantation, the recipient will be brought to theatre and the explant will commence. Explantation, implantation and reperfusion of the liver will be carried out in using standardised techniques by the on-call transplant surgeon. The liver will remain on the machine until after the explantation has taken place at which point it will be flushed by 2L of cold HTK immediately prior to implantation.

5.5.5.5 Concomitant therapy/medications

Patients will receive immunosuppression according to hospital protocols and other medications as necessary for their co-morbidities and current clinical condition. Their post-operative care will be the same as if they had undergone a standard liver transplant.

5.5.5.6 Objectives and Outcome measures

5.5.5.6.1 Primary

There are 2 linked primary objectives and respective outcome measures:

Primary Objective: (A) - Establish the feasibility of NMP-L to increase the number of transplantable livers.

Primary Outcome measure: (A) – "Rescue rate" i.e. the proportion of rejected livers that can be used for transplantation having been deemed viable following a period of machine perfusion.

Primary Objective: (B) – To achieve successful transplantation of previously rejected donor livers following viability testing using NMP-L.

Primary Outcome Measure: (B) – 90-day patient survival, calculated as the number of patients alive 90-day post NMP-L treated marginal liver transplantation (numerator) divided by the total number of NMP-L treated marginal liver transplants performed (denominator).

5.5.5.6.2 Secondary

Secondary Objective (1) – Assessment of liver graft function following transplantation (by incidence of primary non-function, and early allograft dysfunction)

Secondary Outcome Measures (1) – Liver function tests; 90-day graft survival; 12-month patient; and graft survival.

Secondary Objective (2) – Assess morbidity associated with receipt of extended criteria graft that had previously been rejected.

Secondary Outcome Measures (2) – Adverse event rates and severity, graded according to the Clavien-Dindo classification(40) (Appendix 1); Requirement of renal replacement therapy; incidence of biliary complications (including incidence of ischemic type biliary lesions diagnosed on MRCP at 6 months); incidence of vascular complications; biopsy-proven acute rejection; reoperation rate; length of intensive therapy unit stay; and length of hospital stay.

Secondary Objective (3) – Assess the physiological response to reperfusion of the perfused grafts

Secondary Outcome Measures (3) – Post-reperfusion syndrome, defined as a decrease in mean arterial pressure (MAP) of more than 30% from the baseline value for more than one minute during the first five minutes after reperfusion (assessed in the context of inotrope use).

Secondary Objective (4) – Identify impact upon quality of life after transplantation with these liver grafts.

Secondary Outcome Measures (4) – Quality of life by delivery of the EQ-5D-5L questionnaire at baseline, day 30 and 6 months post-transplant.

5.5.5.7 Analytical methods

5.5.5.7.1 Histopathology

Two independent liver histopathologists from UHBFT will perform all the histopathological assessments. Both will be blinded to the graft type, and the primary and secondary outcome measures although the presence or absence of a post-reperfusion biopsy means they will know

whether a graft has met the criteria for transplantation. The histological analysis will be established using haematoxylin and eosin at two levels as well as, periodic acid Schiff (PAS), periodic acid Schiff diastase (PASD), haematoxylin van Gieson (HVG), reticulin, orcein, rhodanin and Perls stains of formalin-fixed paraffin-embedded liver tissue.

5.5.5.7.2 Perfusion, clinical and laboratory data

Donor and patient demographics as well as intraoperative data will be collected. BMI was defined as weight in kilograms divided by the square of the height in metres (kg/m^2). In non-heart beating (DCD) donation, FWIT is defined as the time between the systolic blood pressure of the donor dropping below 50mmHg until the point of aortic perfusion. Cold ischemic time is defined as the time between aortic perfusion and the start of NMP-L. Donor risk index (DRI) and Balance of Risk (BAR) will be calculated as per the relevant literature(11, 41).

The perfusate fluid will undergo point-of-care biochemical testing every 30 minutes as previously described. Perfusate will be taken at the time points described in Table 5.2 and tested for transaminase, urea, albumin and factor V levels. Patient's blood samples will be analysed for full blood count, urea, creatinine and electrolytes, liver function tests, international normalised ratio (INR), prothrombin time, amylase, C reactive protein and plasma glucose using standard laboratory methods (Roche Modular system, Roche Ltd, Lewes, UK) both pre- and post-operatively. Research recipient blood and urine samples will also be taken as part of work package 2 (WP2) that will enable immune cell profiling as well as lipodomic, proteomic and metabolomic testing.

5.5.5.7.3 Patient questionnaires

Quality of life (QoL) will be assessed by delivery of the EQ-5D-5L questionnaire (UK (English) © 2009 EuroQol Group EQ-5D™ is a registered trademark of the EuroQol Group) at baseline, day 30 and 6 months' post-transplant. EQ-5D-5L is a 5-level version of the EQ-5D descriptive system (M. Herdman et al. Qual Life Res DOI 10.1007/s11136-011-99031). The 5L retains the 5-dimensional (5D) structure of the original EQ-5D-3L but the levels on each dimension were expanded to 5 based on qualitative and quantitative studies conducted by the EuroQol Group. Index-based values ('utilities') enable the calculation of quality-adjusted life years (QALYs) which help inform economic evaluations of health care interventions.

5.5.5.8 Statistical justification and outcome analysis

5.5.5.8.1 Sample size justification

For (A) feasibility of NMP-L to rescue discarded liver grafts, it is anticipated that NMP-L will achieve a desirable organ recovery rate of at least 50%, with an undesirable rate of 30% or less as this would not be considered economically feasible. The significance level (α) is set at 0.05, corresponding to the probability of incorrectly rejecting the hypothesis given it is true (Type I error), and the power is set at 0.90 (Type II error rate, $\beta = 0.10$), corresponding to the probability of correctly deciding the NMP-L treatment is successful given the true response rate is greater than 50%.

Using a Simon's two-stage design(38):

Interim assessment stage 1A of accrual: 24 marginal grafts will be perfused and assessed in the first stage. Grafts will be transplanted depending on the criteria achieved. The procedure will be considered infeasible if there are fewer than 8 recovered livers. If more than 8 livers are transplanted, we will proceed to Stage 2A.

Final stage 2A of accrual: Up to additional 29 marginal grafts will be perfused. We would consider the procedure feasible if there are at least 22 recovered livers out of 53 perfused livers.

For (B) for viable livers transplanted following NMP-L, a desirable 90-day patient survival rate is at least 88%, with an undesirable rate of 73% (15% lower). The mean 90-day patient survival rate for 'standard' liver transplants is 93%(42). An optimal three-stage design(39) will be used to test the null hypothesis that the mean 90-day patient survival rate will be less than 73% ($P \leq 0.73$), versus an alternative hypothesis - that the 90-day patient survival rate will be at least 88% ($P \geq 0.88$). The significance level is set at 0.20 (target $\alpha=0.2$), giving a 0.2 probability to conclude that a single transplantation is viable when it truly is not viable. The power is set at 80% (target $\beta=0.2$), giving a 0.2 probability to conclude that a single transplantation is not viable when it truly is viable.

Interim assessment stage 1B: Following transplantation in 3 patients, the trial will stop early (concluding $P \leq 0.73$) if there are fewer than 2 patients achieving 90-day survival. If 2 or more patients reach the primary end point of 90-day survival, an additional 8 transplantations will be performed.

Interim assessment stage 2B: Following transplantation in 11 patients (combined first and second stages) the trial will stop early (concluding $P \leq 0.73$) if there are seven or fewer successes. If 8 or more patients reach the primary end point, an additional 11 transplantations will be performed.

Final stage 3B: Following transplantation in 22 patients in all three stages, the trial will be successful if at least 18 patients reach the primary end-point of 90-day survival. The trial schema is provided in Figure 5.2.

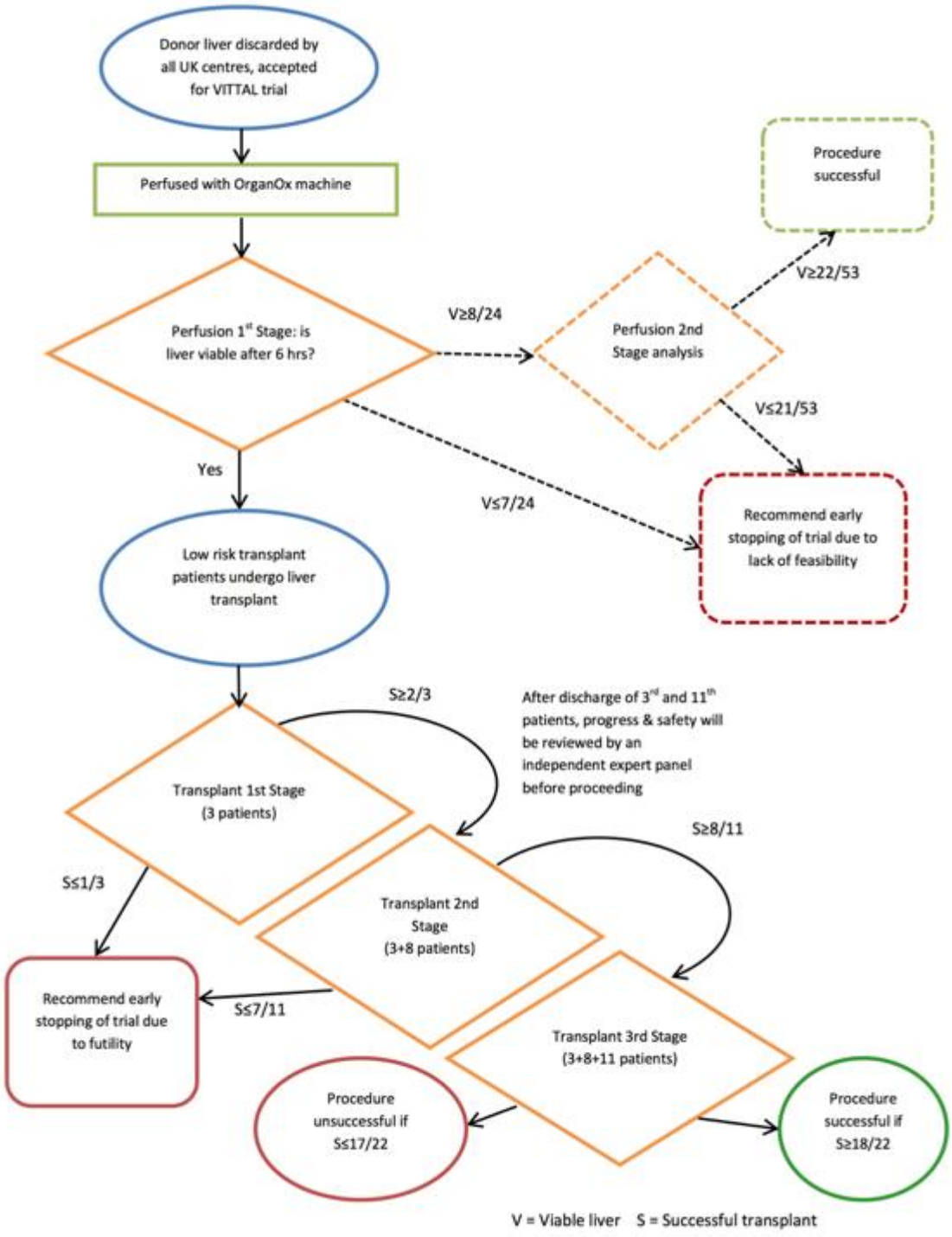

Figure 5.2 Trial schema.

VITTAL, Viability testing and transplantation of marginal livers

5.5.5.9 Analysis of outcome measures

5.5.5.9.1 Primary analysis

To assess (A) the feasibility of NMP-L, the rescue rate will be calculated as the number of perfused marginal grafts meeting the criteria for viability (numerator) divided by the total number of perfused marginal grafts (denominator).

$$Rescue\ Rate = \frac{Number\ of\ viable\ perfused\ marginal\ grafts}{Total\ number\ of\ perfused\ marginal\ grafts}$$

To assess (B) – achievement of successful transplantation of previously rejected donor liver following viability testing using NMP-L. We will evaluate 90-day patient survival rate, as an indicator of liver function and/or viability following transplantation of marginal liver grafts following NMP-L. The 90-day patient survival rate will be calculated as the number of patients alive at 90-day post-transplant with a VITTAL graft, divided by the total number VITTAL-patients transplanted.

$$90\ day\ patient\ survival\ rate = \frac{Number\ of\ patients\ alive\ at\ 90\ days\ post\ transplantation}{Total\ number\ of\ transplanations\ performed}$$

For (A), all livers undergoing NMP-L treatment will be included for evaluation in the interim and final analyses. For (B), all transplantations performed will be included for evaluation in the interim and final analyses. The rate outcomes will be reported together with confidence intervals using the Wilson (1927) method(43).

5.5.5.9.2 Planned Interim Assessments

As we have utilised adaptive designs, there are planned formal interim assessments for both (A) feasibility of NMP-L and (B) successful transplantation of rescued livers, with clear "Go" / "No go" decisions as detailed earlier. Ideally, recruitment (i.e. transplantation) would stop whilst interim analyses of the primary outcome measures are performed. For (A) this could happen immediately, however for (B) this would result in a pause of over 3 months hence the pragmatic approach for such adaptive designs is to continue recruitment whilst they are being conducted.

To maximise patient safety, for (B) at the end of the first stage (transplantation of the first 3 patients), recruitment will be paused to allow the DMC to assess the initial safety data. Once all 3 patients are discharged, if the DMC considers the patients to be recovering well, with liver function that would be expected at this stage, recruitment can continue prior to the patients reaching the primary end-point of 90-day survival. A follow-on report will be sent to the DMC once the third patient reaches the primary endpoint. For the second stage (transplantation of 11 patients), safety data will be sent to the DMC for review after discharge of all 11 patients however recruitment need not stop at this point. A follow-on report will again be sent once the 11th patient reaches the primary endpoint.

Additional DMC meetings will be conducted upon request if the success criteria are not met. If recruitment is fast, prompt reviews will be necessary to ensure the utility of interim decisions.

5.5.5.9.3 Secondary Analysis

For all secondary outcome measures, analyses will be mainly descriptive. Continuous exploratory measures will be summarised via means, medians, standard deviations and ranges.

Categorical measures will be summarised with number and proportion in each category. To model repeated measures over time (e.g. quality of life), a linear mixed effects model (considering subject correlation) using parametric and more flexible models may be considered. Time to event outcomes will be assessed using the method of Kaplan and Meier. Median survival with corresponding 95% confidence interval will also be reported where appropriate. The assessment of graft function post-transplantation by incidence of primary non-function and early allograft dysfunction will be carried out by comparing results with a contemporary matched recipient group of patients obtained from a prospectively maintained database, with adjustment for potential confounders.

The contemporary matched recipient group will be matched using the following:

- Patient Characteristics: age, sex, BMI, MELD, UKELD, aetiology
- Donor Liver Characteristics: DCD or DBD, sex

5.5.5.10 Conduct of trial

5.5.5.10.1 Donor liver selection

Suitable donor liver grafts will be selected from October 2016. Grafts will be retrieved with the intention to transplant and rejected as previously described.

5.5.5.10.2 Graft inclusion criteria

Rejected donor liver grafts must meet *all* of the following inclusion criteria to be eligible for inclusion in the VITTAL trial;

- Liver from a donor primarily accepted with the intention for clinical transplantation
- Rejected by all the other UK transplant centres via normal or fast-track sequence
- Cold ischaemic time less than 16 hours for DBD and 10 hours for DCD grafts

- One of the following parameters which would denote the marginal condition of the liver
    - Donor risk index greater than 2.0(11)
    - Graft macrovesicular steatosis greater than 30%
    - BAR score greater than 9(44)
    - Donor warm ischaemic time greater than 30 minutes
    - Anticipated cold ischaemic time greater than 12 hours for DBD or 8 hours for DCD liver grafts
    - Suboptimal liver graft perfusion documented by a photo of macroscopic appearance
    - Donor transaminases (ALT or AST) above 1000 IU/mL

5.5.5.10.3 Graft Exclusion Criteria

Livers meeting any of the following criteria would not be suitable for the VITTAL trial:

- Grafts from patients with active Hepatitis B, C or HIV infection
- Livers with macroscopic appearance consistent with cirrhosis
- Livers with advanced fibrosis
- DCD grafts with donor warm ischaemic time (systolic blood pressure less than 50mmHg to aortic perfusion) more than 60 minutes
- Excessive cold ischaemic times (DBD more than 16 hours / DCD more than 10 hours)
- Paediatric donor (<18 years)
- ABO incompatibility

5.5.5.10.4 Recipient inclusion criteria

Suitable potential VITTAL graft recipients will be identified during the listing process. Patients will be told that they are potentially suitable to receive a graft from the VITTAL trial and will

be given the patient information sheets to read more about the trial. If already listed, potential recipients will be identified on the list, contacted and sent the same documentation. If they wish to take part a minimum of verbal consent will be taken. Enrolling in the trial will in no way impact upon the chance of them receiving a standard 'transplantable' graft. Patient's with all aetiologies of chronic liver disease will be considered for inclusion. Listed patients must meet all of the following inclusion criteria to be eligible for participation in the VITTAL trial:

- Adult primary liver transplant recipient
- Patient listed electively for transplantation
- Low to moderate transplant risk candidate, suitable for marginal graft, as assessed by the UHBFT liver transplant listing MDT meeting (these are usually candidates with low UKELD score, without cardiovascular comorbidities, with good functional and nutrition status, with patent portal vein and with no history of previous major upper abdominal surgery, e.g. patients transplanted for liver cancer)
- There is no lower limit for MELD or UKELD. Upper UKELD is discussed in the exclusion criteria below.

5.5.5.10.5 Recipient exclusion criteria

Subjects who meet *any* of the following exclusion criteria are excluded from participating in the VITTAL trial:

- "High-risk patients" and recipients not considered suitable for a marginal graft (these are mainly patients with high UKELD score (>62 as per the NHSBT LAG criteria for graft sharing in high risks recipients in the North East of the UK with cardiovascular comorbidities or renal insufficiency, with poor nutrition and performance status or history of major upper abdominal surgery, e.g. patients listed for liver re-transplantation) [http://www.odt.nhs.uk/ search "Liver Allocation Policy"]

- Patients with complete portal vein thrombosis diagnosed prior to the transplantation
- Liver re-transplantation
- Patients with fulminant hepatic failure
- Patients undergoing transplantation of more than one organ
- Contraindication to magnetic resonance imaging (i.e. pacemaker fitted)

5.5.5.10.6 Adverse events reporting and analysis

The collection and reporting of Adverse Events (AEs) will be in accordance with the Research Governance Framework for Health and Social Care and the requirements of the National Research Ethics Service (NRES). Definitions of different types of AE are listed in (Appendix 1). The reporting period for AE's will commence at visit 1 and end at the 24-month follow-up. The Investigator should assess the seriousness and causality (relatedness) of all AE's experienced by the patient (this should be documented in the source data) with reference to the protocol. This will include abnormal laboratory findings which are reported as clinically significant. All AE's, device deficiencies and ADE's will be reported using the applicable eCRF form. AE's will be reported in accordance with Clavien-Dindo classification of surgical complications(40). Anticipated AE's include those related to any form of major surgery; infection (chest, urine, blood, bile, wound, abdominal), fluid collection (abdominal, pleural), renal dysfunction, cardiac failure, respiratory failure, and those related to the disease process and transplantation; early allograft dysfunction, rejection, hospitalisation for pre-existing condition that has not deteriorated, clinically significant abnormal laboratory finding or other abnormal assessments that is associated with the condition being studied (unless judged by the investigator as more severe than expected for the patient's condition). The investigator will exercise his/her medical judgment in deciding whether an abnormal laboratory finding or other abnormal assessment is clinically significant. However, if in the opinion of the investigator,

the frequency or severity of the event is greater than would be expected then it must be reported. Device deficiencies that did not lead to an adverse event, but could have led to a medical occurrence if suitable action had not been taken, or intervention had not been made or if circumstances had been less fortunate, will also be recorded and reported.

5.5.5.10.7 Those events not being reported

The following are considered routine during or after liver transplantation and will not be reported as AE's.

- Initial admission to Intensive Care following liver transplant
- Elevation of AST and/or ALT <2000 iu/ml within 48 hours of liver transplant
- Transfusion of ≤ 5 units of packed red cells
- Transfusion ≤ 8 units of fresh frozen plasma
- Transfusion ≤ 2 adult doses of platelets

In addition to the above, medical and scientific judgement should be exercised in deciding whether expedited reporting is appropriate in other situations, such as important medical events that may not be immediately life threatening or result in death or hospitalisation but may jeopardise the patient or may require intervention. Any death occurring during the protocol defined follow up period (within 90 days), whether considered device-related or not, must be reported as an SAE within 24 hours of the local investigator becoming aware of the event. If a death occurs in a patient receiving a transplant the cause of death will be investigated and reviewed by the Trial Management Group (TMG) and clinical team caring for the patient. Entry of patients in to the study would be temporarily suspended until these investigations are complete.

5.5.5.11 Study visit overview

The VITTAL trial involves a minimum of four patient visits which all coincide with standard admissions either for surgery or for outpatient follow-up. There are no additional trial-specific visits. The schedule for the study visits and data collection is summarised in Table 5.3. Visit 1 encapsulates admission for transplant and the post-operative period if the transplant proceeds. Visits 2, 3 and 4 are scheduled for 30 day, 90 day and 180 day follow-up respectively. All patients will undergo MRCP during visit 4 to investigate the occurrence of ischemic-type biliary lesions which also marks trial end-point. Patients will continue to be followed up at 12 months and 24 months as part of their standard post-transplant care and data will be collected at these time-points for long-term reporting.

Table 5.3 Patient Schedule of Events

Patient Registration	**Screening**	**Visit 1 Transplant** Day 0	**Visit 2** Day 30 (+/- 3days)	**Visit 3** Day 90 (+ 3 days)	**Visit 4** Day 180 (+ 30 days)	**Extended follow up** 12 month + 24 month (+/- 30 days)
Informed consent	**X**					
Eligibility assessment	**X**	**X**				
Patient history	**X**	**X**				
Standard routine blood tests*	**X**	**X**	**X**	**X**	**X**	**X**
MELD (automatically calculated)		**X**				
UKELD (automatically calculated)		**X**				
Trial specific additional patient samples blood and urine		**X**	**X**	**X**	**X**	
PBMC Collection		**X**	**X**	**X**	**X**	
Liver Biopsy 4 (see table 2)		**X**				
Quality of Life questionnaire (EQ-5D-5L)		**X**	**X**		**X**	
Patient Resource Log at Visit 1 discharge		**X**				
Adverse/ Clinical events	**X**	**X**	**X**	**X**	**X**	**X**
Concomitant medications	**X**	**X**	**X**	**X**	**X**	**X**
MRCP					**X**	

* Standard routine blood tests - Full blood count (FBC), urea, electrolytes, liver function tests, AST, GGT, eGFR, international normalised ratio (INR)

5.5.5.12 Storage of samples

Patient blood samples taken as part of their standard of care will be processed and stored according to UHBFT procedures. Perfusate, patient serum, plasma, urine samples and mononuclear cell preparations collected during visits 1-4 will be stored frozen in 0.5–1.0 mL aliquots at – 80°C at the Institute of Biomedical Research, University of Birmingham. Liver biopsy tissue specimens will be collected and the formalin fixed paraffin embedded segments will be processed by staff in the department of cellular pathology at UHBFT. After sectioning and staining, tissue blocks will be stored at the Institute of Biomedical Research. All samples will be collected in accordance with national regulations and requirements including standard operating procedures for logistics and infrastructure. Samples will be taken in appropriately licensed premises, stored and transported in accordance with the Human Tissue Authority guidelines and trust policies.

5.5.5.13 Data handling, quality assurance, record keeping and retention

Data will be managed according to the standard operating procedures of the Cancer Research UK Clinical Trials Unit (CRCTU) at the University of Birmingham, UK. The CRCTU is fully compliant with the Data Protection Act 1998 and the Guidelines for Good Clinical Practice (GCP). The CRCTU will monitor the trial and provide annual reports to the MHRA. The trial is registered with the Data Protection Act website at the University of Birmingham. Donor and patient details will be kept anonymous (specific study identification codes will be used for each study donor). Anonymised donor data will be used in future publications arising from the study. Patients will be identified using only their unique registration number, patient initials on the Case Report Form and correspondence between the Trials Office and the participating site. In addition, the patients are requested to give permission for the Trials Office to be sent a copy of their signed Informed Consent Form which will not be anonymised. This will be used to

perform in-house monitoring of the consent process. Identifiable data will only be made available to authorised staff of the study sponsor, its authorised representatives and regulatory authorities. All patients will be consented specifically to enable data to be shared as detailed above. Confidentiality will otherwise be maintained throughout the trial and thereafter and data will be anonymised. On completion of the trial, data will be transferred to a secure archiving facility at the University of Birmingham, where data will be held for a minimum of 15 years and then destroyed.

5.5.5.14 Electronic case report forms (ECRFs)

ECRFs have been designed to capture as much, donor, perfusion and patient data as possible and feasible. The liver registration form and donor history form detail all that is relevant regarding the quality of the graft itself. The perfusion form enables collection of the perfusion parameters, biochemical data and the outcome of the perfusion. The patient registration and visit 1 forms will capture the demographics of the recipient as well as track the operative and post-operative course. Visits 2-4 are for patient follow-up.

5.5.5.15 Trial organisational structure

The University of Birmingham will act as single sponsor this single centre study. The trial is being conducted under the auspices of the CRCTU, The University of Birmingham according to their local procedures. The Trial Management Group (TMG) will be responsible for the day-to-day running and management of the trial. Members of the TMG include the chief investigator, co-investigators, project manager, trial management team leader, senior trial coordinator, trial coordinator, lead trial statistician, and trial statistician. The TMG will have regular meetings during recruitment. The Data Monitoring Committee (DMC) will consist of independent clinicians Professor James Neuberger, Mr Gabi Oniscu, and Professor Jacques

Pirenne as well as an independent statistician, Mr Andrew Hall. Data analyses will be supplied in confidence to the independent DMC, which will be asked to give advice on whether the accumulated data from the trial, together with the results from other relevant research, justifies the continuing recruitment of further patients. The DMC will operate in accordance with a trial specific charter based upon the template created by the Damocles Group. The DMC will meet at 2 scheduled time points after the interim analyses as previously described (Figure 5.2). An emergency meeting may also be convened if a safety issue is identified. The DMC will report directly to both the VITTAL Trial Management Group (Chief Investigator) who will convey the findings of the DMC to the trial steering group and funders/sponsor as appropriate or when specifically requested by these parties.

5.5.5.16 Sources of funding

The VITTAL trial is funded by a grant awarded by the Wellcome Trust Health Innovation Challenge Fund (awarded December 2015).

5.5.5.17 Trial status

Recruitment for the trial opened in October 2016 and recruitment is expected to last 24 months.

5.5.6 Discussion

The consequence of the escalating demand for liver transplantation is increasing waiting list mortality and in many countries, patients are more likely to die whilst waiting for an organ than in the first year after their transplant(45). The outcomes of high-risk livers are inferior to standard grafts and the difference is most noticeable within the initial 90 days. Indeed, severe early allograft dysfunction or primary non-function often trigger post-transplant sepsis and multi-organ failure and as consequence, livers with marginal features are often declined and discarded.

Our preliminary experience and pilot transplant series showed NMP-L can provide objective information regarding liver function and the VITTAL trial aims to produce robust data and validate our initial observations.

Several challenges were identified when designing the VITTAL trial with the foremost being to create a sound definition of a discarded liver. There is an undeniable variation in utilisation of high-risk livers among the UK transplant centres which has been recognised and highlighted by NHSBT. The organisation published "Taking organ transplantation to 2020", a strategy that aims to create greater consistency in the acceptance of organ offers and utilisation of marginal livers across all centers(46). To address this issue for this study purposes, every declined liver offered for enrolment into VITTAL has to meet also at least one of a list of predefined, constant inclusion measures, adopted in combination with a two-consultant system of macroscopic liver quality assessment.

The most important factor to consider whilst designing a trial that pushes the current boundaries of high-risk livers utilisation is patient safety. Although we opted for liberal liver graft selection

inclusion criteria, only low to moderate risk recipients are eligible to take part in this trial. Such an approach has been shown previously to be the safest and the most successful strategy for utilisation of high-risk organs(47, 48). The intended recipients will be risk stratified and selected by the Liver Unit's liver transplant multi-disciplinary team. Another important trial safety feature is its three-stage adaptive design, introducing two interim safety analyses after completion of 3 and 11 transplants respectively.

There are undoubtedly some livers that will not be salvageable or ever safe to transplant. It is important for the purposes of the trial to include organs that fail to meet the defined viability criteria, to compare these with transplantable high-risk livers. The research work package linked with the trial was designed to identify sensitive point of care liver quality tests and propose novel biomarkers or panels associated with viable livers.

The primary end-point of 90-day patient survival has been chosen as it is a nationally accepted, monitored and continuously audited outcome following liver transplantation. Obviously, the graft survival rate is important and for the trial to truly be successful, patients who reach the primary end-point should have a VITTAL graft still *in-situ*. This will be considered when the DMC monitor the results at the interim analyses.

As well as the study design, challenges with trial logistics were also identified. One of the previously unseen difficulties after discussion with the haematology team, is the issuing of packed red cells matched to the intended recipient, potentially before the patient is admitted to hospital, to avoid delaying the start of the perfusion. When patients are listed, they undergo a blood cross-matching process to identify blood group and the presence of antibodies. This sample is not held for longer than 7 days by the hospital and so if they are admitted for a

transplant or require blood products for some other intervention, they have a new sample sent before those products are issued. In the case of the VITTAL trial, a perfusion may need to commence before the patient is admitted to hospital as they may have to travel some distance. Minimising the cold ischemic time of marginal grafts is paramount to improve the chances of graft salvage. Therefore, in this scenario, blood is issued for the trial based on the results of the original sample and a repeat is sent when the patient is admitted to check they have not subsequently developed new antibodies. Blood product traceability is an important consideration and the blood products are documented to have been used in the device perfusate only and have not been used for recipient transfusion.

5.5.7 Ethics and dissemination

The VITTAL Clinical trial is an academic investigator-led study involving a CE marked medical device. The device is being used outside its current CE mark and therefore has been reviewed by the MHRA UK and received a "clinical investigation: no objection" (CI/2016/0031) letter: 3rd August 2016. In addition, the study has undergone national ethical review in the UK and received national ethical approval from the London – Dulwich Research ethics committee (16/LO/1056) and the Health Research Authority. In addition to the above national regulatory approvals the study has been reviewed by the National Health Service Blood and Transfusion service (NHSBT) and received all appropriate local institution/NHS R&D approvals. The trial management team are also fully engaged in an academic collaboration with the device manufacture OrganOx as part of the management of this study.

The trial management team are fully committed to publishing (within 12 months of the end of the study) the results of this study in accordance with best clinical practice in an open access, peer reviewed medical journal irrespective of outcome. Any dissemination of results, or

publicity will be provided in a format which will not allow individual patients to be identified and confidentiality will be maintained throughout the process. The study management will be conducted in accordance with all applicable clinical trial regulations and managed centrally by the D3B trial management team – part of the CRUK clinical trials unit based in Birmingham, in accordance with the quality management system. The results of the study will also be made available directly to study participants and specialist patient groups.

5.5.8 Summary

The presented VITTAL trial is the first clinical trial designed to objectively assess function of declined livers using NMP-L and subsequently transplanting viable grafts. It is hoped that the trial will identify a proportion of discarded organs that can be successfully transplanted and the generated data will provide objective and validated information that can be subsequently implemented in the process of acceptance and allocation of high-risk donor livers. This novel approach should improve consistency and increase utilisation of marginal liver grafts without compromising recipient safety.

5.5.9 Acknowledgments, financial support and sponsorship

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5.5.10 Authors' contributions

DFM (chief investigator) and HM (principal investigator) had the original concept of the VITTAL trial following on from a successful pilot study. CY (lead statistician), DFM, HM, AK (trial statistician) and RWL designed the VITTAL trial. RWL, MW, AK, CY and HM wrote the protocol. RWL, MW and DB completed the IRAS applications. DB, HM, AK, CY, RWL and DFM reviewed all protocol versions. RWL, MW and DB (senior trials co-ordinator) submitted all REC, MHRA, HRA, NHSBT and local R&D applications. AK, CY and DFM devised the statistical analysis plan. TP, PM, JI, KR, HM and DFM are the transplant surgeons involved in the trial. RWL and MW wrote the patient information sheets, external trial information and patient CRFs. RWL, HM and DFM wrote the manuscript and all authors reviewed the final version.

5.5.11 Conflict of interest

RWL and YB receive salary as Wellcome Trust research fellows.

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CHAPTER 6 - VITTAL: THE RESULTS

6.1 TRANSPLANTATION OF DISCARDED LIVERS FOLLOWING VIABILITY TESTING WITH NORMOTHERMIC MACHINE PERFUSION

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Authors: Mergental H[1,2,3*,] Laing RW[1,2,3*], Kirkham AJ[4], Perera MTPR[1], Boteon YL[1,2,3], Attard J[1,2,3], Barton D[5], Curbishley S[2,3], Wilkhu M[5], Neil DAH[2,3,6], Hübscher SG[2,3,6], Muiesan P[1], Isaac JR[1], Roberts KJ[1,3], Abradelo M[1], Schlegel A[1,3], Ferguson J[1,2], Cilliers H[1], Bion J[7], Adams DH[1,2,3], Morris C[8], Friend PJ[8,9], Yap C[4,10*], Afford SC[2,3*], Mirza DF[1,2,3*]

* These authors contributed equally

Author affiliations:

[1] Liver Unit, Queen Elizabeth Hospital, University Hospitals Birmingham NHS Foundation Trust (UHBFT), Birmingham, United Kingdom

[2] National Institute for Health Research (NIHR), Birmingham Biomedical Research Centre, University of Birmingham and University Hospitals Birmingham NHS Foundation Trust, United Kingdom

[3] Centre for Liver and Gastrointestinal Research, Institute of Immunology and Immunotherapy, University of Birmingham, United Kingdom

[4] Cancer Research UK Clinical Trials Unit, University of Birmingham, Birmingham, United Kingdom

[5] D[3]B team, Cancer Research UK Clinical Trials Unit, University of Birmingham, Birmingham, United Kingdom

[6] Department of Cellular Pathology, Queen Elizabeth Hospital, University Hospitals Birmingham NHS Foundation Trust (UHBFT), Birmingham, United Kingdom

[7] Department of Intensive Care Medicine, University of Birmingham, Birmingham, United Kingdom

[8] OrganOx Limited, Oxford, United Kingdom

[9] Nuffield Department of Surgical Sciences, University of Oxford, Oxford, United Kingdom

[10] Clinical Trials and Statistics Unit, The Institute of Cancer Research, United Kingdom

6.6.1 Abstract

There is a limited access to liver transplantation, however, many organs are discarded based on subjective assessment only. Here we report the VITTAL clinical trial outcomes, using normothermic machine perfusion (NMP) to objectively assess livers discarded by all UK centres meeting specific high-risk criteria. Thirty-one livers were enrolled and assessed by viability criteria based on the lactate clearance to levels ≤2.5mmol/L within 4 hours. The viability was achieved by 22 (71%) organs, that were transplanted after a median preservation time of 18 hours, with 100% 90-day survival. During the median follow up of 542 days, 4 (18%) patients developed biliary strictures requiring re-transplantation.

This trial demonstrates that viability testing with NMP is feasible and in this study enabled successful transplantation of 71% of discarded livers, with 100% 90-day patient and graft survival; it does not seem to prevent non-anastomotic biliary strictures in livers donated after circulatory death with prolonged warm ischaemia.

(Funded by the National Institute for Health Research Wellcome Trust; ClinicalTrials.gov number NCT02740608)

6.6.2 Introduction

Liver transplantation is a lifesaving treatment for selected patients with end-stage liver disease, primary liver cancer and fulminant hepatic failure. The incidence of liver disease has risen by 500% over the last 4 decades, however access to transplantation is limited by the shortage of donor organs(1). As a consequence, 240 patients (19%) waiting for liver transplantation in the United Kingdom either died or were removed from the waiting list in 2016-17(2). Data from the United States shows a similar pattern, comprising 32% of those listed for transplant (3,629 patients) within 3 years of listing(2, 3). The demand for liver grafts has driven the wider use of extended criteria donors(4). However, these are associated with an increased risk of primary non-function or delayed failure(5-9), and the acceptance of these higher-risk organs varies widely(10). Because of these inferior outcomes, and the difficulty of predicting organ viability, many potential donor organs remain unutilised. The high waiting list mortality justifies the utilisation of more marginal grafts, but current practice requires risk mitigation by matching high-risk livers to lower-risk recipients to achieve patient survival rates that are acceptable(11). Furthermore, the determination of suitability of a graft for transplantation largely depends on a surgeon's subjective assessment of the graft's appearance, using criteria that are known to be unreliable(12).

Organ preservation currently relies upon cooling to ice temperature to reduce cellular metabolism, and infusing specialist solutions to limit cellular damage. Oxygen deprivation and accumulation of by-products of anaerobic metabolism limit the duration of storage and result in ischaemia-reperfusion injury at the time of implantation. This process is more severe in marginal organs(13). Normothermic machine perfusion (NMP) has been shown to reduce preservation-related graft injury compared to static cold storage in transplantable livers, according to current selection criteria, in a prospective European trial, which also demonstrated

increased utilisation of organs(14). In NMP, the liver is supplied with oxygen, nutrients and medication at physiological temperature and pressures, maintaining conditions that support homeostasis, normal metabolic activity and objective assessment of function in real-time. Experimental data has shown that end-ischaemic NMP facilitates replenishment of adenosine triphosphate and glycogen levels. Based on increasing clinical experience, viability criteria have emerged; these are objective parameters, measurable during NMP(15). Whilst the feasibility of this approach has been demonstrated in a proof-of-concept series, it has not been validated in a rigorous clinical trial(16, 17).

We therefore conducted this prospective, non-randomised, adaptive phase 2 trial in a large single centre, to evaluate the potential of NMP to provide objective assessment of the viability of livers currently deemed unsuitable for transplantation, and to transplant those that met predetermined criteria. The primary clinical objective underlying this project was the increased and safe utilisation of livers which are currently discarded.

The trial demonstrates that viability testing with NMP is feasible, and the objective assessment enables successful transplantation of 71% of perfused discarded livers, with 100% 90-day patient and graft survival. The intervention does not seem to prevent the development of non-anastomotic biliary strictures in DCD livers with prolonged donor warm ischaemic times.

6.6.3 Methods

6.6.3.1 Study design

This study was a prospective, open label, phase 2 adaptive single-arm trial comprising high-risk livers meeting two-tier inclusion criteria. The first-tier was being considered as unsuitable for transplant by all UK transplant centres within a nationwide fast-track offering scheme. The trial was performed at a single-institution (Queen Elizabeth Hospital, Birmingham, UK) with experience in NMP and utilisation of high-risk grafts(5, 18). The second-tier eligibility required at least one of seven specific criteria that confirmed the high-risk status of every enrolled liver (Table 7.4). To minimise risks of high post-transplant complications or mortality for the study participants, the trial used an adaptive design with two interim safety analyses (Supplementary Figure S7.1). The study was funded by the Wellcome Trust, and granted approval by the National Research Ethics Service in London-Dulwich (REC reference 16/LO/1056, Protocol number RG 15–240) and the Medicines and Healthcare Products Regulatory Agency. The project was endorsed by the Research, Innovation and Novel Technologies Advisory Group committee of the National Health Service Blood and Transplant. The study was registered at ClinicalTrials.gov (reference number NCT02740608), the protocol has been published(19), and can be seen in Chapter 6.

6.6.3.2 Discarded liver inclusion criteria and the study logistics

The study considered all potential donors with a diagnosis of brainstem death or Maastricht category III and IV donors after circulatory death, aged up to 85 years, initially retrieved with the intent for transplantation but subsequently declined by all UK transplant centres based on the retrieving or transplant surgeon's assessment. If our centre was the last in the fast-track offering sequence, the liver had to be deemed untransplantable by two consultant surgeons independently. The surgeons were paired together to create an overall low threshold for using

marginal livers, ensuring any liver that could be used without viability testing was transplanted, thereby minimising bias. For the liver to be eligible it also had to meet at least one defined high-risk criterion (see Tables 6.1 and 6.2). Consent for research was provided by the donor's next of kin.

Table 6.1 Study inclusion and exclusion criteria.

Graft inclusion criteria	Liver from a donor primary accepted with the intention for a clinical transplantation Liver graft was rejected by all the other UK transplant centres via normal or fast-track sequence (see Appendix 3 for list of UK centres) One of the following parameters capturing the objectivity of the liver high-risk status: ▪ Donor risk index >2.0 (Feng, 2006) ▪ Balanced risk score >9 (Dutkowski 2012) ▪ Graft macrosteatosis >30% ▪ Donor warm ischaemic time (defined as the period between the systolic blood pressure <50 mmHg to the time of commencing donor aortic perfusion) in DCD donors >30 minutes, ▪ Peak donor aspartate and alanine transaminases >1,000 IU/mL (AST/ALT) ▪ Anticipated cold ischaemic time >12 hours for DBD or 8 hours for DCD livers ▪ Suboptimal liver graft perfusion as assessed by a consultant transplant surgeon and documented by photography.
Graft exclusion criteria	Grafts from patients with active Hepatitis B, C or human immunodeficiency virus infection Livers with cirrhotic macroscopic appearance Livers with advanced fibrosis DCD grafts with donor warm ischaemic time (systolic blood pressure <50mmHg to aortic perfusion) more than 60 minutes Excessive cold ischaemic times (DBD >16 hours / DCD >10 hours) Paediatric donor (<18 years old) Blood group ABO incompatibility
Recipient inclusion criteria	Primary adult liver transplant recipient Patient listed electively for transplantation Low to moderate transplant risk candidate suitable for marginal graft, as assessed by the UHB Liver Unit liver transplant listing multi-disciplinary team meeting.
Recipient exclusion criteria	High-risk transplant candidates not suitable for a marginal graft Patients with complete portal vein thrombosis diagnosed prior to the transplantation Liver re-transplantation Patients with fulminant hepatic failure Blood group ABO incompatibility Patient unable to consent Patients undergoing transplantation of more than one organ Contraindication to undergo magnetic resonance imaging
Criteria for transplantation	Lactate ≤2.5mmol/L *and* 2 or more of the following within 4 hours of starting perfusion Evidence of bile production pH ≥7.30 Metabolism of glucose HA flow ≥150mL/min and PV flow ≥500mL/min Homogenous perfusion

Abbreviations: DCD, donation after circulatory death; DBD, donation after brainstem death; ALT, alanine aminotransferase; AST, aspartate aminotransferase

NOTE: Donor risk index is calculated from age, race, cause of death, height and the predicted cold ischaemic time (Feng et al 2006); balanced risk score is calculated using model for end-stage liver disease score (MELD), whether or not the recipient is having a re-transplant or is on intensive care, recipient age, donor age and cold ischaemic time (Dutkowski et al 2011).

6.6.3.3 Study participants

Eligible participants were those listed electively for primary liver transplantation and deemed to be low to moderate transplant risk candidates, suitable to receive a high-risk graft, as assessed by the unit's transplant waiting list multi-disciplinary team. Candidates were required to have a patent portal vein, no significant comorbidities (cardiovascular diseases including active angina, a history of ischaemic heart disease, congestive heart failure, cerebrovascular events, symptomatic valvular heart disease or cardiac arrhythmias; pulmonary conditions including pulmonary hypertension or established diagnosis of pulmonary dysfunction), a UK end-stage liver disease(20) (UKELD) score ≤62 and no history of major upper abdominal surgery. Each participant was fully informed of being offered a marginal graft and gave written consent for the trial in advance of the organ offer, after having at least 24 hours to consider their participation.

6.6.3.4 The study intervention and liver viability assessment

All livers were cold-preserved with University of Wisconsin solution and commenced NMP using the OrganOx Metra device after arrival at the transplant centre. The protocol stipulated an NMP duration of between 4 and 24 hours. Serial perfusate, bile and tissue samples were taken at regular time intervals. For a liver to be considered viable it had to metabolise perfusate lactate to levels ≤2.5mmol/L within 4 hours of commencing the perfusion, in addition to meeting at least 2 of the following additional criteria: evidence of bile production, maintenance of perfusate pH ≥7.30, metabolism of glucose, maintenance of stable arterial and portal flows (≥150mL/ min and ≥500mL/min respectively), and homogeneous perfusion with soft consistency of the parenchyma(16).

If a liver was considered viable, the transplant was set up and performed. At the point of recipient hepatectomy, the NMP team disconnected the organ from the device, flushed it with 3 litres of histidine-tryptophan-ketoglutarate solution at 4°C and handed it over for immediate implantation. Post-transplant management followed the unit's standard protocol, with immunosuppression comprising tacrolimus, azathioprine or mycophenolate mofetil, and low dose steroids. Each patient underwent a magnetic resonance cholangiopancreatography (MRCP) at 6 months unless the investigation was clinically indicated earlier. Liver quality was determined retrospectively through histological analysis of parenchymal biopsies which were assessed for pre-existing liver disease, steatosis, glycogen content and features of preservation-reperfusion injury.

6.6.3.5 Outcome measures

The co-primary outcomes consisted of A) feasibility of NMP in discarded organ recovery and B) achievement of successful transplantation. The perfused organ recovery rate was the proportion of perfusions leading to transplantation. Successful transplantation was defined as 90-day patient survival - a nationally accepted, monitored and continuously audited outcome measure.

The key secondary outcome measures included assessment of the liver graft function (by incidence of primary non-function and early allograft dysfunction(21)), liver function test results, 90-day graft survival, intensive therapy unit and post-transplant in-hospital stays, incidence of vascular complications, and anastomotic and non-anastomotic biliary strictures as assessed by MRCP at 6 months. Perioperative data collection included haemodynamic stability, incidence of post-reperfusion syndrome and blood-product requirements. Post-transplant adverse events and complication severity were graded according to the Clavien-

Dindo classification(22). The secondary outcomes were compared with contemporary controls (1:2), matched in order of priority for the donor graft type, UKELD Score, donor age and donor sex. Four variables included in the original protocol (model of end-stage liver disease [MELD], recipient age, BMI and the liver disease aetiology) were removed as matching criteria due to confounding, correlation and being overly stringent. There was consistency in the recipient selection for high-risk grafts guided by the unit's protocols and transplant waiting list multi-disciplinary team meetings that assured similar characteristics regarding the cardiovascular comorbidities and surgical risks in the study participants and the matched controls. The pre-planned comparisons with the matched controls group were not powered to demonstrate any differences. Due to the small sample sizes, these results should be interpreted with caution; the controls were included to present the study results within the context of the unit's contemporary outcomes.

6.6.3.6 Statistical analysis

The trial was powered with an emphasis on (A) the feasibility of the intervention using NMP and (B) recipient safety. In terms of the intervention feasibility (A), the aim was to achieve an organ recovery rate of at least 50%, with a rate of 30% or less being considered unacceptable. Using a two-stage design(23), with an interim assessment after 24 livers (continuing if ≥8 livers were recovered), a sample size of up to 53 livers undergoing NMP might be required, with target alpha (one-sided) of 0.05 (actual alpha = 0.047) and target beta of 0.1 (actual beta = 0.098) . NMP was considered feasible for organ recovery if at least 22 livers were recovered from 53 perfused. Though the two statistical inferences are assessing different hypotheses (safety and feasibility), they are linked as 22 transplants are required for the safety testing of the procedure, which is also the minimum number required out of 53 perfused livers to be considered feasible.

For (B), the mean 90-day patient survival rate for patients receiving liver transplants in the UK was 93%(24). For the discarded livers, the desirable and undesirable 90-day overall survival rates were set at 88% and 73% (15% lower) respectively. Using an optimal three-stage adaptive design(25) with two interim assessments after 3 patients (requires ≥2 successes) and 11 patients (≥8 successes), a sample size of 22 patients was required, with alpha (type I error) and beta (type II error) of 0.2. As this was an early phase (non-definitive) trial to assess the safety of this procedure, a relaxed one-sided alpha was used to attain an achievable sample size within the trial duration and cost constraint. The approach was considered successful if there were at least 18 successes out of 22 transplants.

The descriptive statistics data were presented as number and percentages, and median and interquartile range. Due to small numbers, the pre-planned analyses used Kruskal-Wallis test to assess differences in continuous variables between two groups and Fisher's exact test for categorical variables. Kaplan-Meier survival method was used to analyse time-to-event data and conditional logistic regression for matched case-control analysis. All secondary and exploratory analyses were two-sided at 5% significance level, not powered and not adjusted for multiple testing. STATA software package version 15.1 for Windows (StataCorp LLC, USA) was used for all analyses. Results were rounded to a relevant precision, percentages in the text to full numbers and p-values to three decimals. The statistical analysis plan is provided in the Supplementary Information.

6.6.3.7 Data availability

The source data underlying figures and tables included in the manuscript are provided within the Supplementary Information Source Data File and supplementary tables. Additional data

will be provided upon request (details of the request process is available on the Cancer Research UK Clinical Trials Unit website).

6.6.4 Results

6.6.4.1 Characteristics of discarded liver offers and study participants

Over the 16-month study duration from November 2016 to February 2018, there were 185 livers discarded for clinical use and offered for research. Characteristics of those offers and the study inclusion flowchart are provided in Figure 6.1 and 6.2.

One hundred and sixty-four patients on the waiting list were approached for potential participation, of which 53 were consented, and 22 were enrolled in the study and received rescued grafts.

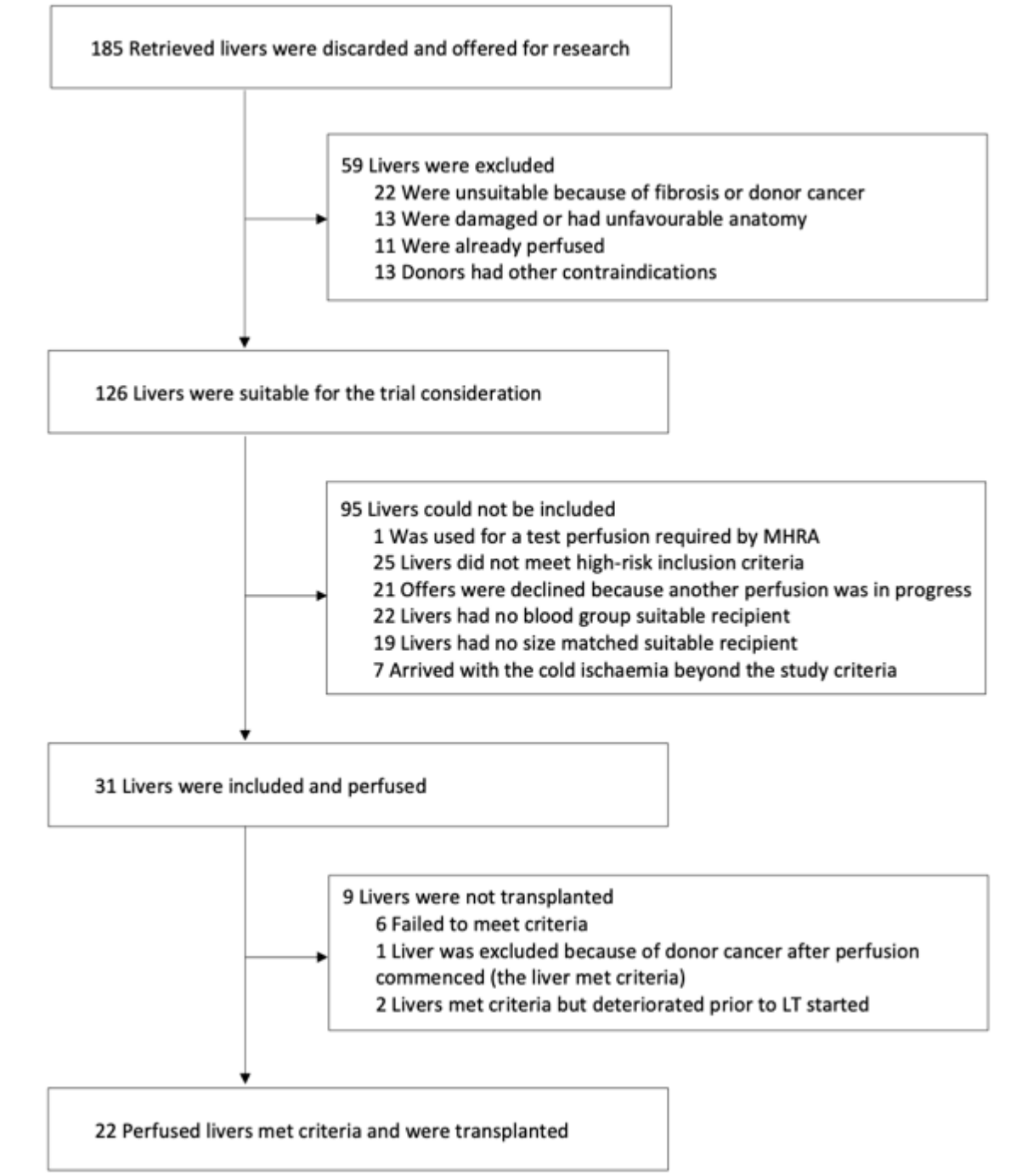

Figure 6.1 Flow diagram of livers that were offered for consideration and inclusion in trial.

This figure shows the study livers inclusion flowchart. Over the 16-month study period there were 185 discarded liver research offers, of which 59 (32%) were not eligible for the trial due to an incidental finding of cancer, macroscopically apparent cirrhosis or advanced fibrosis, severe organ damage or previous machine perfusion. There were 126 livers suitable for the

trial, with steatosis being the leading cause of organ discard with 78 (42%) offers. Stringent donor inclusion criteria were not met in 25 (14%) and on 21 (11%) occasions the research team was already committed to the perfusion of another study liver. A liver was considered for the trial only if it could be allocated to a consented, potential blood group- and size-matched low-risk recipient. Many recipients were apprehensive to participate in such a high-risk clinical trial, and as a consequence, at any given time there were usually only 1-3 patients consented. A significant proportion of approached patients declined to take part, or were transplanted with a standard quality liver before agreeing to take part in this study. Eventually thirty-one livers were enrolled to the trial, of which 22 (71%) grafts met the viability criteria and were successfully transplanted.

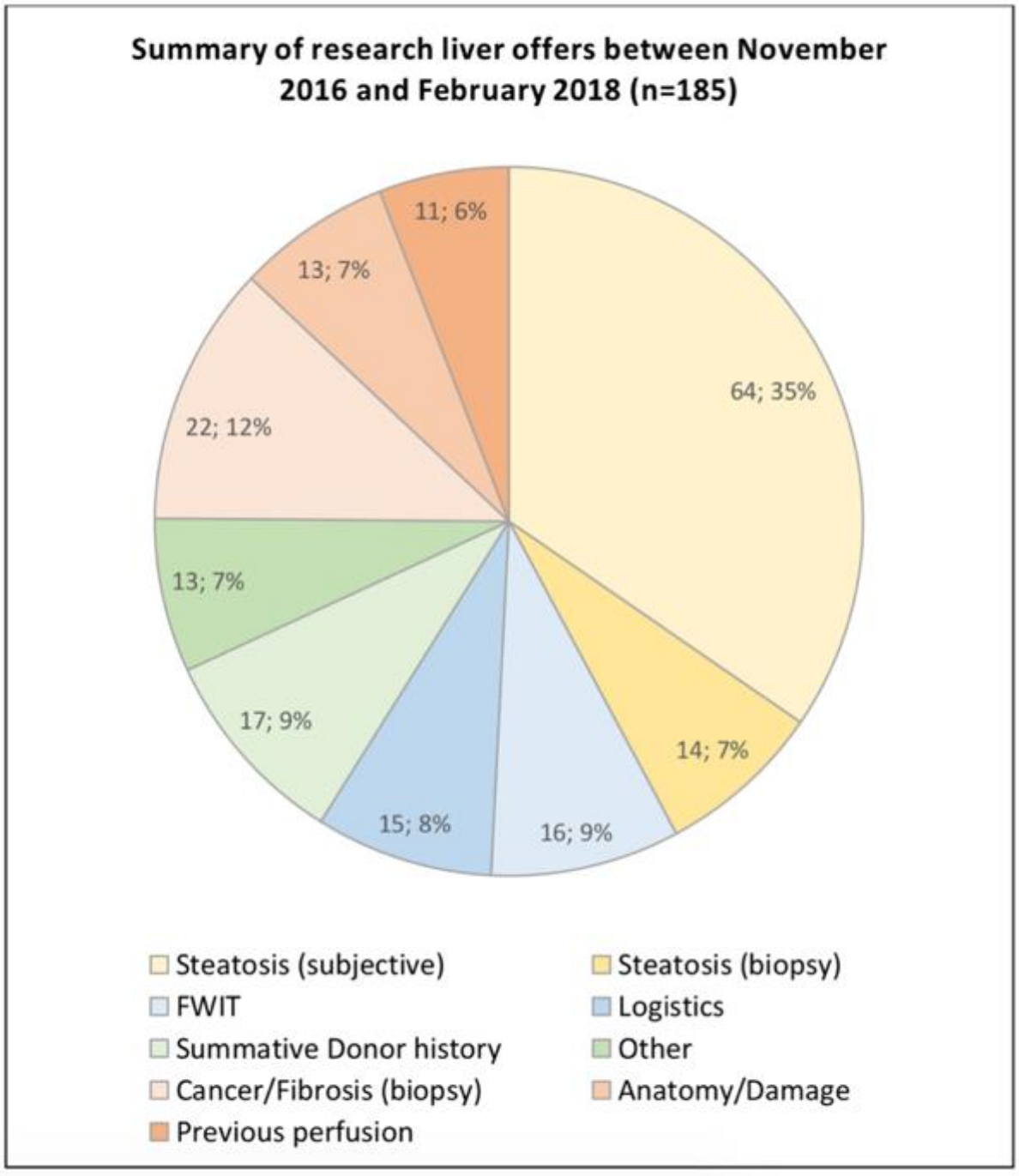

Figure 6.2 Pie chart showing the reasons why the livers offered were rejected for clinical use. This Figure presents a summary of reasons for livers being discarded in the UK between November 2016 and February 2018. A total of 64 livers were discarded for severe steatosis on visual assessment, with 14 discarded for severe steatosis based on urgent liver biopsy. A percentage of livers were declined due to intra-abdominal or lung malignancies (e.g. colonic cancer in donor 22). This did not include primary brain tumours or small renal cell cancers

which are almost always considered for donation. The reasons for logistic discard include the transplant team already being committed to one or more transplantations, lack of a suitable recipient, or too long an anticipated cold ischaemia time due to delays with transportation.

The potential participants were counselled regarding the high-risk nature of the project and unknown long-term outcomes of resuscitated livers. As a consequence, a proportion of patients were understandably reluctant to participate, and therefore the lack of suitable consented recipients was the principal rate limiting factor for inclusion. The number of consented patients at any given time ranged from 1-9; the flow diagram displaying the progress of patients through the trial is shown in Figure 6.3.

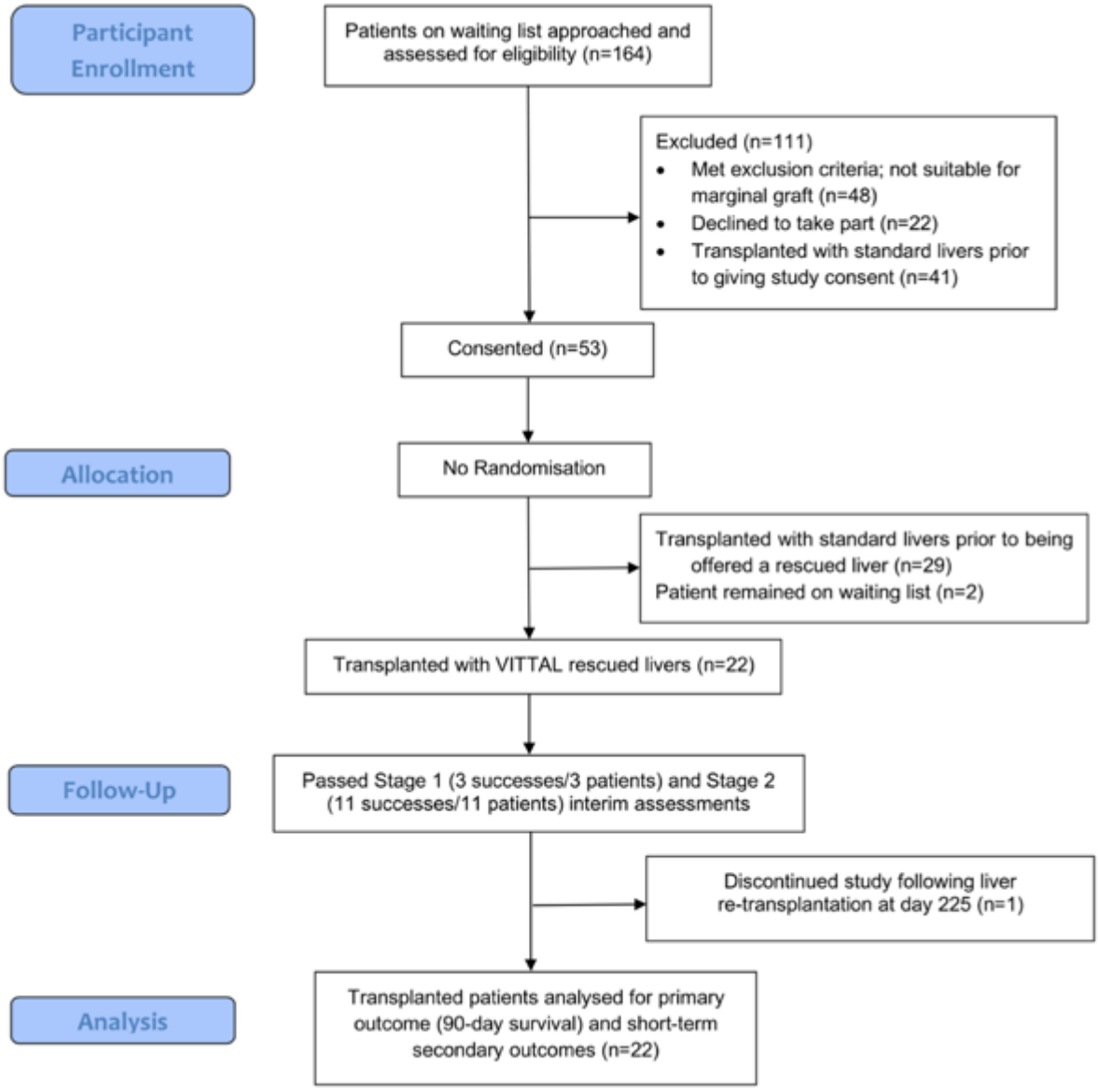

Figure 6.3 CONSORT diagram demonstrating the progress of patients through the trial.

One hundred and sixty-four patients on the waiting list were approached for potential trial participation. Of those, 111 were excluded; 48 patients met exclusion criteria and were not suitable for a marginal liver graft. Twenty-two patients declined to take part and 41 patients either received a transplant before they provided study consent, or were de-listed, or subsequently met exclusion criteria. Eventually 53 patients consented to the study, of which 29 underwent transplantation with a standard quality liver allocated outside the trial. Twenty-two patients were enrolled in the trial and received a salvaged liver.

6.6.4.2 Donor liver characteristics and liver biopsy features

In 8 (26%) donors the liver was the only procured organ. All discarded donor livers entered in the study satisfied one or more of the inclusion high-risk criteria. The livers enrolled in the trial consisted of 17 organs donated after brainstem death (DBD) and 14 after circulatory death (DCD). Many of these organs looked grossly suboptimal, with some degree of steatosis, capsular fibrosis, or rounded edges with multifactorial reasons for discard, that was captured by the donor risk index (DRI) >2.0 in 22 (71%) livers, with the median DRI 2.2 (1.9-2.9). Detailed characteristics are shown in Table 6.2 and Supplementary Table S6.1. Photos of all included livers are presented in Figure 7.4. The transplanted livers were typically smaller than non-viable ones (1.7 vs 2.0 kg, p=0.015; Kruskal-Wallis test), with lower peak pre-mortem donor liver enzymes levels. The median static cold storage time before starting NMP was 7h:44min (6:29-10:25). Only 3 (10%) livers were included in the trial primarily for macrosteatosis >30%, (50%, 80% and 60% macrovesicular steatosis combined with 11hr:55min, 12hr:00min and 6hr:15min cold ischaemia respectively). Glycogen content and steatosis degree did not predict the viability assessment results. The detailed histological finding of each study liver is provided in Supplementary Table S6.2.

Table 6.2 Donor and liver characteristics (median, interquartile range)

Donor characteristics	Non-transplanted (n=9)	Transplanted (n=22)	Overall (n=31)	P-value*
Age in years (range)	57 (52-60)	56 (45-65)	57 (45-63)	0.948
Sex – n (%)				0.696
Female	3 (33.3)	10 (45.5)	13 (41.9)	
Male	6 (66.7)	12 (54.5)	18 (58.1)	
Height (cm)	174 (172-186)	170 (165-175)	170 (166-175)	0.038
Bodyweight (kg)	79 (75-88)	81 (70-90)	80 (70-90)	0.662
Body mass index (kg/m^2)	28.7 (24.8-29.1)	29.3 (26.5-32.4)	28.7 (24.8-32.1)	0.372
Liver weight (kg)	2.0 (1.8-2.4)	1.7 (1.3-1.9)	1.8 (1.4-2.0)	0.015
Peak alanine transferase (IU/ml)	323 (92-1143)	48 (33-159)	83 (36-287)	0.034
Peak gamma-glutamyl transferase (IU/ml)	169 (107-335)	80 (42-111)	92 (57-203)	0.012
Peak bilirubin (µmol/L)	10 (10-18)	11 (7-22)	11 (8-22)	0.768
History of excessive alcohol use – n (%)	5 (55.6)	5 (22.7)	10 (32.3)	0.105
Diabetes mellitus – n (%)	0 (0.0)	2 (9.1)	2 (6.5)	1.000
Donor type - n (%)				1.000
Donor after brain death	5 (55.6)	12 (54.5)	17 (54.8)	
Donor after circulatory death	4 (44.4)	10 (45.5)	14 (45.2)	
Donor warm ischaemic time (minutes)[&]	20.0 (15.5-22.5)[&] n=4	22.5 (19.0-35.0)[&] n=10	21.0 (19.0-25.0)[&] n=14	0.394
Quality of *in situ* flush – n (%)				0.016
Poor	3 (33.3)	4 (18.2)	7 (22.6)	
Fair	4 (44.4)	1 (4.5)	5 (16.1)	
Good	2 (22.2)	17 (77.3)	19 (61.3)	
Cold ischaemic time (minutes)	550 (436-715)	452 (389-600)	464 (389-625)	0.277
Donor risk index[§]	2.3 (2.0-2.7)	2.1 (1.9-3.0)	2.2 (1.9-2.9)	0.728
Histological steatosis assessment – n (%)[$]				0.113
<30% macrovesicular steatosis	2 (22.2)	13 (59.1)	15 (48.4)	
>30% macrovesicular steatosis	7 (77.8)	9 (40.9)	16 (51.6)	
Inclusion criteria[^]				
Donor risk index >2.0	6 (66.7)	16 (72.7)	22 (71·0)	1.000
Steatosis principal reason to discard[+]	1 (11.1)	2 (9.1)	3 (9·7)	1.000
High liver transaminases	3 (33.3)	2 (9.1)	5 (16·1)	0.131
Balanced risk score > 9	Not applicable	2 (9.1)	Not applicable	Not applicable
Extensive cold ischaemic time	2 (22.2)	3 (13.6)	5 (16·1)	0.613
Extensive donor warm ischaemic time	0 (0.0)	3 (13.6)	3 (9·7)	0.537
Poor *in situ* flush	3 (33.3)	4 (18.2)	7 (22·6)	0.384
Perfusion criteria				
Lactate clearance <2.5mmol/L	3 (33.3)	22 (100·0)	25 (80·6)	<0.0001
pH ≥7.30	3 (33.3)	19 (86·4)	22 (71·0)	0.007
Presence of bile production – n (%)	6 (66.7)	18 (81·8)	24 (77·4)	0.384
Bile volume (mL)	10 (2-18)	60 (15-99)	46 (2-90)	0.100
Glucose metabolism	4 (44.4)	20 (90·9)	24 (77·4)	0.012
Vascular flows criteria met	9 (100)	22 (100)	31 (100)	Not applicable
Homogenous liver perfusion	7 (77.8)	22 (100·0)	29 (93·5)	0.077

Note: Body mass index is the weight in kilograms divided by the square of the height in meters. [&] Donor warm ischaemic time is defined as the period from the systolic blood pressure decrease below 50mmHg to commencing the aortic cold flush; this variable applicable only for donors after circulatory death. [§]Donor risk index as described by Feng *et al.* Am J Transplant 2006. [$]The steatosis includes large and medium droplets macrovesicular steatosis assessment obtained from post-transplant paraffin sections (this result was not known at the time of the liver inclusion). [+]This steatosis variable refers to the study inclusion criteria and the results were known before the transplant based on frozen sections histology assessment. [^]Each trial liver had to meet one or more of the following inclusion criteria: donor risk index greater than 2.0; biopsy proven liver steatosis greater than 30%; donor transaminases (aspartate transaminase or alanine transaminase) greater than 1000 IU/mL; warm ischaemic time greater than 30 minutes in donors after circulatory death; extensive cold ischaemic time (defined as the period between the aortic cold flush to the liver implantation, or commencing the normothermic perfusion) greater than 12 hours and 8 hours for donors after brainstem death and circulatory death respectively; suboptimal liver flush documented by photograph and a transplant surgeon assessment; balanced risk score greater than 9. *Due to the small sample sizes and that the statistical comparison tests were not powered, these results should be interpreted with caution.

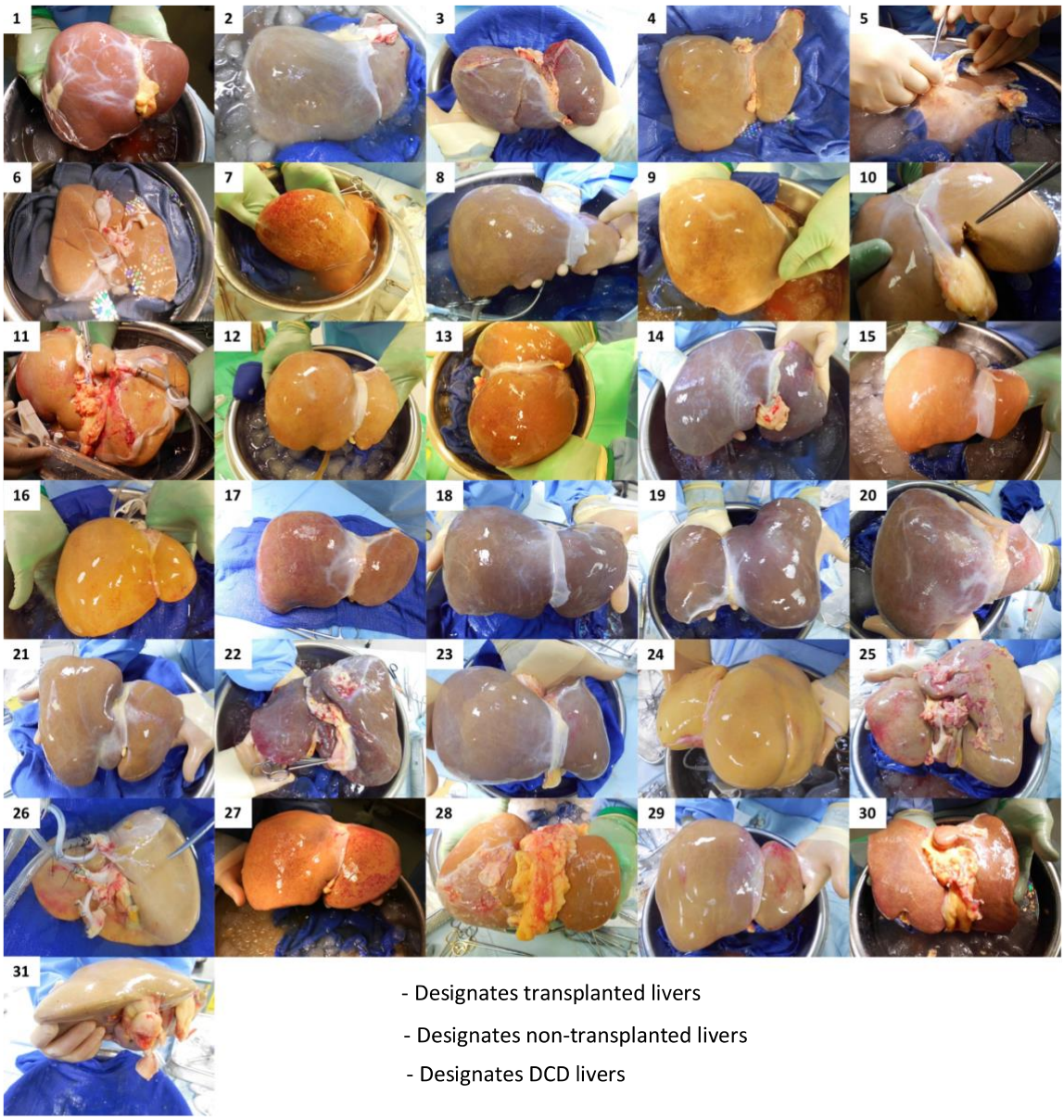

Figure 6.4 Photographs of the study livers.

The figure shows all 31 livers included to the trial. The red frame designates non-transplanted organs and the yellow dot livers donated after circulatory death.

6.6.4.3 Perfusion parameters assessment

During the NMP procedure 25 livers quickly recovered metabolic activity and cleared lactate to the target level (details provided in Figure 6.5). A biopsy of a suspicious donor colonic lesion confirmed malignancy, making one liver unsuitable for transplantation, after meeting the viability criteria. In 3 livers, criteria were initially met, however metabolic function thereafter deteriorated within the first 4 hours, with increasing lactate. In two cases the transplant procedure was not commenced and the livers were discarded. In the third, the explant had begun, and the procedure continued. Overall, 22 (71%) livers met the viability criteria and were transplanted following a median total preservation time of 17h:53min (16:17-21:48; Table 6.3).

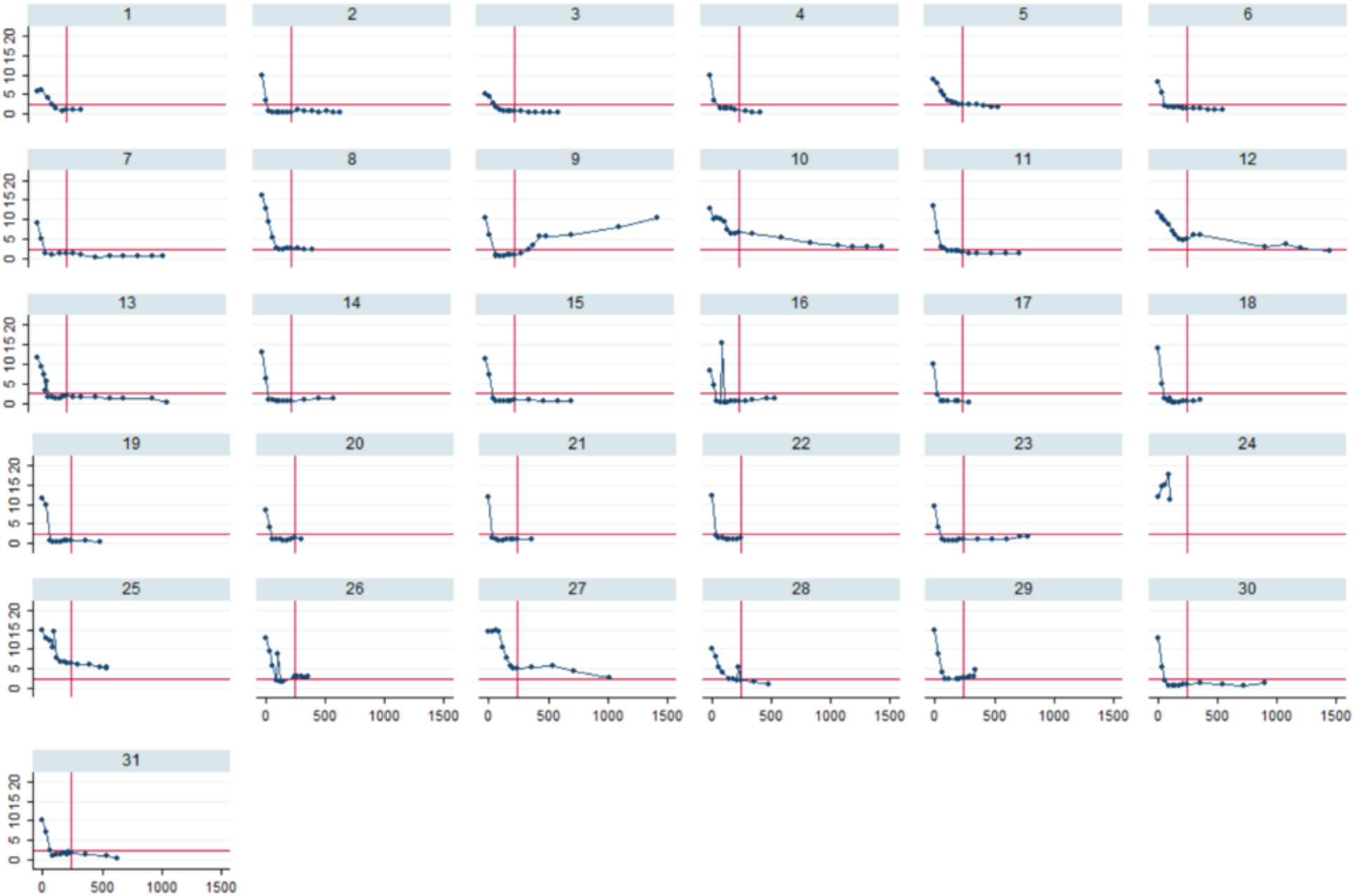
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Figure 6.5 The study liver lactate clearance.

Plots of individual liver arterial lactate (mmol/L [y-axis]) clearance measured during the NMP perfusion (perfusion time (minutes) [x-axis]), showing transplantation eligibility thresholds with red lines for lactate levels less than or equal to 2.5mmol/L. Graphs with grey shading designate livers that were not transplanted. Liver number 22 was from a donor that was unexpectedly diagnosed with a cancer following organ donation.

Table 6.3 Transplant recipient and graft characteristics (median, interquartile range)

Recipient characteristics	**Trial patients (n=22)**
Age in years	56 (46-65)
Sex – n (%)	
Female	8 (36.4)
Male	14 (63.6)
Body mass index	28.5 (24.0-31.0)
UK end-stage liver disease score	52 (49-55)
Model for end-stage liver disease score^	12 (9-16)
Transplant indication – n (%)	
Alcohol-related liver disease	8 (36.4)
Non-alcohol steatohepatitis	4 (18.2)
Hepatitis C virus	2 (9.1)
Primary biliary cirrhosis	2 (9.1)
Primary sclerosing cholangitis	6 (27.3)
Hepatocellular carcinoma[&]	3 (13.6)
Need for intra-operative CVVH – n (%)	1 (4.5)

Graft and transplant details	**Overall (n=22)**	**DBD (n=12)**	**DCD (n=10)**	**P-value***
Cold ischaemic time (minutes)	452 (316-600)	507 (408-718)	416 (354-464)	0.075
Implantation time (minutes)	28 (22-35)	30 (26-38)	26 (22-35)	0.390
Machine perfusion time (minutes)	587 (450-705)	629 (509-700)	549 (424-780)	0.598
Total preservation time (minutes)	1073 (977-1308)	1170 (1038-1367)	1000 (874-1097)	0.075
Post-reperfusion syndrome	10 (45.5)	2 (16.7)	8 (80.0)	0.008

Abbreviation: n, number; CVVH, continuous veno-venous haemofiltration; DBD, donor after brainstem death; DCD, donor after circulatory death

Note: Body mass index is the weight in kilograms divided by the square of the height in meters. Donor warm ischaemic time is defined as the period from the systolic blood pressure decrease below 50mmHg to commencing the aortic cold flush. Cold ischaemic time is defined as the time between the start of the cold flush during retrieval until the start of machine perfusion. Early allograft dysfunction consists of the presence of one or more of the following variables: (1) bilirubin $\geq$10mg/dL on postoperative day 7; (2) INR $\geq$1.6 on postoperative day 7; (3) aminotransferase level (alanine aminotransferase or aspartate aminotransferase) >2,000IU/mL within the first 7 postoperative days (Olthoff *et al*, Liver Transplantation, 2010).

^the liver grafts are allocated in the UK based on the UK end-stage liver disease score; the laboratory values of the model for end-stage liver disease score are included for the comparative information only. [&]The presence of hepatocellular cancer is recorded as a complication of the underlying liver disease mentioned above, and does not impact on the liver allocation algorithm. *Due to the small sample sizes and that the statistical comparison tests were not powered, these results should be interpreted with caution.

6.6.4.4 The study patients

The majority (64%) of recipients were men, and median age was 56 (46-65) years. The leading indication for transplantation was alcohol-related liver disease (36%), followed by primary sclerosing cholangitis (27%) and non-alcoholic steatohepatitis (18%). In three (14%) patients the underlying liver disease was complicated by liver cancer. The median UKELD(20) score was 52 (49-55), with a calculated laboratory MELD score of 12 (9-16). Details are provided in Table 6.3 and Supplementary Table S6.3.

6.6.4.5 Co-primary study outcomes

Thirty-one livers were enrolled into the trial for objective assessment by NMP. Twenty-two of these livers met the viability criteria and were transplanted, resulting in a significant successful rescue rate of 71% (22/31, 90% Wilson confidence interval: 56.3% - 82.2%), to conclude that the procedure is feasible. All 22 (100%) transplanted patients were alive at day 90 post-transplantation – greater than the 18/22 required by the trial design.

6.6.4.6 Transplant outcomes

Graft 90-day survival was 100%. Seven (32%) patients developed early allograft dysfunction, and 7 (32%) patients developed Clavien-Dindo complication grade ≥3, including 4 (18%) cases with acute kidney injury requiring renal replacement therapy. The median intensive care and in-hospital stays were 3.5 days (3-4) and 10 days (8-17) respectively. The 1-year patient and graft survival were 100% and 86% respectively. Details are provided in Figure 6.6 and Table 6.4.

Table 6.4 Post-transplant outcomes

	Study patients (n=22)	**Control patients (n=44)**	**Overall (n=66)**	**OR (95%CI); P-value**
Post-transplant outcomes				
Primary graft non-function – n (%)	0 (0.0)	1 (2.3)	1 (1.5)	1.000†*
Early allograft dysfunction – n (%)	7 (31.8)	4 (9.1)	11 (16.7)	5.62 (1.14, 27.79); 0.034ǂ
Renal replacement therapy – n (%)	4 (18.2)	11 (25.0)	15 (22.7)	0.68 (0.19, 2.38): 0.542ǂ
Intensive care unit stay (days)	3.5 (3-4)	2.0 (1-5)	3·0 (2.5)	1.02 (0.95, 1.10); 0.566ǂ
In-hospital stay (days)	10 (8-17)	9 (8-11)	10 (8-13)	1.00 (0.96, 1.05); 0.822ǂ
Clavien-Dindo complication ≥3 - n (%)	7 (31.8)	17 (38.6)	24 (36.4)	0.089†*
90-day graft survival – n (%)	22 (100)	41 (93.2)	63 (95.5)	0.545†*
90-day patient survival – n (%)	22 (100)	44 (100)	66 (100)	Not applicable
1-year graft survival – n (%)^	19 (86.4)	38 (86.4)	57 (86.4)	1.000 (0.18, 5.46); 1.000ǂ
1-year patient survival – n (%)^	22 (100)	42 (95.5)	64 (97.0)	0.55†*
Biliary complication – n (%)§				
Anastomotic biliary stricture^	2 (9.1)	3 (6.8)	5 (7.6)	1.44 (0.19, 11.12); 0.725§ǂ
Non-anastomotic biliary stricture^	4 (18.2)	1 (2.3)	5 (7.6)	8.00 (0.89, 71.58); 0.063§ǂ
	DBD livers (n=12)	**DCD liver (n=10)**	**Overall (n=22)**	**P-value***
Study patient biliary strictures				
Anastomotic within 6 months^ – n (%)	1 (8.3)	0 (0.0)	1 (4.5)	1.000†
Anastomotic within 12 months& – n (%)	1 (8.3)	1 (10.0)	2 (9.1)	1.000†
Non-anastomotic within 6 months^ – n (%)	1 (8.3)@	2 (20.0)	3 (13.6)	0.571†
Non-anastomotic within 12 months& – n (%)	1 (8.3)@	3 (30.0)	4 (18.2)	0.293†

Abbreviations: n, number; OR, odds ratio; CI, confidence interval, DBD, donation after brainstem death; DCD, donation after circulatory death

Note: § The result needs to be interpreted with caution as the control patients did not receive systematic bile duct imaging; in this group one patient developed non-anastomotic biliary strictures, one died 16 months after the transplantation from biliary sepsis, and one is alive with a complex hilar stricture not amenable to any therapeutic intervention. §The figures represent strictures manifested with cholestasis and elevated liver enzymes. ^Data were assessed at scheduled study visits up to and including the 12-month follow-up visit&. @stricture developed in patient suffering from hepatic artery occlusion requiring revascularisation within 24 hours following the transplant. †P-value obtained from Fisher's exact test. ǂP-values obtained from conditional logistic regression. *Due to the small sample sizes and that the statistical comparison tests were not powered, these results should be interpreted with caution.

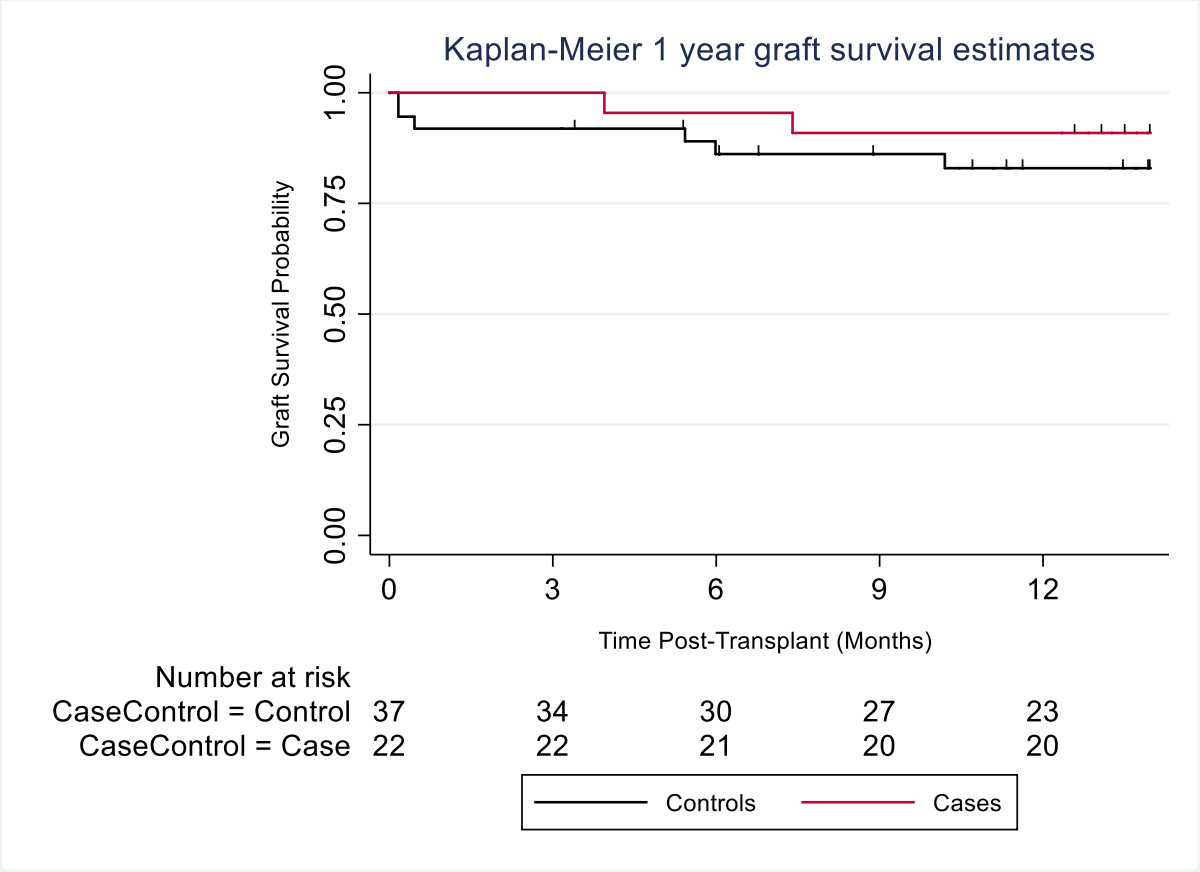

Figure 6.6 Comparison of 1-year graft survival estimate

Conditional logistic regression was carried out on the matched case-control data to determine the relative risk for graft survival at 1 year between matched case-control groups. The median (range) days follow-up data was included in the survival analyses, but the plot was truncated at 12 months. The ticks on the top of each Kaplan-Meier curve relate to the numbers of patients being censored at that particular time point. There are two cases of graft failure in the perfusion group at days 119 and 209; the control group contains five graft failures (two at day 5, one at day 14, one at day 165 and one at day 182). The graft survival was similar in both groups. Findings showed that the odds ratio (relative risk) estimate for graft survival at 6 months was determined as 2.0 (95%CI: 0.2, 17.9; P=0.535). Due to the small sample sizes and that this statistical comparison test was not powered, these results should be interpreted with caution.

6.6.4.7 Vascular and biliary complications

One patient developed an intra-operative hepatic artery thrombosis after receiving a DBD graft that had sustained a hepatic arterial injury during procurement. The artery was reconstructed but post-operatively thrombosed, undergoing emergency revascularisation which achieved long-lasting arterial patency. The graft, however, developed biliary strictures requiring multiple interventions and eventual re-transplantation.

The per-protocol MRCP imaging at 6 months revealed that 2 (9%) patients developed anastomotic, and 4 (18%) patients non-anastomotic biliary strictures that presented with cholestatic symptoms. With the exception of the patient with hepatic artery thrombosis, all biliary strictures affected recipients of DCD grafts. During the study median follow up of 542 days (456-641), four patients underwent liver re-transplantation (at day 120, 225, 375, and 417). The details are provided in Table 6.4 and Supplementary Table S6.3.

6.6.4.8 Comparison of outcomes with contemporary matched controls

Patient and graft survival rates at 12 months (100% and 86% respectively) were similar to the matched controls (96% and 86% respectively). The incidence of early allograft dysfunction was higher in the study group (32% vs 9%, odds ratio 5.6, 95% confidence interval 1.1-27.8, p=0.034; conditional logistic regression). There were no differences in the other assessed parameters, including the need for post-transplant renal replacement therapy, hospital stay, or incidence of Clavien-Dindo grade ≥3 complication rates. The incidence of clinically manifest non-anastomotic biliary strictures was higher in the study group (18% vs 2%, odds ratio 8.0, 95% confidence interval 0.9-71.6; p=0.063; conditional logistic regression), although this result needs to be interpreted with caution as the matched control patients did not receive systematic bile duct imaging. Due to the small sample sizes these comparison results should

be interpreted with caution, and the controls were included to present the study results within the context of the unit's contemporary outcomes. The details are shown in Table 6.4.

6.6.5 Discussion

Utilisation of livers from organ donors is currently a major challenge in liver transplantation(26). Despite a waiting list mortality in Western countries reaching 20-30%, an increasing proportion of extended criteria livers are unused due to concerns of primary non-function and early graft dysfunction(27, 28). The decision to discard donor livers is still largely based upon donor history and subjective assessment by the transplanting surgeon. Standard cold static preservation does not allow for any assessment of liver function, and the only other source of information is liver histology, which is able to diagnose severe large droplet fatty change, a well-recognised risk factor for non-function(28). This study has demonstrated that moving from subjective evaluation to objective testing during NMP might salvage a high proportion of those livers that are currently discarded. The need to improve the method by which high-risk livers are assessed was illustrated in this study by the absence of significant differences in the donor characteristics between transplanted and discarded livers.

The present trial is the first to systematically investigate objective viability criteria in livers that met specific high-risk features in organs initially considered “untransplantable”(11, 29). One major challenge addressed in the VITTAL trial design was that each discarded liver had to also fulfil one or more pre-defined objective high-risk criteria, as the considerations for liver transplantability are always multi-factorial, including the recipient condition, logistical aspects, and the surgeon’s (or transplant centre’s) experience and risk-taking attitude. The utilisation of marginal livers in the UK was facilitated by the centre-based liver allocation system, allowing the use of high-risk organs in any patient on the waiting list. All enrolled organs were simultaneously fast-track offered to all UK transplant centres following the initial decline, and the fact that none of the seven centres were comfortable using any of the livers included in this trial confirms that these organs were uniformly perceived to be of very poor quality. Our team

genuinely aimed to push the boundaries of utilisation of the highest risk organs by accessing the benefit of rigorous peer-review and continual oversight within the framework of a clinical trial. We included only organs that our team did not feel comfortable to use otherwise, and this attitude was reflected by the two-tier liver inclusion process embedded in the trial design, and by the fact that 25 livers, that would very likely meet the transplantability criteria, were not considered for the study inclusion. Some of the study livers might have been transplantable if the cold ischaemia was very short and a suitable recipient was waiting, but currently the majority of these organs are discarded. With the introduction of the National Allocation system, logistical constraints exacerbated by static cold storage are increasingly common and prevent the utilisation of a rising proportion of marginal livers. In these circumstances, NMP mitigates the reperfusion process, allowing assessment of the organ during perfusion without exposing patients to the risk of primary non-function. Additionally, livers discarded due to haemodynamic instability (during procurement or during the process of brain stem death itself), high liver transaminases or poor *in situ* flush, benefited from perfusion in a controlled, near physiological environment thereby facilitating their recovery. The potential to recondition the liver in the interval between retrieval and implantation has hitherto not been possible.

An intervention which increases successful utilisation of high-risk livers will transform access to transplantation to meet predicted increasing demand, particularly given trends in donor demographics and declining organ quality(4). Whilst organ donation in the UK has increased from 676 to 1149 donors per annum between 2008 and 2018, the proportion of retrieved livers that were discarded has nearly doubled (from 8% to 15%; data from the UK Organ Donation and Transplantation Registry), indicating reluctance of surgeons to accept these organs for their increasingly sicker recipients. In 2017-18, not only were 174 retrieved livers discarded, but 425 livers from solid organ donors were not even considered suitable for retrieval (11% of

DBD and 52% of DCD); it is reasonable to assume that many of these would be suitable for testing. Salvaging a proportion of these retrieved but discarded organs would add a good number of transplantable livers annually in the UK, significantly reducing waiting list mortality.

International comparisons demonstrate regional variations in donor demographics and there is evidence that in countries with higher initial organ acceptance rates there is also a higher discard rate, particularly for older donors(30, 31). Viability testing provides objective evidence of liver function with clearance of metabolic acidosis, vascular flows, glucose parameters and bile production; these give the transplant surgeon the confidence to use these organs safely, and minimises the physical and emotional impact of non-transplantation for patients.

In the presented study the NMP was commenced following a median cold storage time reaching 8 hours. Whilst this approach may simplify adoption of the NMP technology without compromising outcomes in transplantable livers(32), recovery of organs from donors with multiple high-risk features might be further facilitated by limiting cold ischaemia through commencing the perfusion immediately after procurement in the donor hospital(14). Inevitably there will always be livers that are not suitable for transplantation, demonstrated by 30% of offers with macroscopic cirrhosis, biopsy-proven fibrosis or an incidental finding of donor cancer. A similar proportion of the livers, however, did not meet any of our high-risk criteria and were therefore considered “too good” for inclusion. It is reasonable to assume that NMP assessment would have provided the reassurance needed to justify transplantation in this group as well.

Improvements in transplant logistics is one of the major advantage of NMP(14, 32, 33), and the study allowed for the machine perfusion duration to be between 4 hours (time needed for the viability assessment) and 24 hours (maximum recommended time by the perfusion device manufacturer). Once the liver met the viability criteria we aspired to commence the transplantation as soon as possible; however, the perfusion was often extended to allow for a day-time procedure, or to facilitate transplant logistics in the unit. From our experience, 4-6 hours' perfusion seems to be sufficient for adequate assessment and replenishment of the organ's energy resources. Due to recirculation of metabolites accumulated in the organs during cold ischaemia, the high-risk organs probably do not benefit from prolonged perfusion. The impact of NMP duration on livers initially exposed to prolonged cold ischaemia is an area of our ongoing research interest.

Transplant surgeons in many countries are expanding the donor pool with the use of organs donated after circulatory death(34), In the context of liver transplantation, the longevity of these organs might be compromised by development of non-anastomotic biliary strictures(8). The incidence of clinically manifest non-anastomotic biliary strictures in the DCD grafts cohort was 30% (3 out of 10 grafts), higher than the study matched controls group, but similar to other reported high-risk DCD series(35). In concordance with the European prospective normothermic preservation trial, our results suggested that MRCP findings are likely to over-estimate the incidence of biliary complications(14). The per-protocol investigation at the 6-month time point would identify over 80% of the clinically relevant biliary strictures and asymptomatic irregularities with varying clinical significance(35). The presented findings are accurate, as the images were correlated with clinical reviews and liver function tests through the median follow-up of 542 (range 390-784) days. Nevertheless, it is clear that end-ischaemic NMP does not prevent the development of non-anastomotic biliary strictures in high-risk DCD

organs, and our outcomes suggest that extending the donor warm times beyond the currently widely accepted limit of 30 minutes is not advisable. This finding was not anticipated at the time of trial design or during the conduct of the trial and only became evident during the longer term follow up of these grafts beyond the primary end point of 90 days. Further work is needed to identify new limits (e.g. donor characteristics, warm ischaemia time, cold ischaemia time) and to define perfusion biomarkers that predict this complication and avoid futile transplantation. Recently published research suggests that the composition of bile produced during perfusion (pH, bicarbonate and glucose concentration) is predictive of ischaemic cholangiopathy(17). Sub-analysis of bile samples and determination of biliary endothelial health is the subject of ongoing research. Evolving novel perfusion strategies might enable the use of DCD grafts exposed to prolonged warm ischaemia(14, 36, 37).

The other limitations of our study include the sensitivity of the cut-off lactate value, the non-randomised trial design, and exclusion of high-risk transplant recipients. Regarding the former, following previous experience, we set the lactate viability threshold to less than 2.5mmol/L within 2 hours of NMP(15, 16). To maximise utilisation, this trial extended the assessment period to 4 hours. Two livers in the trial were discarded following a rise of the perfusate lactate after meeting the 2-hour target. The significance of this is uncertain, although it is notable that a third liver with a similar pattern of lactate clearance was transplanted and experienced a substantial period of early allograft dysfunction with a post-transplant peak ALT of 2074 IU and AST of 3031 IU. Concerning the design, the trial was conducted as a non-randomised study, as transplanting discarded livers with an expected high incidence of primary non-function as controls would be ethically unacceptable. We expect further advances to be achieved through the identification of specific biomarkers that correlate with long-term graft outcomes, in the context of large NMP series or registries. Lastly, as we did not want to

compound risks, the study did not include higher risk recipients deemed not suitable to receive marginal organs at the unit's multi-disciplinary liver transplant listing meeting. The majority of participants who decided to participate did so after a long period waiting on the list, with progressive deterioration that was not necessarily reflected by their waiting list position. The feasibility of using livers rescued by NMP for the high-risk recipient is currently under investigation.

In conclusion, this trial demonstrated that NMP provides a way of objectively assessing high-risk organs, and allowed transplantation in a significant proportion of currently unutilised livers without any incidence of primary non-function. The use of perfusion technology was associated with increased graft utilisation, considerably extended preservation time and greatly improved transplant logistics. Adoption of functional assessment of high-risk livers can increase access to life saving transplantation and reduce waiting list mortality.

Supplementary Table S6.1 Study livers overview

Liver number	Graft type	Inclusion criteria met								Viability criteria met	Total bile production (ml)	Liver transplanted	Additional Risk Scores		
		DRI	BAR	Steatosis	CIT*	WIT§	Flush	Enzymes	TOTAL				UK-DLI	ET-DRI	UK-DCD
1	DCD	Y (2·7)	NA	N	Y 9:10	N (11)	N	N	2	Y	18	N (anatomy)~	2·29	3·31	4
2	DBD	N (1·6)	N (5)	N	N 7:20^	NA	N	N	0	Y	0	Y	1·04	2·38	-
3	DBD	Y (2·3)	Y (10)	N	N 5.59	NA	N	N	2	Y	46	Y	1·81	2·78	-
4	DBD	N (1·7)	Y (11)	N	Y 13:13	NA	N	N	2	Y	64	Y	1·05	2·12	-
5	DBD	Y (2·5)	N (3)	N	N 10:25	NA	N	N	1	Y	>99	Y	1·23	2·92	-
6	DBD	Y (2·1)	N (3)	N	Y 14:50	NA	N	N	2	Y	60	Y	1·18	2·42	-
7	DBD	Y (2·1)	N (3)	N	N 6:30	NA	N	Y (1812)	2	Y	>99	Y	0·99	2·52	-
8	DCD	Y (3·0)	N (3)	N	Y 9:33	N (23)	N	N	2	Y	90	Y	1·98	3·78	7
9	DCD	Y (2·1)	NA	N	N 6:00	N (20)	N	Y (1383)	2	N	>99	N (lactate drifted)	1·67	2·92	5
10	DCD	Y (2·9)	NA	N	N 7:16	N (20)	N	N	1	N	10	N	1·72	3·41	7
11	DBD	N (1·6)	N (4)	N	N 5:24	NA	N	Y (1041)	1	Y	60	Y	1·60	1·92	-
12	DCD	Y (2·9)	NA	N	Y 9:59	N (25)	N	N	2	N	0	N	2·00	3·68	5
13	DCD	Y (2·2)	N (2)	N	N 6:29	N (17)	Y	N	2	Y	0	Y	1·42	3·03	4
14	DCD	Y (2·9)	N (5)	N	N 7:09	Y (40)	N	N	2	Y	0	Y	1·70	3·41	10
15	DCD	Y (3·2)	N (1)	N	N 5:32	N (22)	N	N	1	Y	>99	Y	1·88	3·63	7
16	DBD	N (1·8)	N (8)	Y (50%)	N 11:55	NA	N	N	1	Y	0	Y	1·41	2·68	-
17	DBD	N (1·9)	N (6)	N	Y 12:00	NA	Y	N	2	Y	0	Y	1·10	2·22	-
18	DCD	Y (2·1)	N (7)	N	N 7:44	Y (35)	N	N	2	Y	75	Y	1·95	3·19	8
19	DCD	Y (2·5)	N (2)	N	N 7:00	Y (46)	N	N	2	Y	>99	Y	1·57	3·14	8
20	DCD	Y (3·1)	N (2)	N	N 5:54	N (19)	Y	N	2	Y	48	Y	2·65	3·72	4
21	DCD	Y (3·2)	N (4)	N	N 6:52	N (23)	N	N	1	Y	15	Y	2·52	4·51	9
22	DBD	Y (2·3)	NA	N	N 8:36	NA	N	Y (1143)	2	Y	18	N (donor cancer)	1·13	4·25	-
23	DCD	Y (3·1)	N (3)	N	Y 10:00	N (18)	Y	N	3	Y	>99	Y	2·33	3·50	6
24	DBD	Y (2·0)	NA	Y (80%)	Y 12:00	NA	N	N	3	N	0	N	1·18	2·96	-
25	DBD	Y (2·4)	NA	N	Y 13:24	NA	Y	N	3	N	2	N	1·38	2·77	-
26	DBD	N (1·2)	NA	N	N 6:00	NA	Y	Y (1811)	2	Y	30	N (lactate drifted)	0·72	2·15	-
27	DBD	N (1·9)	NA	N	N 11:55	NA	Y	N	1	N	3	N	0·93	2·71	-
28	DCD	Y (3·8)	N (4)	N	N 5:34	N (20)	N	N	1	Y	56	Y	2·77	3·76	9
29	DBD	N (1·7)	N (3)	Y (60%)	N 6:15	NA	N	N	1	Y	15	Y	1·16	2·03	-
30	DBD	Y (2·1)	N (3)	N	N 7:46	NA	N	N	1	Y	>99	Y	1·35	2·76	-
31	DBD	Y (2·1)	N (3)	N	N 7:33	NA	N	N	1	Y	63	Y	1·01	2·68	-

Abbreviations: BAR, balance of risk score; DRI, donor risk index; Y, Yes; N, No; NA, not applicable; CIT, cold ischaemic time; WIT, donor warm ischaemic time (DCD only); DCD, circulatory death donor; DBD, brainstem death donor; UK-DLI, United Kingdom donor liver index; ET-DRI, Eurotransplant Zone donor risk index; UK-DCD, United Kingdom DCD score. RED denotes principle criterion for inclusion and PINK denotes other high risk criteria that were met.

Notes: *Time expressed in hours:minutes. § Donor warm ischaemic time is defined as the period from the systolic blood pressure decrease below 50mmHg to commencing the aortic cold flush and is expressed in minutes. Highlighted inclusion criteria cells designate the criterion was met. ^The liver was a fast-track offer from another centre following the initial recipient intra-operative death, where the short notice precluded commencement of the transplantation within 12 hours of CIT. ~The liver was not used because of multiple arterial reconstructions and poor vessels quality. Donor risk index is calculated from age, race, cause of death, height and the predicted cold ischaemic time (Feng *et al* 2006); BAR is calculated using model for end-stage liver disease score (MELD), whether or not the recipient is having a re-transplant or is on intensive care, recipient age, donor age and cold ischaemic time (Dutkowski *et al* 2011); UK-DLI is calculated using donor age, sex, height, donor type, bilirubin, smoking history, and whether the liver was split (Collett *et al* 2017); ET-DRI is calculated using donor age, cause of death, whether whole or split liver, regional or national share, gamma glutamyl transferase and whether a rescue offer (Braat *et al* 2012); UK-DCD score is calculated using donor age, donor body mass index, duration of functional warm ischaemic time, cold ischaemic time, recipient age, MELD score, and re-transplant status (Schlegel *et al* 2018). Additional risk score data supplied by RW Laing and steatosis percentages supplied by DAH Neil.

Supplementary Table S6.2 Histology findings

Liver Number	Liver Type	Steatosis (Prior to NMP)		Glycogen depletion			Bile duct injury		Other
		LARGE DROPLET	SMALL/MED DROPLET	Pre-NMP	4hrs NMP	Post NMP	Pre-NMP	After liver implantation	
1	DCD	nil	mild	severe	severe	severe	moderate	not applicable	Moderate to severe siderosis
2	DBD	nil	nil	severe	severe	moderate	min	none	
3	DBD	nil	mild	mild	mild	moderate	mild	mild-mod	
4	DBD	nil	severe	mild	mild	mild	mild-moderate	mod-severe*	
5	DBD	nil	mild	nil	nil	nil	minimal	mod-severe#	
6	DBD	mild	moderate	nil	mild	nil	minimal	minimal	Minimal siderosis
7	DBD	mild	severe	severe	severe	nil	minimal	minimal	
8	DCD	mild	mild	mild	nil	nil	mild-moderate	mild -moderate*	
9	DCD	nil	severe	severe	severe	moderate	mild-moderate	not applicable	Mild siderosis
10	DCD	mild	severe	mild	mild	moderate	mod	not applicable	
11	DBD	mild	severe	severe	severe	mild	mild	moderate	
12	DCD	nil	moderate	severe	severe	severe	mod-severe	not applicable	
13	DCD	nil	nil	nil	nil	nil	minimal	mild	
14	DCD	nil	mild	moderate	mild	mild	mod-severe	severe#	
15	DCD	mild	moderate	severe	severe	mild	mild	mod-severe	Mild siderosis
16	DBD	mild	severe	severe	severe	moderate	mild-moderate	moderate	Mild siderosis
17	DBD	nil	nil	nil	nil	nil	severe	severe	Minimal siderosis
18	DCD	nil	mild	severe	moderate	severe	mod-severe	severe	
19	DCD	nil	nil	moderate	mild	nil	moderate	minimal	
20	DCD	nil	mild	severe	moderate	moderate	minimal	mild-mod#	Moderate siderosis
21	DCD	mild	moderate	mild	mild	mild	mild	severe	
22	DBD	nil	nil	mild	nil	nil	minimal	not applicable	Not transplanted because malignancy found in donor, perfusion stopped at 4 hours.
23	DCD	mild	moderate	mild	nil	moderate	mild	severe	
24	DBD	severe	severe	moderate	severe	severe	minimal	not applicable	
25	DBD	mild	severe	mild	nil	moderate	mild	not applicable	Steatohepatitis + mild fibrosis
26	DBD	nil	severe	moderate	severe	severe	mild	not applicable	
27	DBD	moderate	moderate	mild	severe	moderate	mild	not applicable	Steatohepatitis + mild/moderate fibrosis
28	DCD	nil	nil	moderate	nil	mild	moderate	severe#	
29	DBD	nil	severe	severe	severe	moderate	mild	mod-severe	Mild fibrosis, Mild siderosis
30	DBD	nil	mild	severe	severe	mild	moderate	mild-moderate	Minimal siderosis
31	DBD	mild	moderate	mild	mild	mild	nil	no biopsy taken	Minimal siderosis

Abbreviations: NMP, normothermic machine perfusion; DCD, donation after circulatory death; DBD, donation after brainstem death

Note: Large droplet fat is defined as macrovesicular steatosis with a single large fat droplet within the hepatocyte cytoplasm displacing the nucleus. Small and medium droplet macrovesicular steatosis is defined as fat droplets, usually multiple, within the cytoplasm of the hepatocyte which do not displace the nucleus. Both types of steatosis and glycogen depletion are graded by the % of hepatocytes containing fat droplets or not containing glycogen respectively based on thirds, with up to 5% being considered negative i.e. none: 0-5%; mild: 5-33%; moderate: 34-67%; severe: >67%. Injury to extrahepatic bile ducts was determined by grading injury to deep peribiliary glands (0-3), stromal nuclear loss (0-3) and loss of nuclei in the media of arteries/arterioles (0-3), the extent of haemorrhage (0-4) and presence of thrombi (Y/N) (op den Dries et al 2014 and Hansen et al 2012). An overall bile duct injury grade was assigned based on the severity/presence of each e.g. minimal = mild of one or two features, severe = moderate to severe in all etc. Rows with gray background designates transplanted livers. *Designates livers that developed anastomotic biliary strictures. #Designates livers that developed symptomatic non-anastomotic biliary strictures.

Supplementary Table S6.3 Post-transplant recovery and follow up

Liver Number	Donor type	Total preservation time (Hours:Minutes)	Post-reperfusion syndrome	Early allograft dysfunction	Peak ALT /AST	Renal replacement therapy (days)	Clavien-Dindo complication grade	ITU stay	In hospital stay	Anastomotic / Non-anastomotic biliary strictures	Graft survival up to the last follow up (days)	Patient survival up to the last follow up (days)	Re-Tx
1	DBD	18:55	No	Yes	1176/3165	No	2	2	8	No/No	784	784	No
2	DBD	17:05	No	No	507/322	No	2	4	8	No/No	647	647	No
3	DBD	21:07	No	No	230/247	No	2	2	7	Yes/No	641	641	No
4	DBD	20:05	No	Yes	688/641	No	4	4	30	No/Yes*	225	708	Yes
5	DBD	23:50	No	No	614/1038	No	1	2	11	No/No	656	656	No
6	DBD	24:13	No	No	289/215	No	2	6	8	No/No	472	472	No
7	DCD	17:03	Yes	Yes	921/2510	No	3	3	7	Yes/No	634	634	No
8	DBD	17:09	No	No	824/1095	No	2	4	10	No/No	456	456	No
9	DCD	25:32	Yes	Yes	306/216	No	2	6	17	No/No	620	620	No
10	DCD	17:29	Yes	Yes	2339/3612	Yes (3)	4	3	19	No/Yes	375	650	Yes
11	DCD	18:17	Yes	No	529/716	No	2	2	10	No/No	611	611	No
12	DBD	21:48	No	Yes	477/1543	No	2	4	13	No/No	558	558	No
13	DBD	17:27	No	No	166/240	No	2	4	10	No/Yes^	517	517	No
14	DCD	14:38	Yes	No	594/331	No	2	3	11	No/Yes^	513	513	No
15	DCD	16:17	No	No	392/495	No	2	3	9	No/No	561	561	No
16	DCD	11:21	Yes	No	57/166	Yes (37)	4	38	47	No/Yes	120	509	Yes
17	DCD	13:59	Yes	No	394/677	No	2	3	10	No/Yes^	525	525	No
18	DCD	24:00	Yes	No	255/423	Yes (29)	4	18	32	No/Yes^	442	442	No
19	DCD	14:34	No	No	338/327	No	3	3	17	No/Yes	417	417	Yes
20	DBD	15:03	Yes	Yes	2074/2836	No	2	3	14	No/No	390	390	No
21	DBD	23:46	Yes	No	273/626	Yes (3)	4	7	10	No/No	403	403	No
22	DBD	18:53	No	No	827/1385	No	2	4	6	No/No	423	423	No

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; DBD, donor following brainstem death; DCD, donor following circulatory death; ITU, intensive treatment unit; Re-Tx, re-transplant required.

Note: *Designates patient with hepatic artery thrombosis requiring early revascularisation. ^Designates asymptomatic non-anastomotic biliary strictures without cholestasis.

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PART III – MANIPULATION OF PERFUSATE AND THE USE OF NORMOTHERMIC MACHINE PERFUSION TO DELIVER THERAPEUTICS

CHAPTER 7 - THE USE OF AN ACELLULAR OXYGEN CARRIER IN NMP-L

7.1 THE USE OF AN ACELLULAR OXYGEN CARRIER IN A HUMAN LIVER MODEL OF NORMOTHERMIC MACHINE PERFUSION

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Authors: Laing RW[*1,2], Bhogal RH*[1,2], Wallace LL[2], Boteon YL[1,2], Neil DAH[1], Smith A[1], Stephenson BTF[2], Schlegel A1, Hübscher SG[1], Mirza DF[1,2], Afford SC[*2] and Mergental H[*1,2].

Affiliations:

[1] Liver Unit, Queen Elizabeth Hospital, University Hospital Birmingham NHS Foundation Trust, Birmingham, United Kingdom.

[2] National Institute for Health Research, Birmingham Liver Biomedical Research Unit and Centre for Liver Research, Institute of Immunology and Immunotherapy, Institute for Biomedical Research, College of Medical and Dental Sciences, University of Birmingham, United Kingdom.

7.7.1 Abstract

Background

Normothermic machine perfusion of the liver (NMP-L) is a novel technique that preserves liver grafts under near-physiological conditions whilst maintaining their normal metabolic activity. This process requires an adequate oxygen supply, typically delivered by packed red blood cells (RBC). We present the first experience using an acellular haemoglobin-based oxygen carrier (HBOC) Hemopure in a human model of NMP-L.

Methods

Five discarded high-risk human livers were perfused with HBOC-based perfusion fluid and matched to 5 RBC-perfused livers. Perfusion parameters, oxygen extraction, metabolic activity and histological features were compared during 6 hours of NMP-L. The cytotoxicity of Hemopure was also tested on human hepatic primary cell line cultures using an in-vitro model of ischemia reperfusion injury.

Results

The vascular flow parameters and the perfusate lactate clearance were similar in both groups. The HBOC-perfused livers extracted more oxygen than those perfused with RBCs (O_2ER 13.75 vs 9.43 % x10^5 per gram of tissue, p=0.001). *In vitro* exposure to Hemopure did not alter intracellular levels of reactive oxygen species and there was no increase in apoptosis or necrosis observed in any of the tested cell lines. Histological findings were comparable between groups. There was no evidence of histological damage caused by Hemopure.

Conclusion

Hemopure can be used as an alternative oxygen carrier to packed red cells in NMP-L perfusion fluid.

7.7.2 Introduction

The rising incidence of chronic liver disease has resulted in increased demand for liver transplantation(1, 2). This can be met by the progressive utilization of high-risk organs from extended criteria donors. The quality of these livers is already compromised at the time of organ recovery, and deteriorates further during static cold storage (SCS) thereby increasing the risk of early graft dysfunction and/or primary non-function(3). Machine perfusion is a novel technology that can minimize preservation-associated liver injury and several groups have already reported promising results from pilot series of patients transplanted with machine perfused grafts(4-7). Oxygen requirements during hypothermic or sub-normothermic machine perfusion are relatively low due to reduced liver metabolic activity and these can be met by supplying a high fraction of inspired oxygen (FiO2) dissolved in the perfusion fluid(8).

To date, all clinical transplant series using organs preserved by normothermic machine perfusion of the liver (NMP-L) have used red blood cells (RBC) as oxygen carriers(9-14). Whilst blood-based perfusion fluid is physiological, it has also several potential disadvantages including immune-mediated phenomena, blood-borne infectious transmission, RBC haemolysis, use of a precious resource and logistical difficulties associated with using cross-matched blood(15-18).

Acellular oxygen carriers have been developed and tested as an alternative to packed red cell transfusions(19, 20). Hemopure (haemoglobin glutamer-250 [bovine]; HBOC-201, Haemoglobin Oxygen Therapeutics LLC, Cambridge, MA) is a polymerized bovine haemoglobin-based oxygen carrier (HBOC) of low immunogenicity and an oxygen carrying capacity similar to that of human haemoglobin at normothermic temperatures(20, 21). Fontes

et al recently reported successful sub-normothermic machine perfusion of the liver using Hemopure in combination with a colloid in a porcine liver transplant model(22).

Here we present the first experience using an acellular HBOC-based perfusion fluid in human livers during normothermic machine perfusion.

7.7.3 Materials and methods

7.7.3.1 Study design

The study was performed on 10 rejected donor livers offered to our centre for research between August 2014 and July 2016. Five organs were perfused with a Hemopure-based perfusion fluid (HBOC group) and 5 with a packed red blood cell-based fluid (RBC group) and underwent 6 hours of NMP-L. The HBOC and RBC livers were matched according to type of organ donation (donor after brain death or circulatory death) and function based on the unit's developed viability testing protocol. All 3 viable RBC livers were successfully transplanted(14). Ethical approval for the study was granted by the London-Surrey Borders National Research Ethics Service committee as well as Loco-Regional and NHSBT Ethics Committees (reference 13/LO/1928 and 06/Q702/61). The tissue used for the cellular isolation and *in vitro* toxicity experiments was obtained from fully consenting adult patients undergoing hepatic explant or resection at the University Hospital Birmingham.

7.7.3.2 Normothermic machine perfusion of the liver

Normothermic machine perfusion was performed using the Liver Assist device (Organ Assist, Groningen, The Netherlands) which perfuses both hepatic arterial and portal venous systems as described previously(14).

7.7.3.3 Hemopure

This bovine haemoglobin product is processed to eliminate RBC constituents, bacterial endotoxins, viruses and the prions responsible for variant Creutzfeldt-Jakob disease and bovine spongiform encephalopathy. The result is a sterile glutaraldehyde-polymerized bovine haemoglobin (30-35g Hb per 250 mL) which is added to a modified Ringer's lactate solution. Hemopure has an average molecular weight of 250 kDa and can be stored at 2 - 30°C for up to

3 years(23).

7.7.3.4 Perfusion fluid constitution

We used a perfusion fluid developed by our team for resuscitation of discarded livers(24). This consisted of 3 units of group-specific Rhesus-negative donor packed RBCs obtained from the local blood bank, or an equivalent volume of Hemopure. The remaining perfusion fluid constituents are detailed in Table 7.1 and the biochemical starting compositions of the fluids are shown in Table 7.2. Details of exact fluid constituents can be found in the Appendix as supplementary material.

Table 7.1 Perfusion fluid constitution

Oxygen carrier	
Packed red blood cells *or* Hemopure	3 units 4 bags (same volume as 3 units RBC)
Drug	**Amount** *(Initial fluid added to circuit)*
Human albumin solution 5%	1000 ml
Heparin	10,000 IU[1]
Sodium bicarbonate 8.4%	30 ml[2]
Calcium gluconate 10%	10 ml
Vancomycin	500 mg
Gentamicin	60 mg
	Continuous infusions
Epoprostenol	2 µg/ml, commenced at 4 ml/hour and titrated as necessary
	Intermittent drug administration
Aminoplasmal 10%[3]	50 ml bolus every 6 hours
Dextrose 10%	Infusion as necessary according to perfusate glucose concentration

Note: [1]bolus repeated every 3 hours; [2]bolus 10-30ml administrated if perfusate pH<7.00; [3]with added vitamins (Cernevit and Vitamin K)

Table 7.2 Comparison of biochemical composition of RBC-based and HBOC-based perfusion fluids prior to the start of perfusion

	RBC-Based Perfusate	**HBOC-Based Perfusate**	**p-value**
pH	7.379 (7.256-7.458)	7.685 (7.463-7.785)	**0.008**
PCO2 (kPa)	4.31 (0.86-7.78)	0.81 (0.74-2.23)	0.087
PO2 (kPa)	28.26 (24.60-30.36)	56.43 (45.11-64.07)	**0.008**
BE (mmol/L)	-13.7 (-19.1- -1.7)	-10.7 (-12.1- -9.7)	0.691
CHCO3- (mmol/L)	17.3 (3.7-40.4)	8.1 (7.1-11.7)	0.691
Na+ (mmol/L)	138.6 (116.8-157.2)	150.9 (148.3-153.6)	0.206
K+ (mmol/L)	8.79 (6.31-12.90)	1.90 (1.80-1.90)	**0.008**
Cl- (mmol/L)	112.4 (76.0-117.0)	109.0 (108.4-113.4)	**0.008**
Ca2+ (mmol/L)	0.637 (0.573-0.700)	1.000 (0.900-1.000)	0.786
tHb (g/L)	84.4 (75.5-100.2)	57.3 (55.8-58.5) [1]	**0.008**
O2Hb (%)	97.8 (94.8-98.0)	81.1 (79.3-82.9)	**0.008**
COHb (%)	1.0 (0.9-1.9)	0.1 (0.1-0.1)	**0.008**
H.Hb (%)	0.7 (0.6-3.6)	17.6 (16.8-18.6)	**0.008**
MetHb (%)	0.5 (0.4-0.6)	1.8 (1.6-2.0)	**0.008**
SO2 (%)	99.3 (96.4-99.4)	82.0 (81.0-82.9)	**0.008**
Hct(c) (%)	22.6 (16.9-30.1)	17.2 (16.8-17.5)	0.079
Glu (mmol/L)	8.0 (6.8-10.5)	3.5 (3.5-5.6)	**0.008**
Lactate (mmol/L)	7.7 (6.7-9.2)	5.4 (5.3-5.8)	**0.008**

[1]After 6 hours the median (range) value of Hb (g/L) was 59.3 (49.6-64.1)

7.7.3.5 Assessment of liver physiology and sample collection protocol

The macroscopic appearance of the liver was assessed throughout the course of NMP-L. The perfusion and sampling protocol included recording of arterial and venous circuit flow rates (ml/min for hepatic artery, L/min for portal vein), pressure (mmHg), resistance (mmHg·min/L) and temperature (˚C) at 30-minute intervals. At the same intervals, we sampled arterial and hepatic venous perfusion fluid that was immediately assessed using a Cobas b 221 point of care system (Roche Diagnostics, USA). Determination of organ viability was as per our criteria for organ viability used in a pilot series of transplantation using discarded donor livers(6) (see supplementary material for more information).

7.7.3.6 Histological Assessment

Liver biopsies were taken prior to the start of NMP-L and after 6 hours of perfusion. Biopsies were assessed for pre-existing acute or chronic liver injury. The percentages of large and small droplet macrovesicular steatosis, coagulative necrosis, subtle zone 3 changes of detachment of hepatocyte plates from the sinusoidal endothelium and glycogen depletion was determined(25).

7.7.3.7 Perfusate and tissue analysis

Perfusates and tissues were snap-frozen at different time points for subsequent analyses. This included analysis of tissue adenosine triphosphate (ATP) content, and analysis of the perfusate for levels of transaminases and 8-hydroxy-2'-deoxyguanosine (8-OH-dG) – an established marker of oxidative stress. All perfusates underwent haemoglobin depletion using Hemoglobind (BioTech Support Group LLC, Monmouth Junction, NJ) as per the manufacturer's instructions, except using a 1:8 ratio to ensure removal of all free haemoglobin.

7.7.3.8 Primary human hepatocyte, human sinusoidal endothelial cell and human biliary epithelial cell isolation.

The isolation of primary human hepatocytes(26), sinusoidal endothelial cells(27) and biliary endothelial cells(28) has been previously described, the detailed protocols for which are supplied In the Appendix as online supplementary material.

7.7.3.9 In vitro model of ischemia reperfusion injury

Cells were incubated in the standard media for each cell type or 50:50 mix of standard media with Hemopure (the same concentration as is present in the perfusion fluid). In experiments, human hepatocytes, HSEC and BEC were grown for 3 days in standard media, in 6-well plates coated with rat type 1 collagen, at 37°C in 5% CO_2. We utilized a model of warm *in vitro* ischemia reperfusion injury (IRI) that we have described previously(29), the details of which are within the supplementary material.

7.7.3.10 Assessment of reactive oxygen species production, apoptosis and necrosis

Reactive oxygen species (ROS) production, apoptosis and necrosis were determined using a three-color assay as previously described(30). All data are expressed as Median Fluorescence Intensity (MFI). Taken together these 3 markers give a comprehensive assessment of the magnitude of IRI in primary human liver cells.

7.7.3.11 Statistical analysis

Categorical data is presented as numbers and percentage and were compared with Fischer's exact test. Continuous variables are expressed as mean and standard deviation or median with range (where appropriate) and were compared using t-tests or two-tailed Mann-Whitney U test. A p-value of <0.05 was deemed significant and was rounded to three decimal places for the

presentation of results. All statistical analyses were performed using Prism 6 for Mac software (Graphpad Software Inc, La Jolla, CA, USA).

7.7.4 Results

7.7.4.1 Donor characteristics

The majority of livers (8 out of 10) were from donors after circulatory death (DCD). The median (range) donor age was 48 (25 - 70) years, the donor body mass index 26 (21 – 45) kg/m^2 and the liver weight 1998 (1555-2486) grams. The median static cold storage time was 450 (380 – 754) minutes. The mean donor risk index for the RBC and HBOC groups were 2.21 and 2.36 respectively(31). The most common reason for the organ being declined for transplantation was prolonged donor warm ischemic time in combination with suboptimal macroscopic liver appearance. Examples of these livers can be seen in Figure 7.1 and the detailed characteristics are provided in Table 7.3.

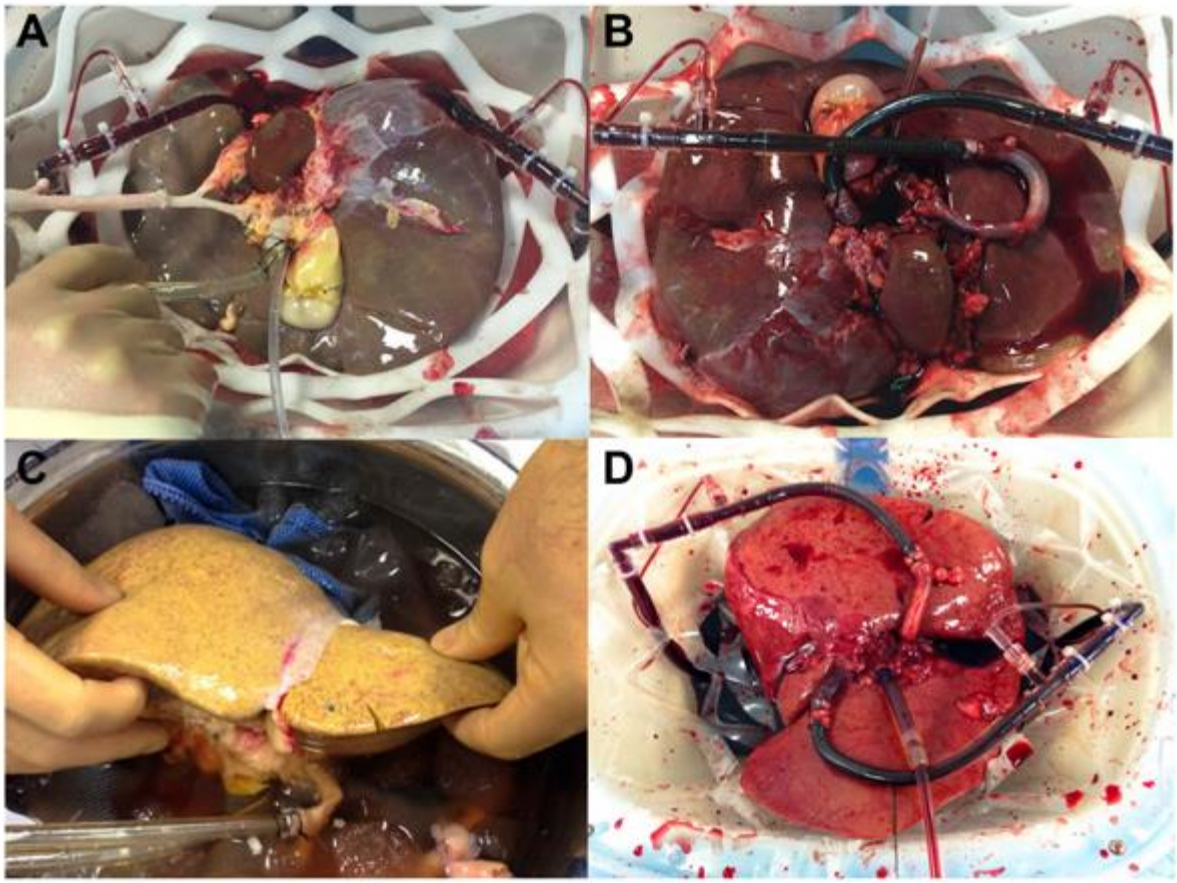


Figure 7.1 Macroscopic liver appearance

Hemopure perfused liver #5 before (A) and 1 minute after (B) commencing the perfusion. This liver was poorly perfused in situ and on the back table during the retrieval process, however performed very well and a homogenous perfusion was achieved almost immediately, helped by the low viscosity of the fluid. Hemopure perfused liver #1 before (C) and 5 minutes after (D) commencing the perfusion. Despite the severely steatotic nature of the graft, a homogenous perfusion was still achieved shortly after almost 7 hours of cold storage.

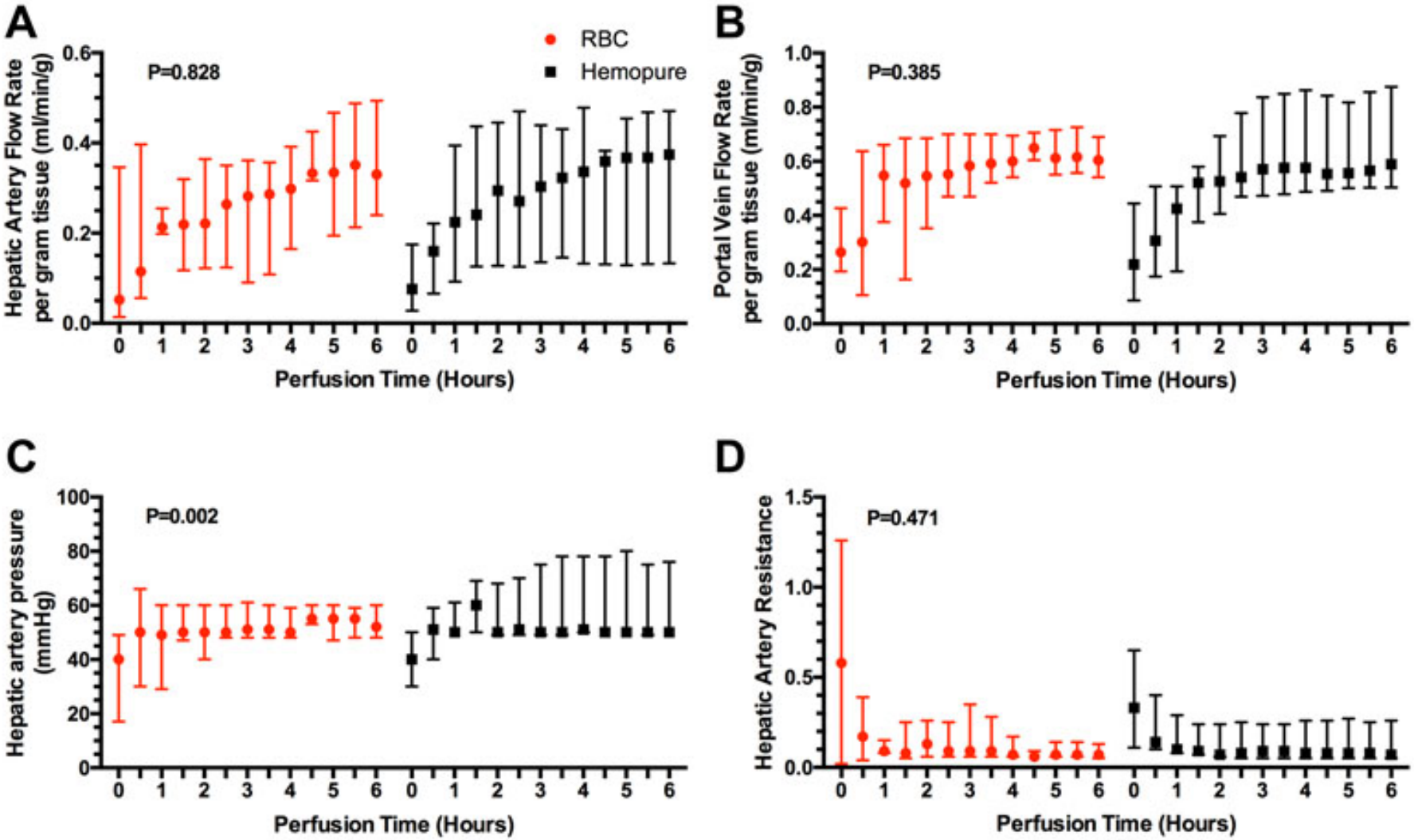
Table 7.3 Donor demographic, liver characteristics and machine perfusion data

	RBC 1	RBC 2	RBC 3	RBC 4	RBC 5	HBOC 1	HBOC 2	HBOC 3	HBOC 4	HBOC 5
Donor information										
Donor type	DBD	DCD	DCD	DCD	DCD	DCD	DCD	DBD	DCD	DCD
Age	46	49	60	46	30	70	35	50	25	60
Sex	male	female	female	male	male	male	male	male	male	male
BMI (kg/m^2)	23	45	36	28	25	29	21	25	26	21
Blood Group	O+	O+	A+	O+	A+	A+	A+	O+	O+	A+
Cause of death	ICH	HBI	HBI	HBI	HBI	ICH	ICH	ICH	Trauma	Trauma
Reason for rejection	Length of ITU stay	WIT and donor history	Steatosis	WIT and appearance	100 minutes agonal period	Steatosis	Poor in-situ perfusion	Donor history of malignancy	Segment VII laceration and patchy perfusion	Donor malignancy (renal)
Liver Characteristics										
Liver weight	1961	1943	1712	2486	1997	2208	2218	2380	1998	1555
Cold ischemic time	380	406	491	453	445	400	453	754	612	446
Donor WIT	-	36	32	31	12	24	20	-	22	21
Donor risk index	1.41	2.86	2.77	2.25	1.76	3.20	2.47	1.58	2.20	2.26
Machine perfusion parameters										
Lactate (mmol/L)										
Highest	>20.0	5.5	13.3	12.4	13.3	9.6	>20.0	10.3	10.4	9.0
Lowest	4.4	1.4	9.0	1.2	0.8	3.8	8.8	0.3	1.2	0.6
Last	>20.0	1.4	9.0	1.2	0.8	5.2	>20.0	0.3	1.6	1.4
Total Bile production (g)	2.6	0	0	11.3	27	0	0	17.6	26.2	24
Mean Arterial flow (mL/min)	277	476	582	654	558	535	256	761	529	616
Mean Portal vein flow (L/min)	1188	926	1112	1015	963	865	1237	1505	1286	1021
Mean Arterial flow per gram liver tissue (mL/min/g)	0.14	0.24	0.34	0.26	0.28	0.24	0.12	0.32	0.26	0.40
Mean O2 ER per gram liver tissue ($x10^5$)	14.57	9.98	8.47	3.86	12.90	11.64	19.80	8.49	16.31	12.02

Abbreviations:
DBD, donation after brain death; DCD, donation after circulatory death; ER, Extraction ratio; HBI, hypoxic brain injury; ICH, intracranial hemorrhage; ITU, intensive treatment unit; WIT, warm ischemic time

7.7.4.2 Machine perfusion parameters

The HBOC group livers established global perfusion rapidly and the liver surface appeared homogenous within the first five minutes. This observation was reflected in the lower initial hepatic arterial resistance and pressure required to achieve the target flow rates within the initial 30 minutes of perfusion (resistance 0.26mmHg·min/L (range 0.20-0.32) in HBOC group versus 0.39mmHg·min/L (0.22-0.56), p=0.667; Figure 7.2 and Table 7.4).


A
RBC
Hemopure
P=0.828
Hepatic Artery Flow Rate per gram tissue (ml/min/g)
Perfusion Time (Hours)
B
P=0.385
Portal Vein Flow Rate per gram tissue (ml/min/g)
Perfusion Time (Hours)
C
P=0.002
Hepatic artery pressure (mmHg)
Perfusion Time (Hours)
D
P=0.471
Hepatic Artery Resistance
Perfusion Time (Hours)

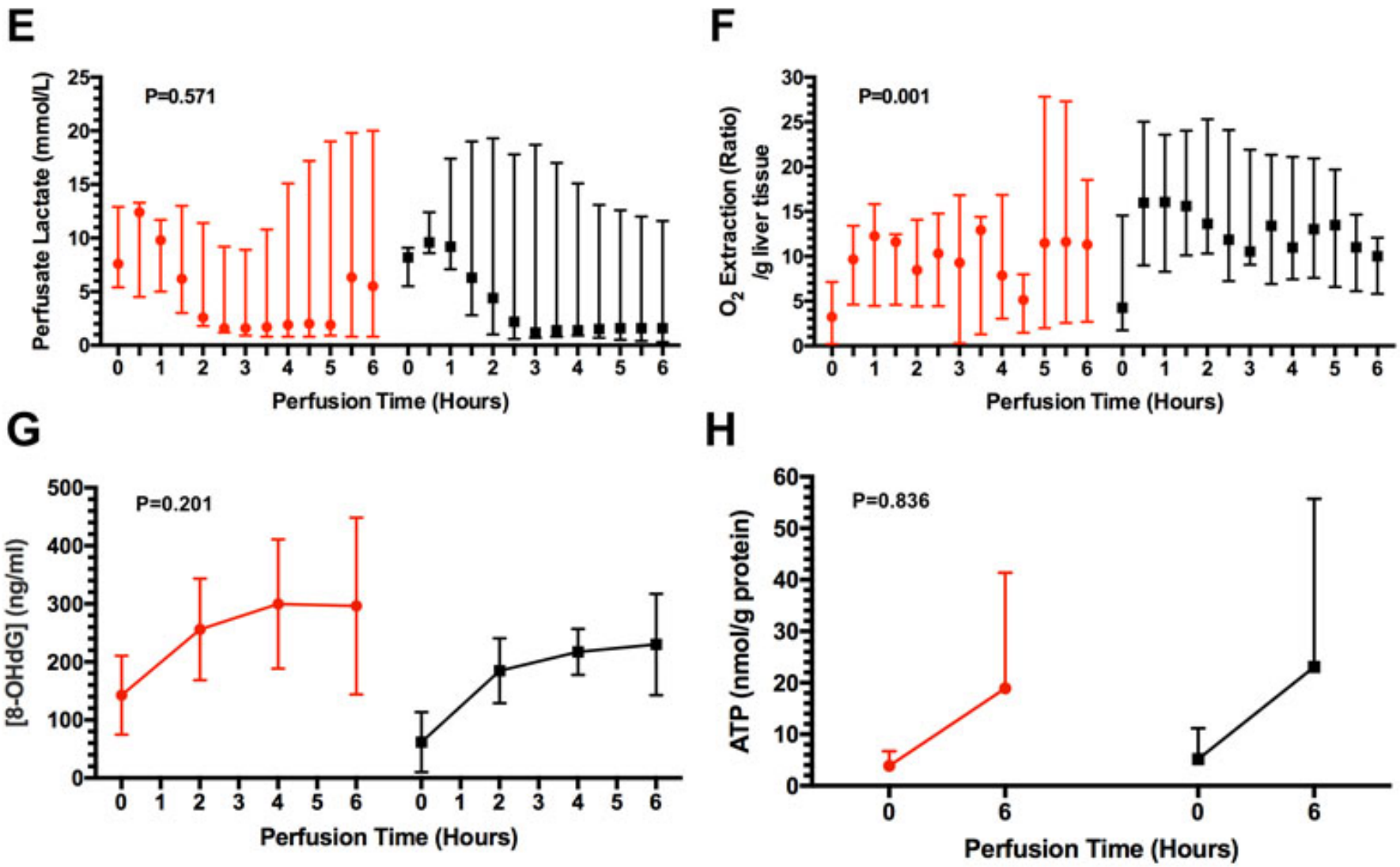

E
Perfusate Lactate (mmol/L)
P=0.571
Perfusion Time (Hours)
F
O2 Extraction (Ratio) /g liver tissue
P=0.001
Perfusion Time (Hours)
G
[8-OHdG] (ng/ml)
P=0.201
Perfusion Time (Hours)
H
ATP (nmol/g protein)
P=0.836
Perfusion Time (Hours)

Figure 7.2 Perfusion parameters of Hemopure perfused grafts.

Hepatic artery flow rates (A) and portal vein flow rates (B) in Hemopure and RBC perfused livers. Hepatic artery pressure (C) and resistance (D) showed slight differences in the pressure settings used, however the resistances over the course of the perfusion were similar. The resistance in cold livers were observably lower (within first 30 minutes as liver warmed) in the Hemopure group, likely due to the low viscosity of the fluid. There were no differences in lactate metabolism (E), 8-OH-2-dG production (G) or ATP replenishment (H). O2ER (F) was increased in livers perfused with Hemopure.

Table 7.4 Perfusion parameters of both perfused groups with associated p-values

	RBC	**HBOC**	**P-Value**
HA Pressure (mmHg)	53.0 (36.5-56.0)	56.6 (41.8-58.2)	**0.002**
HA Resistance T0	0.39 (0.22-0.56)	0.26 (0.20-0.32)	0.667
HA Resistance T0-6.0	0.12 (0.07-0.56)	0.11 (0.10-0.32)	0.471
HA Flow/gram (mL/min/g)	0.26 (0.10-0.36)	0.30 (0.09-0.32)	0.828
PV Flow/gram (mL/min/g)	0.59 (0.29-0.65)	0.63 (0.23-0.67)	0.385
Haemoglobin (g/dL)	73.80 (70.54-85.80)	56.24 (52.80-61.40)	**<0.001**
O_2ER/gram x 10^5	9.43 (3.45-13.67)	13.75 (5.53-17.40)	**0.001**
Lactate level			
Highest	13.3 (5.4-20.0)	10.1 (8.6-19.3)	0.389
Lowest	1.4 (0.8-9.0)	1.2 (0.3-9.1)	0.524
Last	1.5 (0.8-20.0)	1.6 (0.3-11.6)	0.889
Tissue ATP content (nmol/g protein)			0.836
T0 (pre-perfusion)	3.9 (2.9)	5.2 (6.0)	
T6 (post-perfusion)	18.9 (22.5)	23.1 (32.6)	

Abbreviations:
ATP, adenosine triphosphate; HA, Hepatic Artery; HBOC, Hemopure perfusion group; O_2ER, Oxygen extraction ratio; PV, Portal Vein; RBC, Red blood cell perfusion group

Note:
T0 designates the median resistance immediately after perfusion was commenced; T0-6.0 designates the median resistance over the course of the perfusion.

7.7.4.3 Liver viability and oxygen consumption

HBOC perfusion fluid provided sufficient oxygen delivery for livers to perform metabolic functions that indicate their viability (Figure 8.2). Active liver metabolism was also confirmed by the progressive storage of glycogen in hepatocytes (Figure 7.3). There was progressive regeneration of ATP stores over the course of the perfusion and there were no differences between the RBC and HBOC groups (Figure 7.2 panel H). There was an increase in perfusate levels of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) – an established marker of oxidative stress, although this appeared to plateau after the first 2 hours of perfusion and again, there were no differences between the RBC and HBOC perfused groups (Figure 7.2 panel G). There was a significantly higher oxygen extraction observed in the HBOC group compared to the RBC group and this difference was apparent throughout the course of the perfusion (Figure 7.2 panel F).

7.7.4.4 Histological assessment

The viable livers in the Hemopure group had a similar histological appearance to those perfused with packed RBC (not shown) with the majority of hepatocytes showing normal morphology with an intact hepatocyte plate/sinusoidal lining (Figure 7.3.A-D). Following perfusion with Hemopure the vasculature appeared to contain a pink-staining solution (7.3.E) which was not present following RBC-based perfusions and which appeared to be flushed out effectively with 2L 10% dextrose at the end of the perfusion process (Figure 7.3.F). Extrahepatic bile ducts perfused with Hemopure maintained normal morphology (Figure 7.3.C) with a largely intact surface epithelium, viable epithelial lining of the deep peribiliary glands and no loss of stromal nuclei, arterial medial nuclei or evidence of thrombosis. Within both groups, the livers deemed viable (based on perfusion characteristics) demonstrated an increase in glycogen storage (Figure 8.4) or maintained high glycogen stores during perfusion, whilst those which were

deemed non-viable failed to restore glycogen reserves. PAS stain was unaffected by the presence of Hemopure. Importantly, there was no histological evidence of damage caused by Hemopure infusion and livers that were viable according to our criteria, had similar histological features in both RBC and HBOC-infused groups. Although we do not use the scoring system at our centre, when we compared the two groups histologically using our own system or Suzuki's criteria for IRI(32), there were no observable differences.

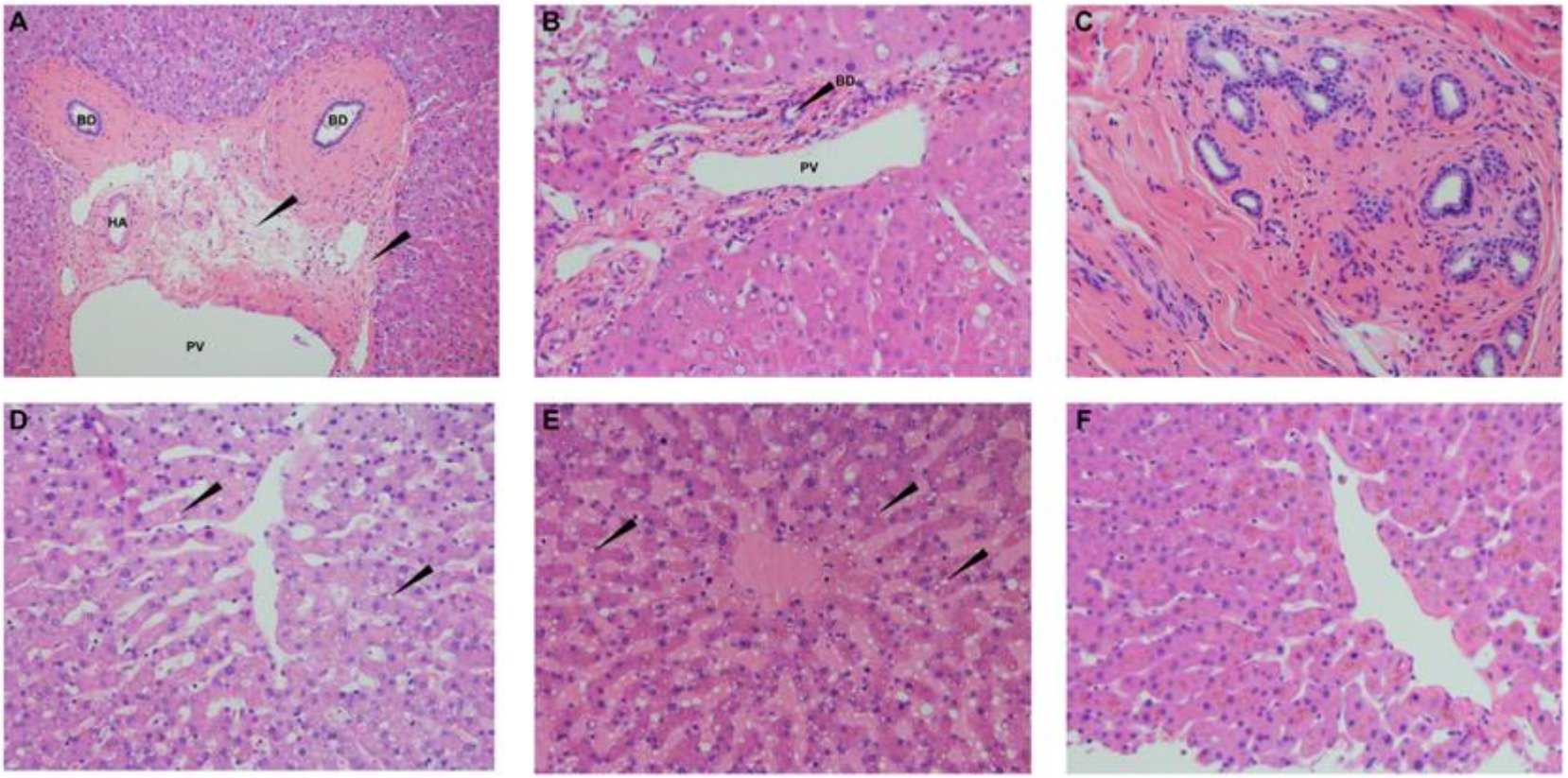


Figure 7.3 H&E sections of Hemopure-perfused livers.

A: H&E stained section of part of a large portal tract following 6 hours of perfusion showing normal bile ducts (BD), artery (HA) and portal vein (PV). There is some portal oedema present (black arrow) (objective x10). **B**: H&E stained section showing an intra-parenchymal portal tract with normal bile duct, artery and vein (objective x20). **C**: H&E stained section of extrahepatic bile duct following 6 hours of perfusion demonstrating normal architecture of the epithelium within the deep peri-biliary plexus (objective x20). **D**: H&E stained section prior to perfusion showing small droplet steatosis (black arrows) with empty sinusoids (objective x20). **E**: H&E stained section following 6 hours of

perfusion showing a similar degree of small droplet steatosis of hepatocytes (black arrows). The Hemopure fluid fills the sinusoids and central vein and stains pink (objective x20). **F**: H&E stained section following 6 hours of perfusion and flushing with 2L 10% dextrose showing the Hemopure has been flushed out of the vasculature. The hepatocytes and sinusoids appear normal (objective x20).

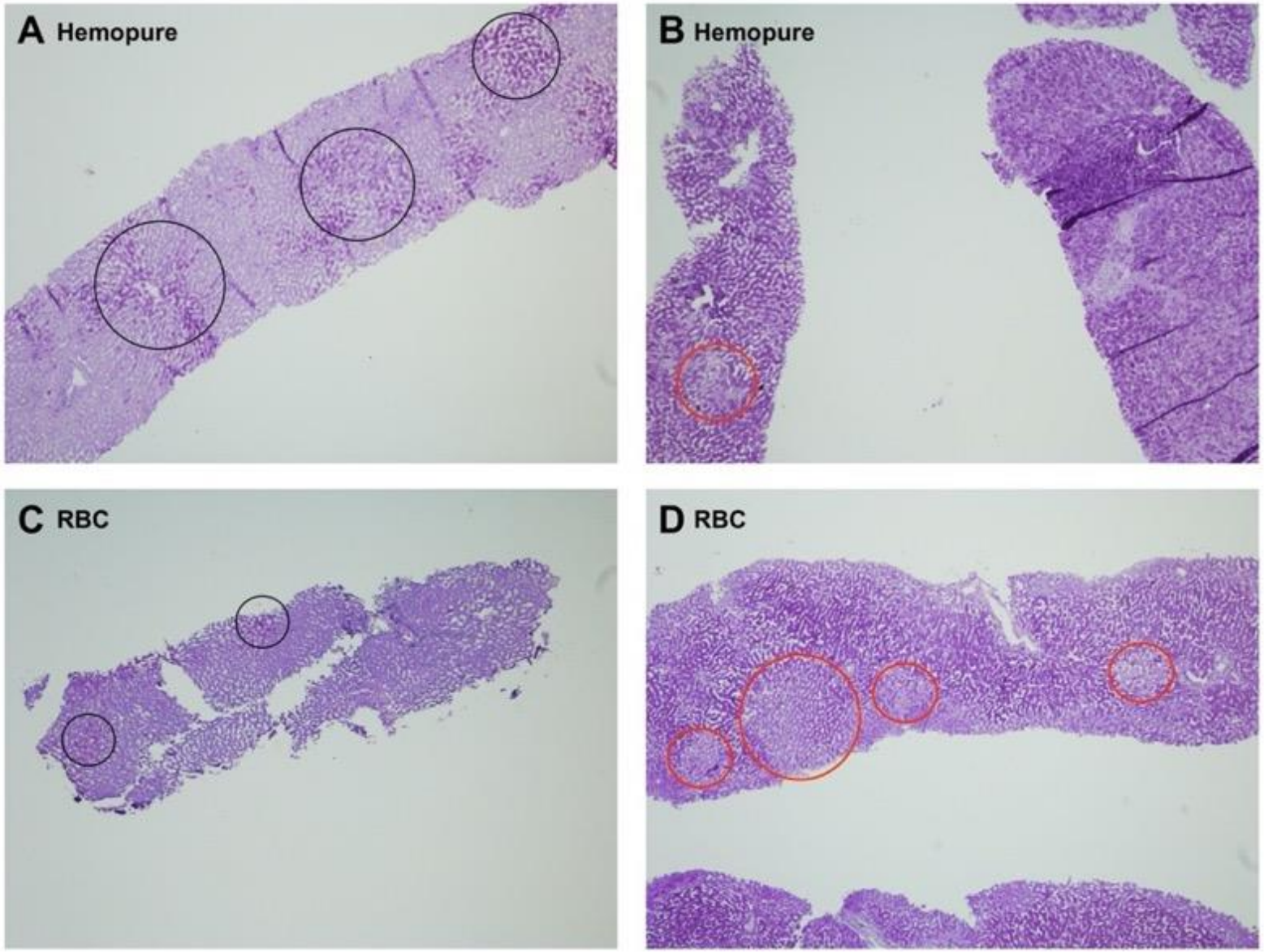


Figure 7.4 PAS sections of Hemopure and RBC-perfused livers.

A and **C**: PAS stained section of Hemopure-perfused (HBOC5) and RBC-perfused (RBC5) livers respectively, showing marked glycogen depletion prior to perfusion (60% and 80% depletion) with black circles showing scanty glycogen stores (objective x4). **B** and **D**: PAS stained section of Hemopure-perfused and RBC-perfused livers respectively, showing increased glycogen (then 15% and 15% depletion) within hepatocytes following 6 hours of perfusion with red circles showing scanty areas which lack glycogen. (objective x4).

Table 7.5 Histological features on liver biopsies

	RBC 1	RBC 2	RBC 3	RBC 4	RBC 5	HBOC 1	HBOC 2	HBOC 3	HBOC 4	HBOC 5
Designated viability	Non-viable	Viable	Non-viable	Viable	Viable	Non-viable	Non-viable	Viable	Viable	Viable
Large droplet steatosis[1] (%)	0	<1	15	<5	0	80	0	0	<1	0
Small droplet steatosis[2] (%)	0	10	40	20	5	70	<1	1	80	0
Glycogen depletion[3] (pre-NMP-L/post-NMP-L)	-	95/10	90/80	90/10	80/15	90/85	60/65	10/10-15	80/50	60/15
Detached hepatocytes[4] (%) (pre-NMP-L/post-NMP-L)	-	1 / 2	20 / 15	-	0 / 1	0/5	0/50	0/0	0/0	0/0
Coagulative Necrosis[5] (%) (pre-NMP-L/post-NMP-L)	-	0 / 5	0 / 5	0 / 30	0 / 0	0/1	0/2	0/0	0/0	0/0
Other finding	Hepatitis with severe cholestasis		Patchy congestion			Steatohepatitis	Congested, did not flush well			

Abbreviations:
HBOC, Hemopure group; NMP-L, normothermic machine perfusion – Liver ; RBC, Red blood cell group

Note: Values designated with "–" are missing; Steatosis determined in the pre-NMP-L biopsy

[1] Large droplet macrovesicular steatosis is defined as a single large fat droplet within the hepatocyte cytoplasm displacing the nucleus. Values are % of hepatocytes containing fat

[2] Small droplet macrovesicular steatosis is defined as fat droplets, usually multiple within the cytoplasm of the hepatocyte which do not displace the nucleus. Values are % of hepatocytes containing fat

[3] Glycogen depletion is graded as % of hepatocytes which do not contain glycogen.

[4] Detached hepatocytes is the % of hepatocytes which have lost cohesion from each other and from the sinusoidal lining

[5] Necrosis is depicted as the percent of total hepatocytes in the biopsy which show classical ischemic-type coagulative necrosis.

7.7.4.5 In vitro cytotoxicity testing of Hemopure

HSEC and BEC did not increase intracellular ROS production during *in vitro* IRI when cultured in standard media (Figure 7.5). Human hepatocytes demonstrated increased ROS accumulation when exposed to hypoxia that was accentuated during H-R as we have previously demonstrated(33). When human hepatocytes, HSEC or BEC were cultured in Hemopure-containing media, there was no significant increase in intracellular ROS production during normoxia, hypoxia or H-R. These results demonstrate that Hemopure does not increase ROS accumulation in isolated primary liver cells during *in vitro* IRI.

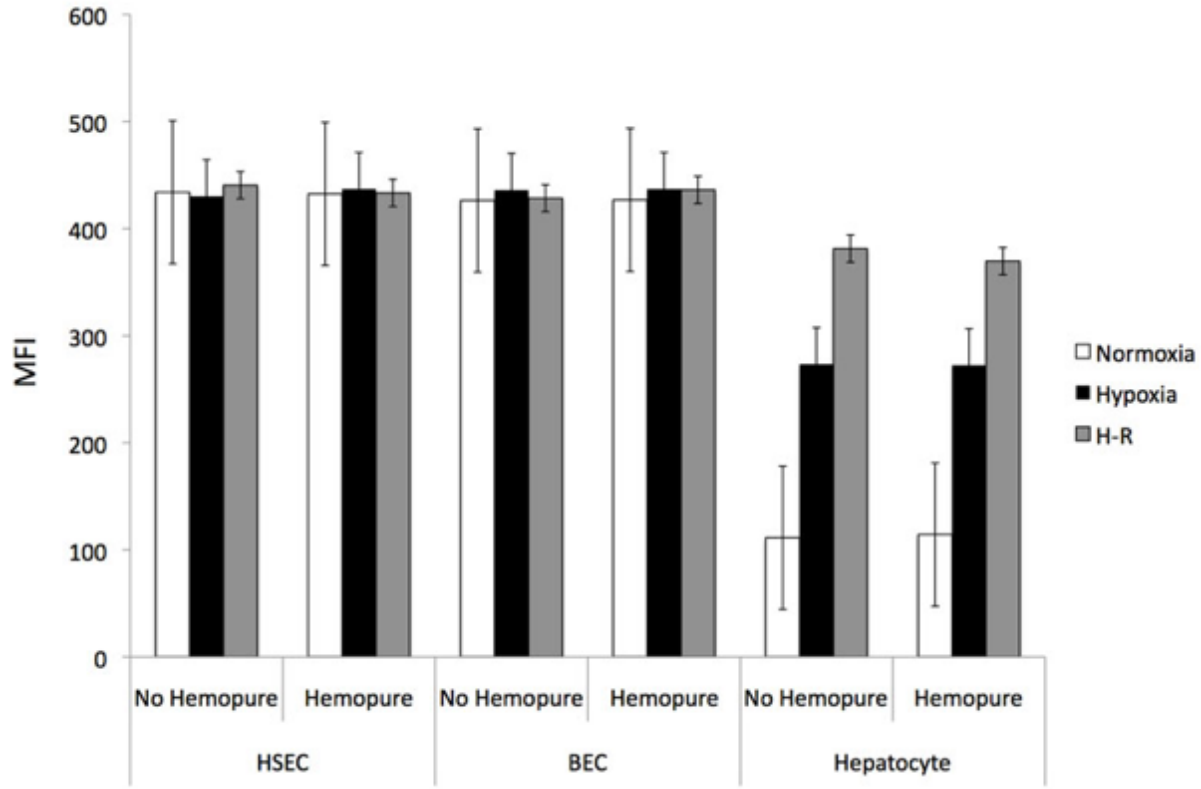


Figure 7.5 In vitro cytotoxicity testing of Hemopure Part I

Isolated human hepatocytes, HSEC and BEC were exposed to the in vitro model of IRI in the presence and absence of Hemopure and the effect upon intracellular ROS accumulation was assessed using 2',7'-dichlorofluorescin. Data are expressed as MFI and calculated as described in the Methods and Materials section (n=3-6).

Our previous work has shown that increases in intracellular ROS increase cell death in parenchymal liver cells primarily via apoptosis but also necrosis(33). As Figure 7.6 demonstrates, when human hepatocytes, HSEC or BEC were cultured in Hemopure during normoxia, hypoxia or H-R there was no increase in apoptosis relative to cells cultured in standard media. There was no increase in necrosis in human hepatocytes, HSEC or BEC during *in vitro* IRI when cultured with Hemopure (data not shown). Hemopure therefore shows no increase in cytotoxicity in primary human liver cells during IRI.

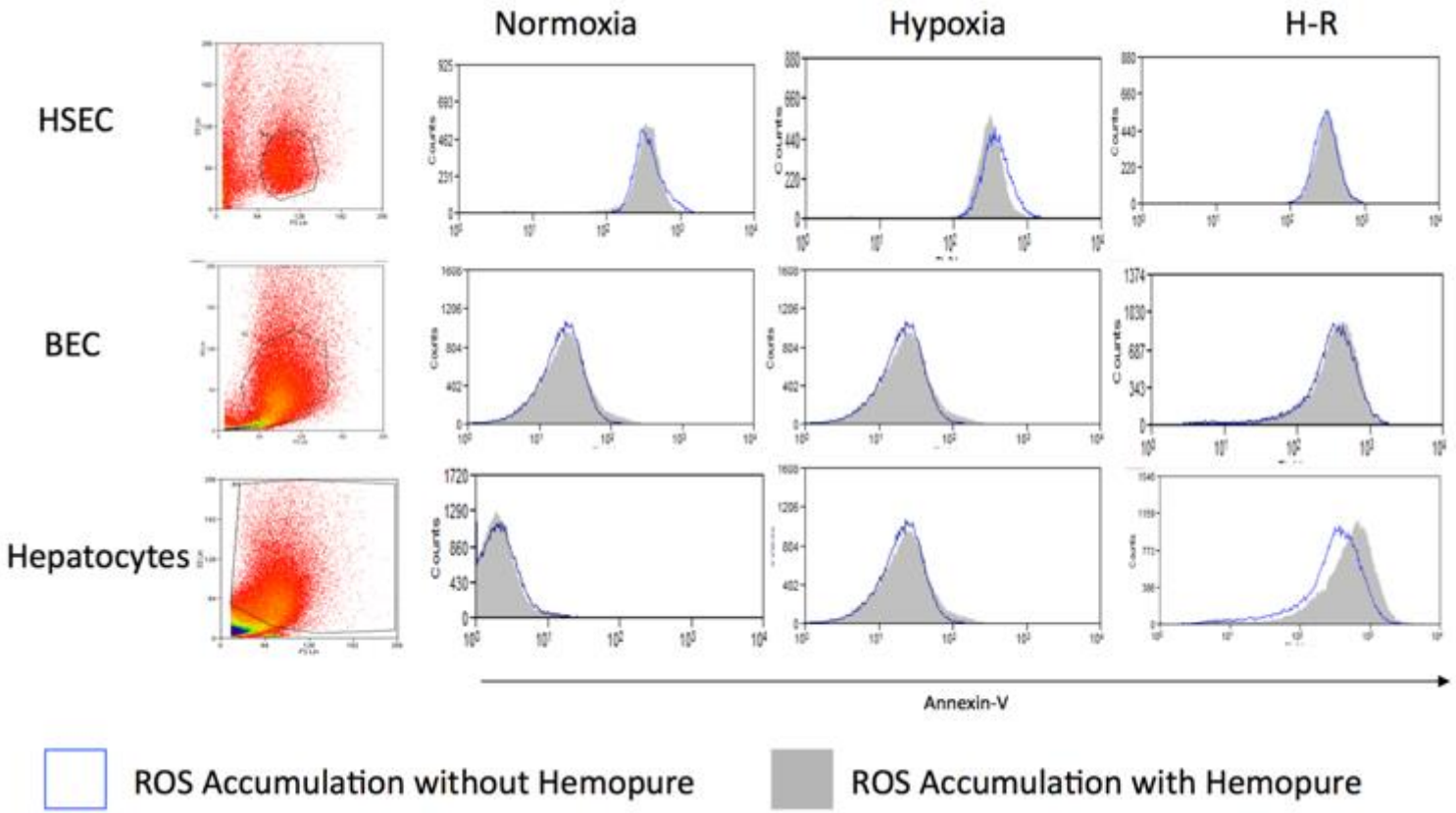


Figure 7.6 In vitro cytotoxicity testing of Hemopure Part II

The bottom panel shows representative flow cytometry plots demonstrating the effects of Hemopure on apoptosis in human hepatocytes, HSEC and BEC during hypoxia. Similar plots were obtained during normoxia and H-R (data not shown).

7.7.5 Discussion

Organ machine perfusion is becoming an increasingly attractive preservation method since experimental studies have demonstrated it mitigate IRI and potentially improve allograft function(34). In particular, data from early clinical transplant series using normothermic machine perfused grafts show promising results and provide a potential means of overcoming the critical shortage of donor organs(9, 10, 14, 35, 36). Regardless of perfusion temperature, it is generally accepted that oxygenation of the perfusate is advantageous(4). Here we show for the first time that Hemopure, an acellular oxygen carrier, has the potential to replace packed red cells as the oxygen carrier of choice in a human NMP-L model. This study was primarily designed to assess the feasibility of Hemopure to replace RBC in a model of viability testing using NMP-L.

In the present experiments, we observed increased oxygen consumption in the HBOC liver group. We believe this to be a result of the physiological and rheological properties of Hemopure. As previously described, the oxygen dissociation curve of Hemopure lies to the right of corpuscular haemoglobin (with a p50 of 40mmHg) and therefore gives up oxygen to tissues more readily[41,42]. This difference was more pronounced at the initial phase of the perfusion, prior to the liver core and the perfusate temperature reaching 37°C, during which Hb-O_2 affinity would normally be increased, giving oxygen to tissues less freely. Across all temperatures therefore, Hemopure will give up more oxygen to tissues than corpuscular haemoglobin. Additional properties such as a molecular diameter approximately 1/1000th the diameter of a red-blood cell and the fact Hemopure is a less viscous fluid, result in a more homogenous perfusion(37) and facilitate the diffusive transport of oxygen in the microcirculation improving tissue oxygenation. Low-viscosity preservation fluids may protect against the development of post-transplant biliary complications however this aspect of NMP-

L requires further research(38-40).

The apparent advantage of a lower O_2 affinity did not translate to a reduction in intracellular ROS when using Hemopure in the IRI model *in vitro* and the reasons for this remain the focus of ongoing research in our laboratory. Crucially, Hemopure did not induce cell death in primary human liver cells during IRI. One of the acknowledged protective mechanisms of NMP-L is attenuation of IRI because the organ replenishes energy stores within an environment free from recipient immune-mediated injury, thereby minimizing ROS accumulation at true reperfusion – a central trigger of allograft necro-apoptosis observed following transplantation(29). Porcine HBOC's have been shown to exhibit anti-oxidant activity *in-vitro* and significantly inhibit hydrogen peroxide-mediated endothelial cell damage and apoptosis(41). They have also been shown to have a protective effect on focal cerebral IRI in an animal model(42).

Whilst third party blood provides good results for NMP-L, there are several reasons why finding alternatives may be an important development for the clinical adoption of machine perfusion in the future. The obvious reason is to avoid any unnecessary blood usage – a scarce resource and vital for major surgical procedures or other therapeutic interventions. Complying with ethical and legislative regulations, acquiring approval for third party blood to be used in NMP-L research is a lengthy process. Using an acellular oxygen carrier would avoid this and overcome other challenges that are associated with the use of blood products such as traceability. Additionally, HBOC's do not require cross-matching and have a long shelf-life at room temperature. In our experience this prevented delays and minimized the cold ischemic time which is a key factor when attempting to utilize extended criteria donor livers. Hemopure can deliver oxygen within the wide range of the conventionally used machine perfusion temperatures (10°C to 37°C), currently being trialled in clinical and research settings(22, 43).

There has been a longstanding interest in developing an efficient and safe alternative to donor blood. Several products have been tested mainly in the pre-clinical setting with promising results although they have not been adopted into routine clinical practice(19, 44). Despite negative reports from a meta-analysis examining the use of five different HBOC products, Hemopure has demonstrated clinical efficacy in trials investigating its use in general, urological, orthopaedic, vascular and cardiac surgery, though it demonstrated some side effects, most commonly hypertension and bradycardia(20, 45-48). Liver machine perfusion with Hemopure *ex-situ* avoids the potential complexities of systemic *in-vivo* interactions and their potential side effects. Histological assessment also showed that flushing the liver at the end of NMP-L effectively removes Hemopure from the liver, so only a very small volume (if any) would reach the recipient circulation.

The main limitation of our study is being unable to assess the effect of true reperfusion during transplantation as the livers were not transplanted. We also chose not to simulate the reperfusion effect by NMP-L with whole blood containing immune cell populations. This model however, provided reassurance that Hemopure does not cause any apparent histological damage and it is able to deliver enough oxygen to fully support human liver metabolism at normothermic condition. Such confirmation was necessary prior to evaluating Hemopure in a clinical transplant setting.

In conclusion, this study suggests that Hemopure-based perfusion fluid is a feasible alternative to the blood-based solution currently used for NMP-L. Hemopure may be logistically, rheologically and immunologically superior to packed red cells when used in a normothermic

perfusion model. Our findings warrant further HBOC-based machine perfusion fluid testing in a pilot clinical trial.

7.7.6 Acknowledgments

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7.7.7 Appendix

Detailed protocols for perfusion fluid, ATP quantification, human primary liver cell isolations, in vitro assessment of reactive oxygen species production, apoptosis and necrosis in a model of ischemia-reperfusion and assessment of donor liver viability.

7.7.7.1 Perfusion fluid constitution

We used a perfusion fluid developed by our team for resuscitation of discarded livers. This consisted of 3 to 4 units of group-specific Rhesus-negative donor packed RBCs obtained from the local blood bank, or an equivalent volume of Hemopure (Haemoglobin Oxygen Therapeutics LLC, Cambridge, MA). This was supplemented with 1000mL of 5% w/v human albumin solution (Alburex 5, CSL Behring GmbH, Germany), 30mL sodium bicarbonate 8.4% (B. Braun Medical Limited, UK) and 10ml calcium gluconate 10%. The circuit was loaded with 10,000IU heparin (Wockhardt, UK), 500mg vancomycin (Wockhardt, UK) and 60mg gentamicin (Cidomycin, Sanofi, UK) prior to connecting the liver. Epoprostenol (8µg/hour; Flolan, GlaxoSmithKline, UK) was infused once perfusion had started. Further supplementation with 50mL of 10% v/v Aminoplasmal (B. Braun Medical Limited, UK), 0.2mL Cernevit (Baxter Healthcare Ltd., UK) and 0.1mg phytomenadione (Konakion, Roche Products Ltd, UK) was given during the perfusion. Extra buffering capacity was provided by aliquots of 8.4% sodium bicarbonate to maintain a pH of greater than 7.20 as necessary during the perfusion.

7.7.7.2 Tissue ATP quantification

To quantify ATP content, 100mg of frozen liver tissue was taken and immediately homogenised in 1ml SONOP Buffer (0.372g EDTA in 130ml ddH2O (adjusted to pH 10.9 with NaOH) = 370ml of 96% Ethanol) using the GentleMacs system. Particulates were removed by

centrifugation at 13,000xg. The protein concentration was determined in the supernatant with the use of a Pierce BCA Protein Assay kit and the concentration adjusted to 300ug/ml protein with the SONOP buffer. Samples were then diluted 10-fold in 100µM Phosphate buffer and ATP concentration determined was using the ATP Bioluminescent Kit (Sigma FL-AA). Concentrations were determined from a calibration curve on the same plate, corrected for amount of protein and expressed as nM/g protein.

7.7.7.3 Protocols for isolation of primary human hepatocytes, sinusoidal endothelial cells and biliary endothelial cells

Human hepatocytes were isolated from liver wedges using a collagenase perfusion technique that we have published previously. Following perfusion, centrifugation was utilized to isolate a highly pure population of human hepatocytes that were plated on rat tail collagen for 72 hours in Williams E media prior to use in experiments.

Human Sinusoidal Endothelial Cells (HSEC) were isolated from liver tissue as previously described. Parenchymal cells were collected after collagenase digestion of liver slices and purified by density gradient centrifugation over Percoll. Endothelial cells were isolated from the resultant heterogeneous cell mixture by positive immunomagnetic selection using antibodies raised against CD31 (Clone JC70A, Dako, Denmark) according to the manufacturer's protocol. All endothelial cells were maintained in complete media comprising Human Endothelial-Serum Free Media basal growth medium (Invitrogen, UK) containing 104U/mL penicillin and 10µl/mL streptomycin, 10ng/mL epidermal growth factor (R&D Systems, UK), 10µg/mL hydrocortisone (Sigma-Aldrich, UK), and 10% heat-inactivated human serum (TCS Biologicals, UK).

Biliary epithelial cells (BEC) were isolated from liver tissue. The liver (30g) was finely diced and incubated with collagenase type 1A (Sigma, St. Louis, MO, USA). The digest was layered onto a 33% and 77% iso-osmotic Percoll gradient and centrifuged at 500g for 30 minutes. The interface layer was collected, washed three times in phosphate buffered saline and incubated with the BEC-specific mouse antihuman monoclonal antibody to human embryonic antigen 125 (TCS Biologicals Ltd., Botolph Claydon, Bucks, UK). BEC were positively selected by incubating with antimouse IgG1-coated Dynabeads (ThermoFisher Scientific, UK) and by magnetic separation. The cells were cultured in plating media containing: Hams F12, Dulbecco's Eagle medium; heat-inactivated foetal calf serum (10% v/v); penicillin, streptomycin (100ng/mL), glutamine (2mM); epidermal growth factor (10ng/mL); hydrocortisone (2µg/mL); choleratoxin (10ng/mL); tri-iodo-thyronine (2nM); insulin (0.124IU/mL). After 1–2 days in culture, the medium was exchanged for media containing 5% v/v foetal calf serum and 10ng/mL hepatocyte growth factor (R&D Systems Ltd., UK).

7.7.7.4 In vitro model of ischemia-reperfusion injury and assessment of reactive oxygen species production, apoptosis and necrosis

Cells kept at ambient oxygen concentrations were designated as being in normoxia. Those exposed to 0.1% O_2 for 24 hours were classified as being in hypoxia akin to ischemia. Those cells that were placed into hypoxia for 24 hours and then exposed to ambient oxygen for 24 hours were classified as having undergone hypoxia-reoxygenation (H-R) akin to reperfusion. This experimental model replicates the IRI environment to which the liver cells are exposed during transplantation[33].

Reactive oxygen species (ROS) production, apoptosis and necrosis were determined using a three-color assay. 2,7-dichlorofluorescin reacts with intracellular ROS to produce a signal

proportional to the intracellular concentration of ROS. Annexin-V is a specific marker of apoptosis due to its recognition of phosphatidylserine on the outer membrane of the cell membrane when the cell has committed to apoptosis. 7-Aminoactinomycin D (7-AAD) is a vital dye and only binds to DNA once there is disruption of the cell wall and is thus a marker of necrosis. Following exposure to the IRI model all cells were stained with each dye alone and in combination. Using the flow cytometry protocol and design we have detailed previously we set gating strategies that enabled the assessment of the above 3 parameters.

7.7.7.5 Assessment of donor liver viability

For a donor liver to have been considered viable and suitable for transplantation, it needed to meet the following criteria – Metabolism of lactate level to less than or equal to 2.5 within 2 hours or convincing evidence of bile production, in combination with two or more of the following: arterial flow >150ml min and portal flow >500ml/min, maintenance of pH >7.30 and homogenous liver perfusion with soft parenchyma consistency.

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CHAPTER 8 - THE TARGETED DELIVERY OF CELLULAR THERAPY TO EXTENDED CRITERIA LIVERS USING NMP-L

8.1 THE DELIVERY OF MULTIPOTENT ADULT PROGENITOR CELLS TO EXTENDED CRITERIA HUMAN DONOR LIVERS USING NORMOTHERMIC MACHINE PERFUSION

Authors: Laing RW[1,2], Stubblefield S[3], Wallace LL[1], Roobrouck VD[4], Bhogal RH[1,2], Schlegel A[2], Boteon YL[1,2], Reynolds GM[1], Ting AE[3], Mirza DF[1,2], Newsome PN[1,2], Mergental H[1,2], Afford SC[1,2]

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Affiliations:

[1] Centre for Liver Research, NIHR Liver Biomedical Research Unit, Institute of Immunology and Immunotherapy, College of Medical and Dental Sciences, University of Birmingham, United Kingdom.

[2] Department of Liver Surgery, Queen Elizabeth Hospital, University Hospitals Birmingham NHS Foundation Trust, Birmingham, United Kingdom.

[3] Athersys Inc., Cleveland, Ohio, United States.

[4] ReGenesys BVBA, Leuven, Belgium.

8.8.1 Abstract

Background

Pre-clinical research with multi-potent adult progenitor cells (MAPC® cells, Multistem, Athersys Inc., Cleveland, Ohio) suggests their potential as an anti-inflammatory and immunomodulatory therapy in organ transplantation. Normothermic machine perfusion of the liver (NMP-L) has been proposed as a way of introducing therapeutic agents into the donor organ. Delivery of cellular therapy to human donor livers using this technique has not yet been described in the literature. The primary objectives of this study were to develop a technique for delivering cellular therapy to human donor livers using NMP-L and demonstrate engraftment.

Methods

Six discarded human livers were perfused for 6 hours at 37°C using the Liver Assist (Organ Assist, Groningen). 50x106 CMPTX-labelled MAPC cells were infused directly into the right lobe via the hepatic artery (HA, n=3) or portal vein (PV, n=3) over twenty minutes at different time points during the perfusion. Perfusion parameters were recorded and central and peripheral biopsies were taken at multiple time-points from both lobes and subjected to standard histological stains and confocal microscopy. Perfusate was analysed using a 35-plex multiplex assay and proteomic analysis.

Results

There was no detrimental effect on perfusion flow parameters on infusion of MAPC cells by either route. Three out of six livers met established criteria for organ viability. Confocal microscopy demonstrated engraftment of MAPC cells across vascular endothelium when perfused via the artery. 35-plex multiplex analysis of perfusate yielded 13 positive targets, 9 of which appeared to be related to the infusion of MAPC cells (including Interleukin's 1b, 4, 5,

6, 8, 10, MCP-1, GM-CSF, SDF-1a). Proteomic analysis revealed 295 unique proteins in the perfusate from time-points following the infusion of cellular therapy, many of which have strong links to MAPC cells and mesenchymal stem cells in the literature. Functional enrichment analysis demonstrated their immunomodulatory potential.

Conclusion

We have demonstrated that cells can be delivered directly to the target organ, prior to host immune cell population exposure and without compromising the perfusion. Transendothelial migration occurs following arterial infusion. MAPC cells appear to secrete a host of soluble factors that would have anti-inflammatory and immunomodulatory benefits in a human model of liver transplantation.

8.8.2 Introduction

The demand for donor livers overwhelms supply and in the UK, 19% of patients die or are removed from the list whilst waiting for a transplant (1). Strategies to improve the quality of high risk donor livers (531 rejected in the UK last year (1)) would increase the pool of transplantable livers and improve patient outcomes.

Multipotent adult progenitor cells (MAPC®) have been proposed as an immune-active treatment for a wide variety of conditions (2). They belong to the family of mesenchymal stem cells (MSC) but show a higher proliferative capacity and a broader differentiation potential (3). A distinct bone-marrow derived cellular population, they meet the formal criteria for designation as stromal stem cells in that they are plastic-adherent and express CD73, CD90, and CD105, in the absence of the hematopoietic markers CD14, CD34, CD45, and HLA-DR (4). They differ from MSC based on cellular phenotype (negative for CD140a, CD140b, alkaline phosphatase and express major histocompatibility complex class I at lower levels), size, transcriptional profile, and expansion capacity (5). Proof of concept of their efficacy has been demonstrated in animal models for the treatment of different conditions including graft versus host disease and in a porcine and human lung model of machine perfusion (6-11). Not only can they impair the induction of CD8+ cytotoxic T-lymphocyte function and supress T-lymphocyte proliferation (12), but MAPC cells and related mesenchymal stem cells (MSC) have been shown to reduce ischaemia reperfusion injury (IRI) and reduce the inflammatory response in solid organs (2, 10, 13, 14). These preclinical studies suggest that MAPC cells could exert their beneficial effects in a solid organ transplant model through immunomodulation by promoting immunological tolerance (9, 15-17).

Transplantation is the only curative option for patients with end-stage liver disease and the

global shortage of suitable donor livers has been extensively reported (18, 19). The UK transplant activity data over the past decade (2008-2018) demonstrates a 54% increase in transplant activity (657 to 1014) (1). The increase in donor numbers over this period has been achieved through a 58% increase in livers donated following brain death (DBD) and a 257% increase in those donated following circulatory death (DCD) (20). Our own data shows a pre-transplant on-list mortality rate for priority patients of up to 40% (unpublished data). It is widely accepted that whilst the use of extended criteria DCD or marginal DBD liver grafts may provide additional organs for transplantation they are known to be associated with additional challenges (21-23). Given the significant clinical impact of these factors, there is an urgent clinical need to attempt to modulate the inflammatory and immune responses they induce.

Normothermic machine perfusion of the liver (NMP-L) is a novel technique whereby a donor liver graft is perfused at physiological temperature and pressure with a complex solution containing an oxygen carrier and other constituents (including colloid, electrolytes etc.) that aims to preserve the graft under physiological conditions ex-situ. It has been shown to be a superior to static cold storage as a method of organ preservation (24, 25), it also provides the unique opportunity to assess organ viability prior to transplantation (26-29). The potential use of NMP-L as a method of delivering cell-based and novel small molecule therapies aimed at improving the condition of extended criteria livers has been proposed (30) and is steadily gaining credence within the transplant community as experimental proof that concept data is emerging (31, 32). Despite examples in animal models, delivery of cellular therapy using machine has not been demonstrated in a human liver model (33-35).

The aims of this study were to a) develop and demonstrate feasibility of NMP-L as a technique for delivering cellular therapy to extended criteria human donor livers; b) determine the best

vascular route for delivery and confirm the presence of cellular engraftment and c) determine parameters that may reflect biologically functional activity imparted by the presence of the therapeutically administered MAPC cells.

8.8.3 Materials and methods

8.8.3.1 Preparation of MAPC cells

MAPC cells were provided by Athersys Inc. (Cleveland, Ohio, USA). The isolation and cultivation of these MAPC cells have been previously described (36). Cryovials containing approximately $10x10^6$ cells labelled with CellTracker™ Red CMTPX dye (Thermo Fisher Scientific Inc.) were thawed and prepared according to clinical protocols immediately prior to infusion into the donor liver (see supplementary information for protocols). Cellular concentrations and viability were determined using trypan blue dye exclusion and $50x10^6$ cells were made up to a final volume of 50ml with 0.9% normal saline ready for infusion. Calculations of number of cells were based on clinical studies where cells were delivered systemically (150-600 million) and this was scaled down due to infusion into the target organ and in this case the right lobe of the liver (16).

8.8.3.2 Source of discarded human livers

The six donor livers included in this study were offered, accepted and retrieved with the initial intention to use them for clinical transplantation. They were procured by one of the UK's National Organ Retrieval Service teams using nationally agreed surgical protocols (National standards for organ retrieval from deceased donors (joint with NHSBT). Available from: http://www.bts.org.uk). Following assessment by either the retrieval or transplanting surgeon, the livers were declined by all UK transplant centres and consent-permitting, subsequently offered for research by the NHSBT co-ordinating office. Ethical approval for the study was granted by the National Research Ethics Service committee in London-Surrey Borders (reference number 13/LO/1928). Consent for the use of donor tissues for research was obtained by the specialist nurses in organ donation from the designated donor's next of kin.

8.8.3.3 Preparation of the donor liver for NMP-L and MAPC cell infusion

On receipt of the donor liver, its preparation for NMP-L was initially analogous to clinical transplantation. A polyethylene Leadercath Arterial catheter (Vygon [UK] Ltd) was placed to permit infusion of cellular therapy into the right lobe either via the hepatic artery or portal vein. For arterial infusion the guidewire was passed through the gastroduodenal arterial stump and gently directed into the main branch of the right hepatic artery. For portal venous infusion the needle supplied was used to puncture the portal vein proximal to the bifurcation and the wire passed down the right portal venous branch. The catheter was then guided over the wire and into the appropriate vessel and secured using 5-0 prolene sutures. Cells were infused directly into the right lobe via either the right hepatic arterial branch or the right portal vein branch to create an internal control and gain information on engraftment of recirculating cells. A 3-way tap was attached to the catheter, flushed with 2ml of Ringer's solution and set to the closed position. The distal end of the catheter was always placed in the main trunk of the right arterial or portal venous branch (Figure 8.1). Following insertion and securing of cannulae, the liver was placed into the machine reservoir and connected to a Liver Assist device (CE marked; Organ Assist, Groningen, The Netherlands) as previously described (29) (37).

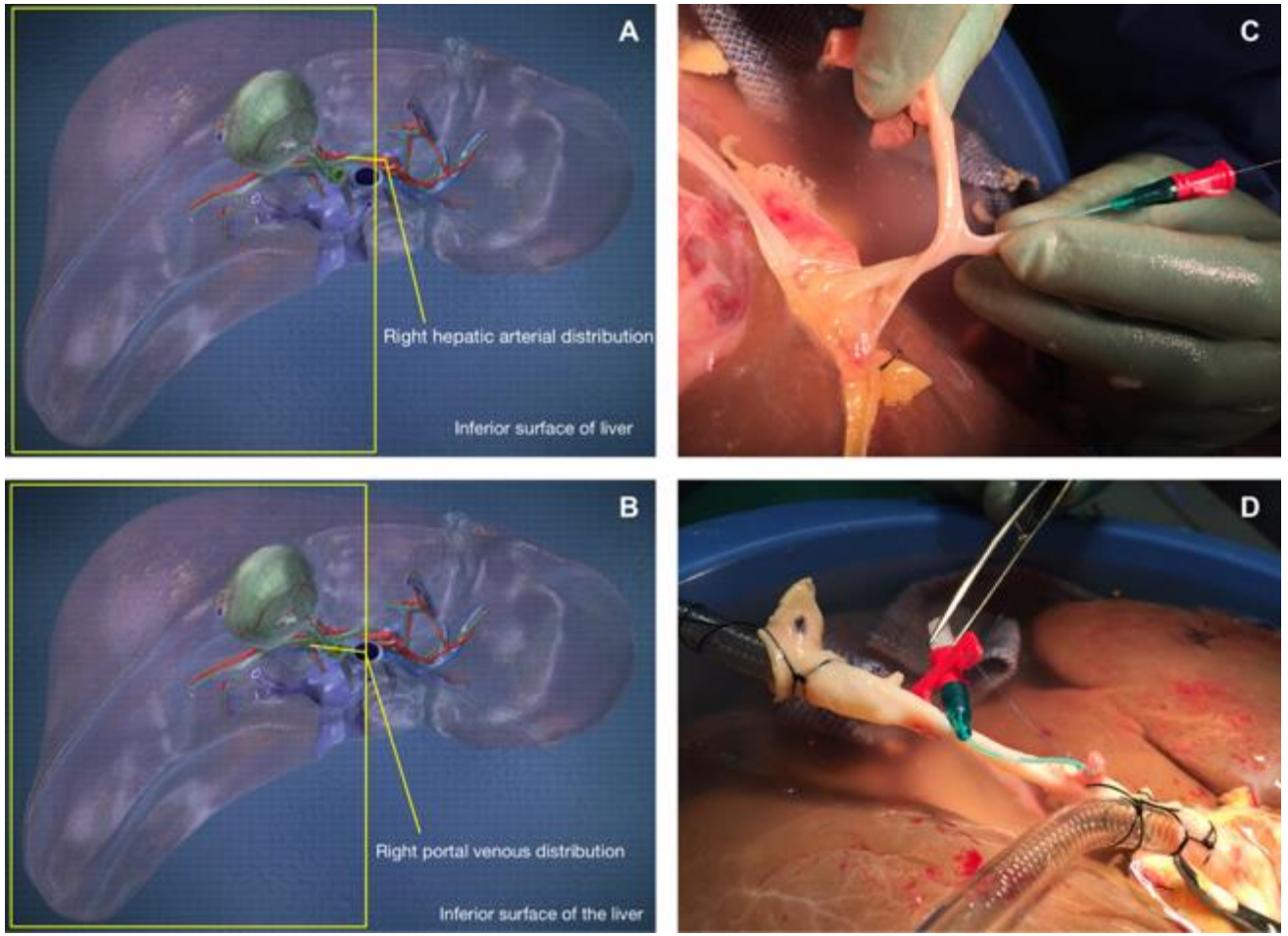


Figure 8.1 Technique for cannulating liver for cellular infusion

Cells were infused via the gastroduodenal arterial stump (C and D) into the right hepatic arterial branch (A) or directly into the right portal venous branch (B).

8.8.3.4 Infusion of MAPC cells

MAPC cells were infused via syringe driver attached to the Vygon Leadercath catheter over 20 minutes into the right lobe via the hepatic artery (HA, n=3; HA1, HA2, HA3 [1 DBD and 2 DCD]) or portal vein (PV, n=3; PV1, PV2, PV3 [1 DBD and 2 DCD]) during the perfusion. The cells were infused as described initially after 4 hours of perfusion (n=2, first HA and PV infusion). Vascular flow characteristics were unaffected by the infusion, therefore subsequent infusions were performed after 1 hour (n=4, 2 HA and PV infusions).

8.8.3.5 Assessment of physiology and sample collection protocol

Flow rates, pressures, resistances and temperatures in the hepatic arterial and portal venous circuits were recorded every 30 minutes and specifically before, during and after cell infusions. Arterial and hepatic venous perfusion fluid was sampled every 30 minutes and immediately assessed using a Cobas b 221 point of care system (Roche Diagnostics, USA). Samples were also processed to permit the freezing of perfusate at -80^{o}C. Livers that metabolised lactate to below 2.5mmol/L within 2 hours were termed "viable" as it is predicted that these livers have the metabolic capacity to function sufficiently following transplantation (28) – a hypothesis that was tested during the clinical pilot study as well as in the VITTAL trial (Viability Testing and Transplantation of Marginal Livers) which is now closed to recruitment (27, 38).

8.8.3.6 Histological Assessment

Liver biopsies were taken from both the left and right lobes; on the back bench prior to the start of NMP-L, pre-cell infusion and at the end of the 6-hour perfusion. Biopsies were fixed in formalin, embedded in paraffin and sections cut at 4μm. The MAPC cells were identified by the CellTracker™ Red CMTPX dye and their biodistribution – related to their route of administration assessed using confocal microscopy. Three-colour confocal microscopy (4',6-

diamidino-2-phenylindole [DAPI] on the blue channel, CMTPX Red on the red channel and CD31 on the green channel (to identify vascular endothelium) was used to demonstrate the presence and location of MAPC cells. The creation of virtual slides through imaging of whole tissue mounts was achieved using the ZEISS AxioScanZ.1 slide scanner and confocal microscopy was performed using the ZEISS LSM780 confocal microscope.

8.8.3.7 Assessment of soluble markers in perfusate samples

8.8.3.7.1 Cytokine and chemokine analysis using multiplex array

Perfusate samples from all perfusions at 4 time-points were analysed using the 34-Plex Human ProcartaPlex™ Panel 1A multiplex kit (ThermoFisher Scientific Ltd.). The target list included Eotaxin/CCL11; GM-CSF; GRO alpha/CXCL1; IFN alpha; IFN gamma; IL-1 beta; IL-1 alpha; IL-1RA; IL-2; IL-4; IL-5; IL-6; IL-7; IL-8/CXCL8; IL-9; IL-10; IL-12 p70; IL-13; IL-15; IL-17A; IL-18; IL-21; IL-22; IL-23; IL-27; IL-31; Interferon gamma-induced protein 10 (IP-10/CXCL10); Monocyte chemoattractant protein-1(MCP-1/CCL2); Macrophage inflammatory protein-1 alpha (MIP-1 alpha/CCL3); MIP-1 beta/CCL4; RANTES/CCL5; Stromal cell-derived factor-1 (SDF1 alpha/CXCL12); TNF alpha; TNF beta/LTA. A "viable" liver that had not received MAPC cells and was transplanted as part of the clinical pilot study was used as a control. The multiplex assay was performed according to the manufacturers guidelines and run on a Luminex® 100™ System. Raw data were analysed using Prism 8.0 for Mac OS X.

8.8.3.7.2 Proteomic analysis of the perfusate

Proteomic analysis of individual perfusate samples from four time-points was performed for each liver and compared to results from all other livers (n=8) previously perfused with standard perfusate that had not received cellular therapy. This was to maximise the probability of

identifying unique proteins in the MAPC cell perfused livers. Haemoglobin depletion of haemolysed samples using Hemoglobind (BioTech Support Group LLC, Monmouth Junction, NJ) was followed by trypsin-based liquid digestion, peptide cleaning, gradient separation and elution into a Linear Trap Quadropole (LTQ) Orbitrap Elite mass spectrometer for liquid chromatography (LC-MS/MS). Scan results were searched against Uniprot database. Protein-protein interactions (PPI's) and functional enrichments (FE's) were determined using the String© database 2017 (https://string-db.org, String Consortium 2020) and Cytoscape© (Cystoscape Consortium (39-41).

8.8.4 Results

8.8.4.1 Donor demographics and perfusion parameters

Six livers were perfused (2 DBD and 4 DCD) with a median donor age of 52.5 (35-71), cold ischemic time of 500 minutes (453-754), and donor risk index of 2.41 (1.58-3.22). Three received cells via the right hepatic artery and 3 via the right portal vein (1 DBD and 2 DCD in each group). The timing of infusions varied also. HA1 and PV1 received cells towards the end of the perfusion (infusions started at 4hrs 40mins and ran over 20 minutes, cells delivered with 1 hour perfusion remaining) and in the remaining four livers (HA2, 3 and PV2, 3) the cells were infused after 40 minutes of perfusion and were delivered fully with 5 hours of perfusion remaining. There were no significant detrimental effects on the perfusion parameters during cellular infusion and neither resistances or flow rates were adversely affected. Of interest, flow rates in the artery transiently increased by approximately 30% during all 3 arterial infusions but flows returned to normal shortly after stopping the infusions (data not shown). Arterial resistance and flow, portal flow and lactate can be seen in Figure 8.2.

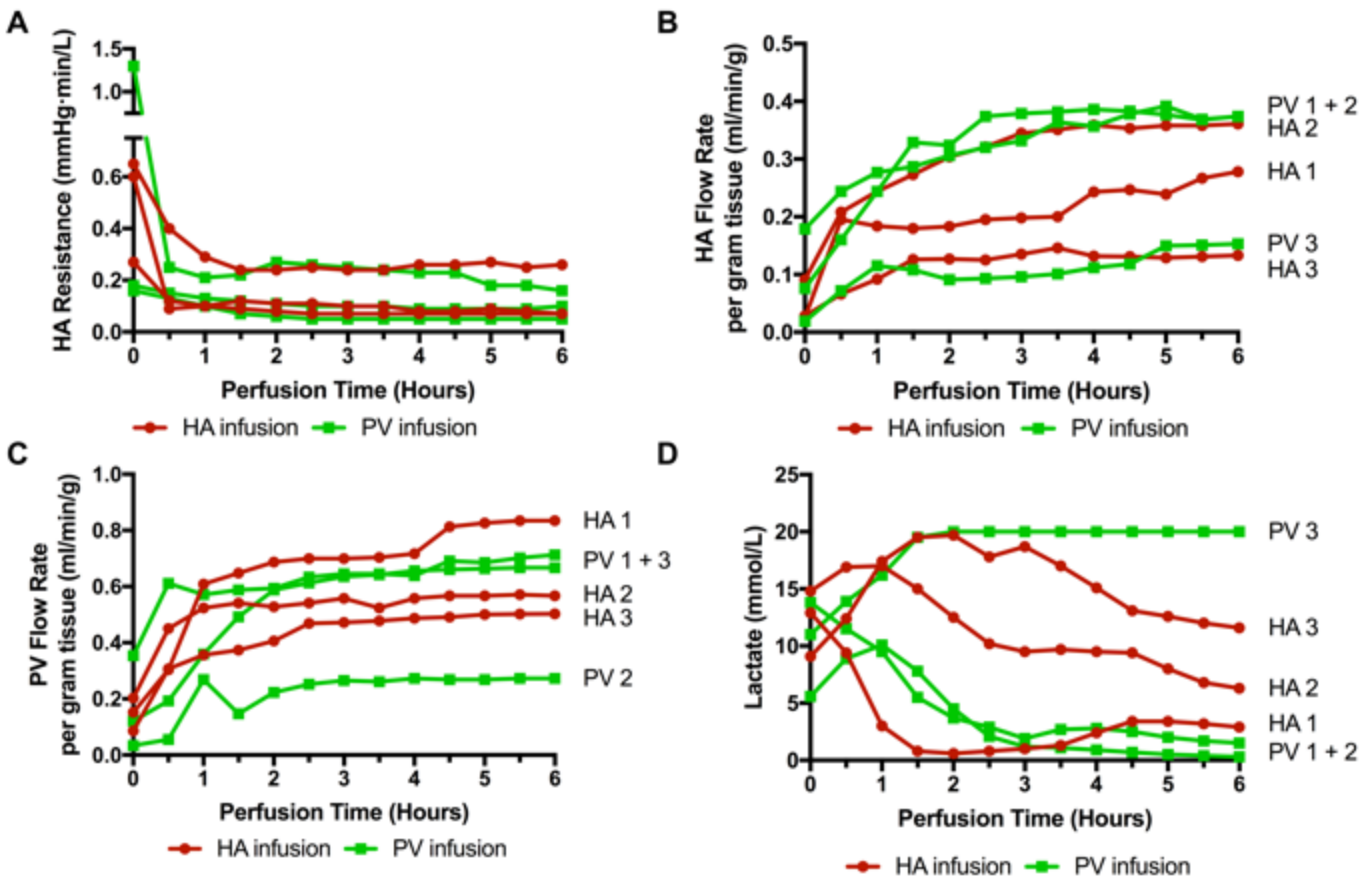


Figure 8.2 Perfusion parameters during 6 MAPC cell perfusions

Perfusion parameters during 6 perfusions (HA1-3 cells infused via right hepatic artery. PV1-3 cells infused via right portal venous branch). A: HA resistance; B: HA flow rate adjusted for liver weight; C: PV flow rate; D: Lactate level over the course of the perfusion. 3 livers met viability

criteria according to our Birmingham Machine Perfusion Group Viability Criteria. Two of the non-viable livers HA3 and PV3 also have very low arterial flow rates due to high intrinsic arterial resistances.

8.8.4.2 Histology and Confocal Microscopy

Histological features were in keeping with efficacy of perfusion and liver quality. Architecture of the liver parenchyma was well maintained in those livers that were deemed viable (Figure 8.3A). Liver PV3 was severely steatotic and H&E stained sections demonstrated large droplet macrovescicular steatosis and loss of cohesion between hepatocytes in liver cell plates suggesting endothelial disruption (Figure 8.3B). In those livers that met viability criteria, increases in glycogen storage were observed (Figure 8.3C and 8.3D). Three-colour confocal microscopy (4',6-diamidino-2-phenylindole [DAPI] on the blue channel, CMTPX Red and CD31 on the green channel (to identify vascular endothelium) was used to demonstrate the presence and location of MAPC cells. Cells were visualised in the right lobe of all 6 livers. MAPC cells were visualised in every low power field of view in central and peripheral biopsies of the right lobe (5 random biopsies each of central and peripheral tissue) and were visualised 1 hour after infusion and five hours after infusion (Figures 8.4, 8.5, 8.6 and 8.7 show confocal images comparing right and left lobes pre and post infusion [4], low power HA vs PV infusion [5], and high power post HA infusion [6] and post PV infusion [7]). MAPC cells were never visualised in the left lobe. Arterially infused cells appeared to cross the CD31 stained vascular endothelium and migrate to within the parenchyma. These cells also appear to undergo some form of conformational change as they are also expressed in the green channel in addition to the red channel as opposed to those cells that remain in the vascular channels and are visible in the red channel only.

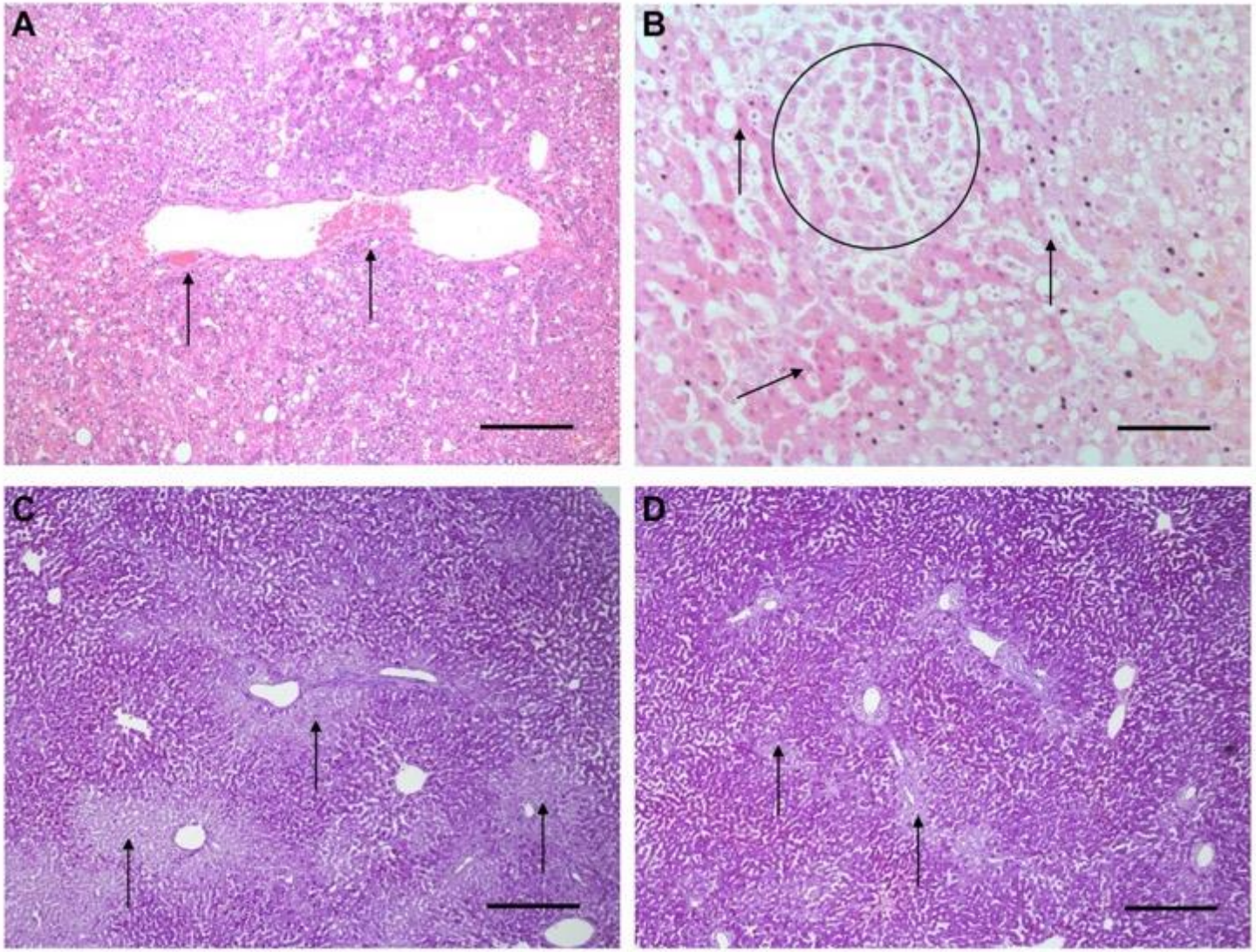


Figure 8.3 Light microscopy images of H&E (A, B) and periodic acid Schiff stains (C, D).

A and B are H&E stained sections from early NMP-Ls showing some histological abnormalities. Architecture of the liver parenchyma was well maintained in those livers that were deemed viable. Liver PV3 (B) was severely steatotic and H&E stained sections demonstrated large droplet macrovescicular steatosis and

loss of cohesion between hepatocytes in liver cell plates suggesting endothelial disruption. In those livers that met viability criteria, increases in glycogen storage were observed (Figure 8.3C and 8.3D).

In HA3 (slide A), portal microvessels (arrows) are seen plugged by disintegrating red cells. Original objective x10. Original objective x10, scale = 100 µm.

B. 1 hour after commencement of perfusion number PV3, loss of cohesion between hepatocytes in liver cell plates is observed (circle). Normal liver cell plates are arrowed. Original objective x20, scale = 50 µm.

C and D are periodic acid Schiff stained sections of liver PV1.

C. PV1 before NMP-L

D. PV1 After 4 hours of NMP-L. Glycogen stains as dark pink, arrows highlight pale glycogen depleted areas. It can be seen that there is less glycogen depleted pale areas after perfusion indicating that the hepatocytes have taken up glucose from the perfusate and metabolise it to glycogen. Original objective x5 for both, scale = 200 µm.

Figures 8.4, 8.5, 8.6 and 8.7.

Confocal microscopy images showing representative tissue sections (as labelled) of livers infused with MAPC cells. Blue channel (Nucleic acid probe DAPI 345nm) (4',6-diamidino-2-phenylindole), Red channel (615nm) CMTPX Red and green channel (FITC 495nm).

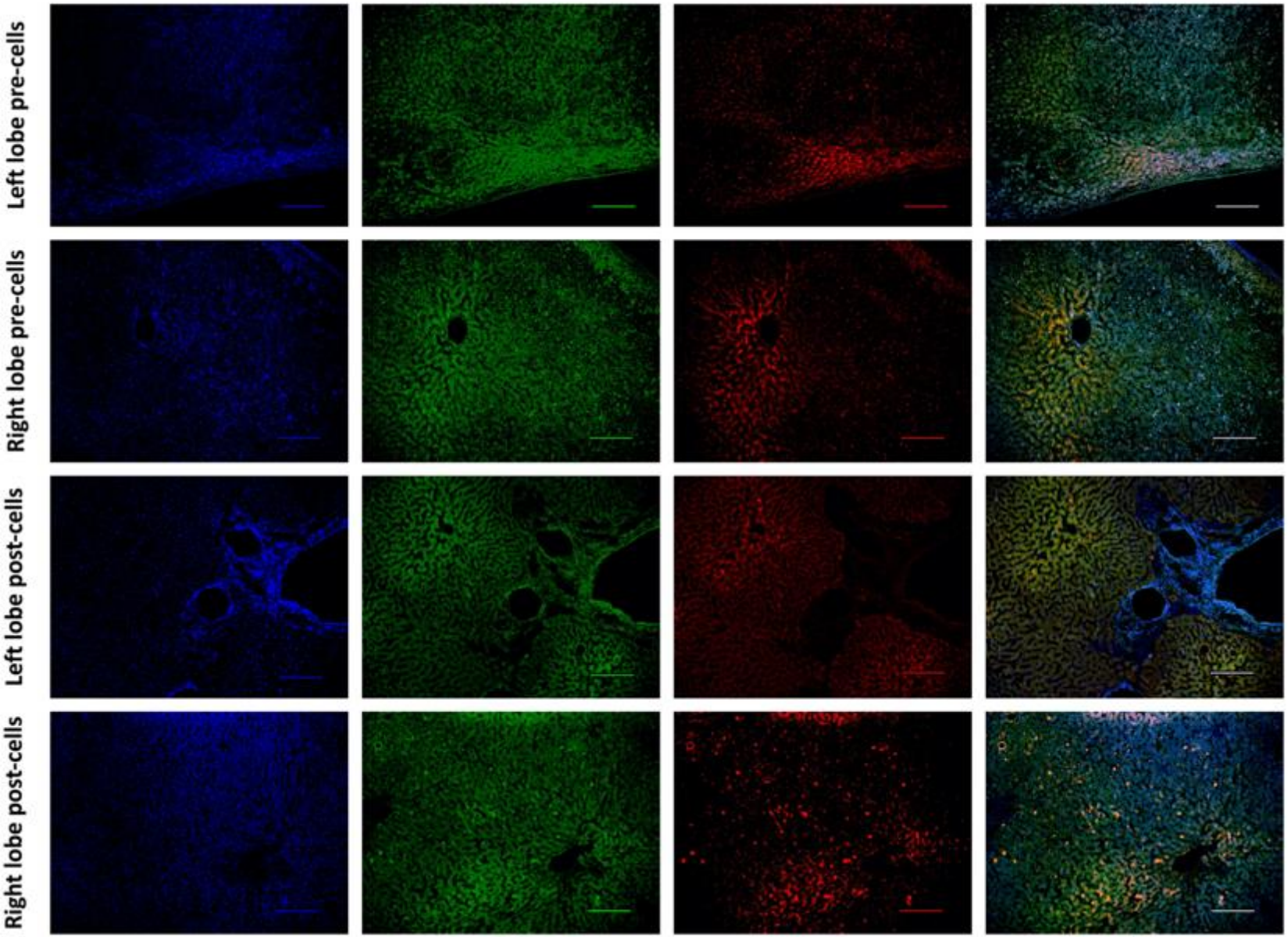
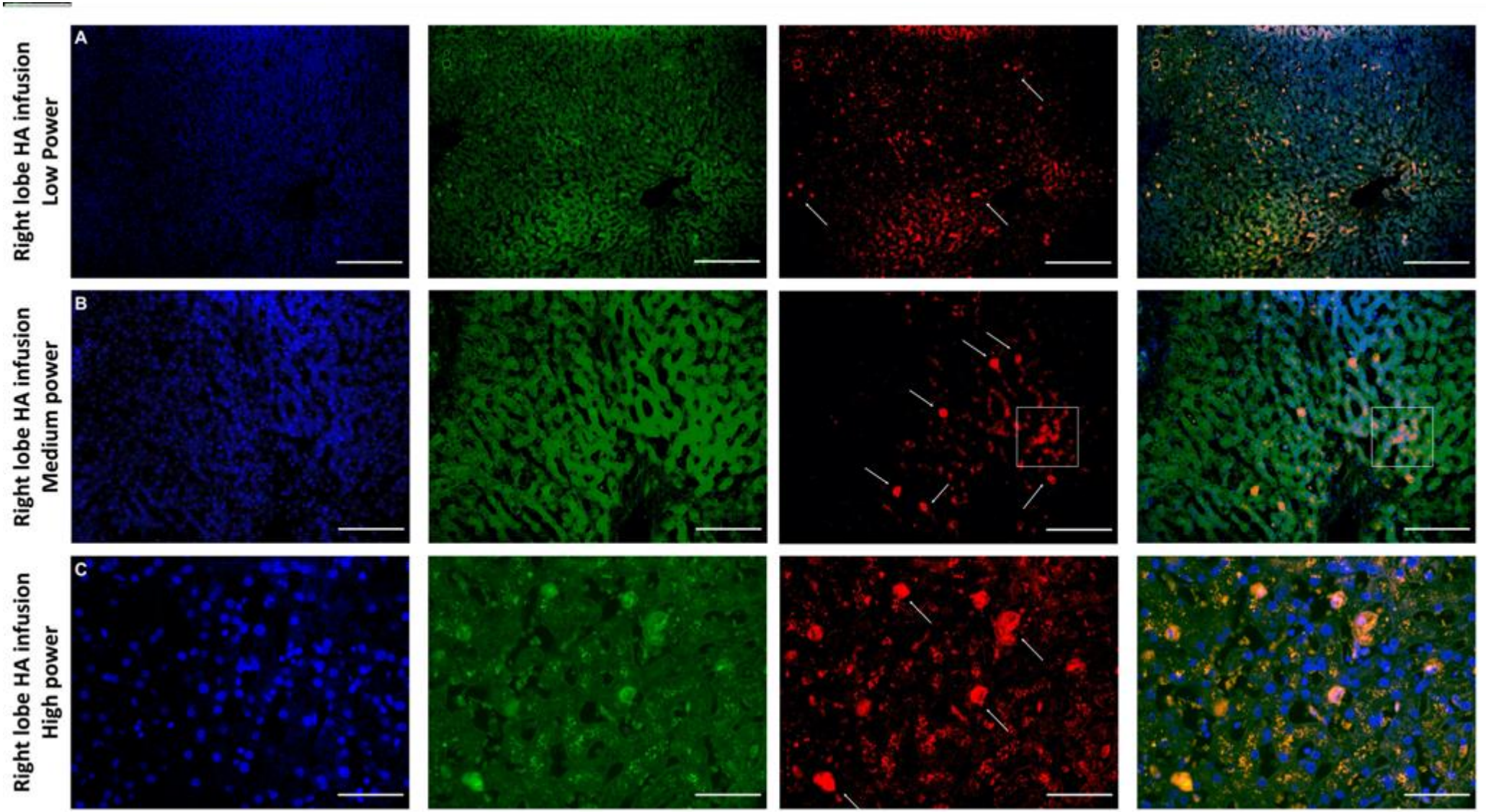


Figure 8.4 Confocal images of left and right lobe before and after MAPC cell infusion

Four confocal microscopy panels (blue channel, green channel, red channel and composite) of representative images of the left lobe (A) and right lobe (B) prior to MAPC cell administration and left lobe (C) and right lobe (D) 4 hours after MAPC administration via the right hepatic artery. The cells are clearly seen in panel D fluorescing in the red and green channels and visible as orange cells in the composite image. Cells were never seen in any of the left lobe biopsies at 1 or 4 hours after MAPC cell administration. A-D Objective x 10, scale =100 μm.


A
Right lobe HA infusion
Low Power
B
Right lobe HA infusion
Medium power
C
Right lobe HA infusion
High power

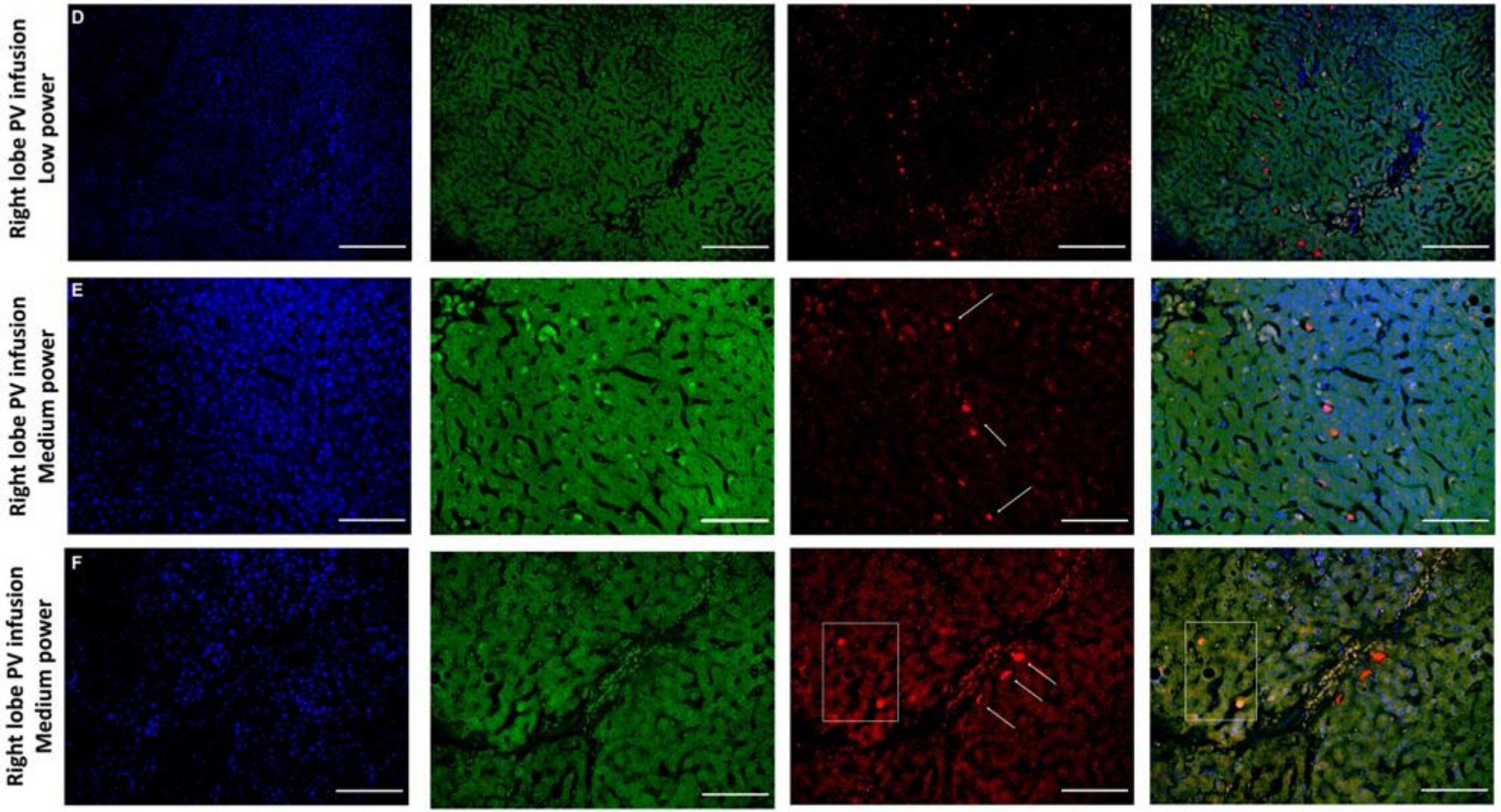


Figure 8.5 Confocal images demonstrating engraftment differences between HA and PV MAPC cell infusion

Six confocal microscopy panels A-F (blue channel, green channel, red channel and composite) of representative images of the right lobe comparing route of delivery of the MAPC cells. A: HA low power; B: HA medium power; C: HA high power; D: PV low power; E: PV medium power; F PV high power. A, B and

C demonstrate widespread delivery of MAPC cells which are visible (arrows) in both the green and red channel images suggesting a possible conformational change following engraftment which is more clearly demonstrated in Figures 6 and 7. The square annotation in panel B shows the autofluorescence commonly seen in the red channel in liver tissue, however the granular pattern is clearly different to the solid appearance of the cells that fluoresce due to the CMTPX stain. C, D and E demonstrate cells arrested within the sinusoids of the liver following administration via the right portal vein. These are much brighter in the red channel and they clearly reside within the vascular channels. In panel F there are two cells which appear similar to those in panels A-C suggesting that they may have started to engraft within the parenchyma, although many remain in the sinusoids. A and D – x10 objective, scale = 100 µm; B and E – x20 objective, scale = 50 µm; C and F x40 objective, scale = 25 µm.

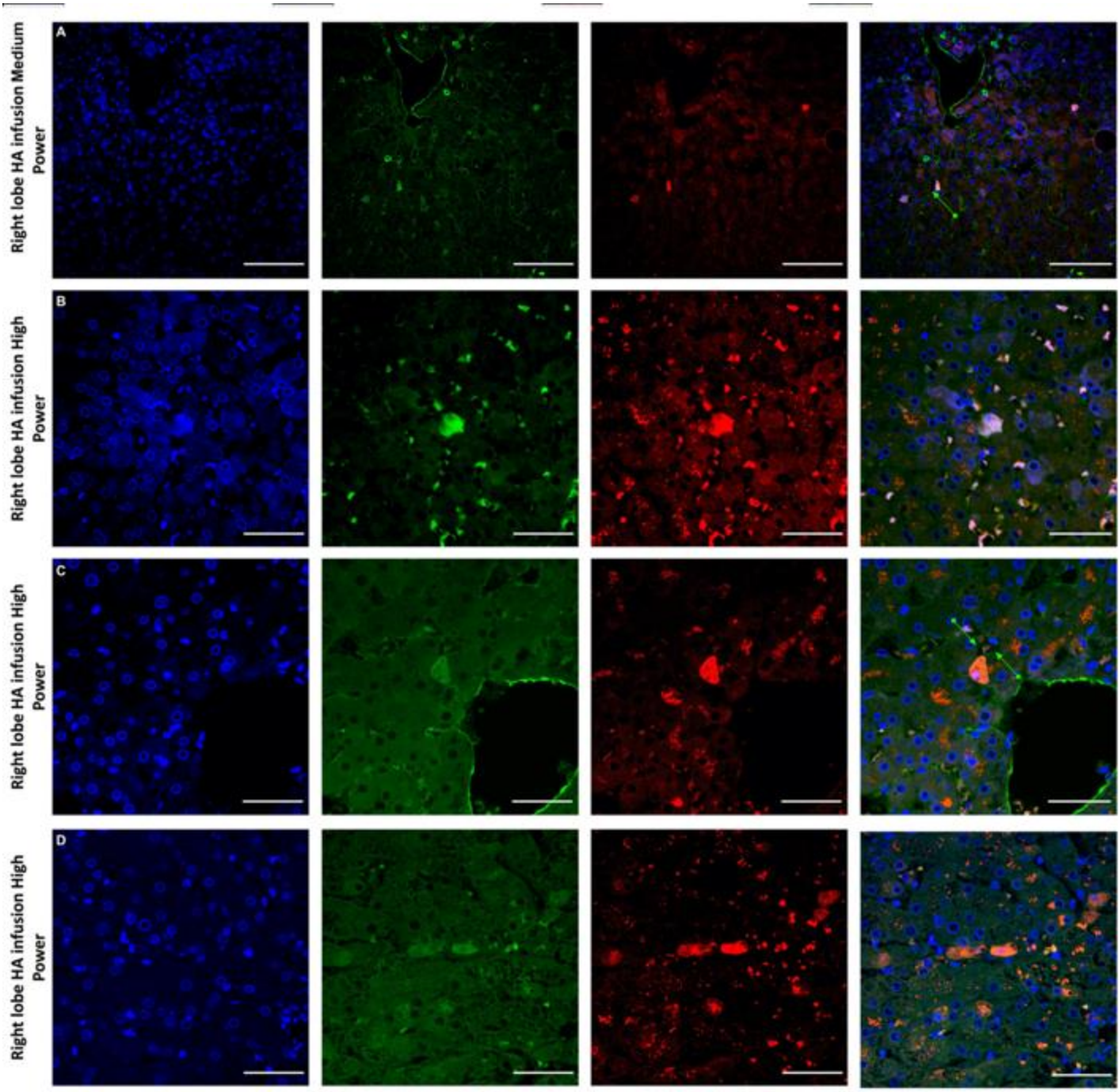


Figure 8.6 High power confocal images following HA MAPC cell infusion

Four confocal microscopy panels (blue channel, green channel, red channel and composite) of representative images of the right lobes of 3 livers infused with MAPC cells via the right hepatic artery after 1 hour (A – medium power) and 4 hours (B-D – high power). Here the green arrows in A and C demonstrate the vascular endothelium stained with CD31 and cells that appear to lie out with the vasculature between the parenchymal cells. These cells also fluoresce in the FITC channel and this may be because they have undergone some form of conformation change during the engraftment process. A x20 objective, scale = 50 μm; B-D x40 objective, scale = 25 μm.

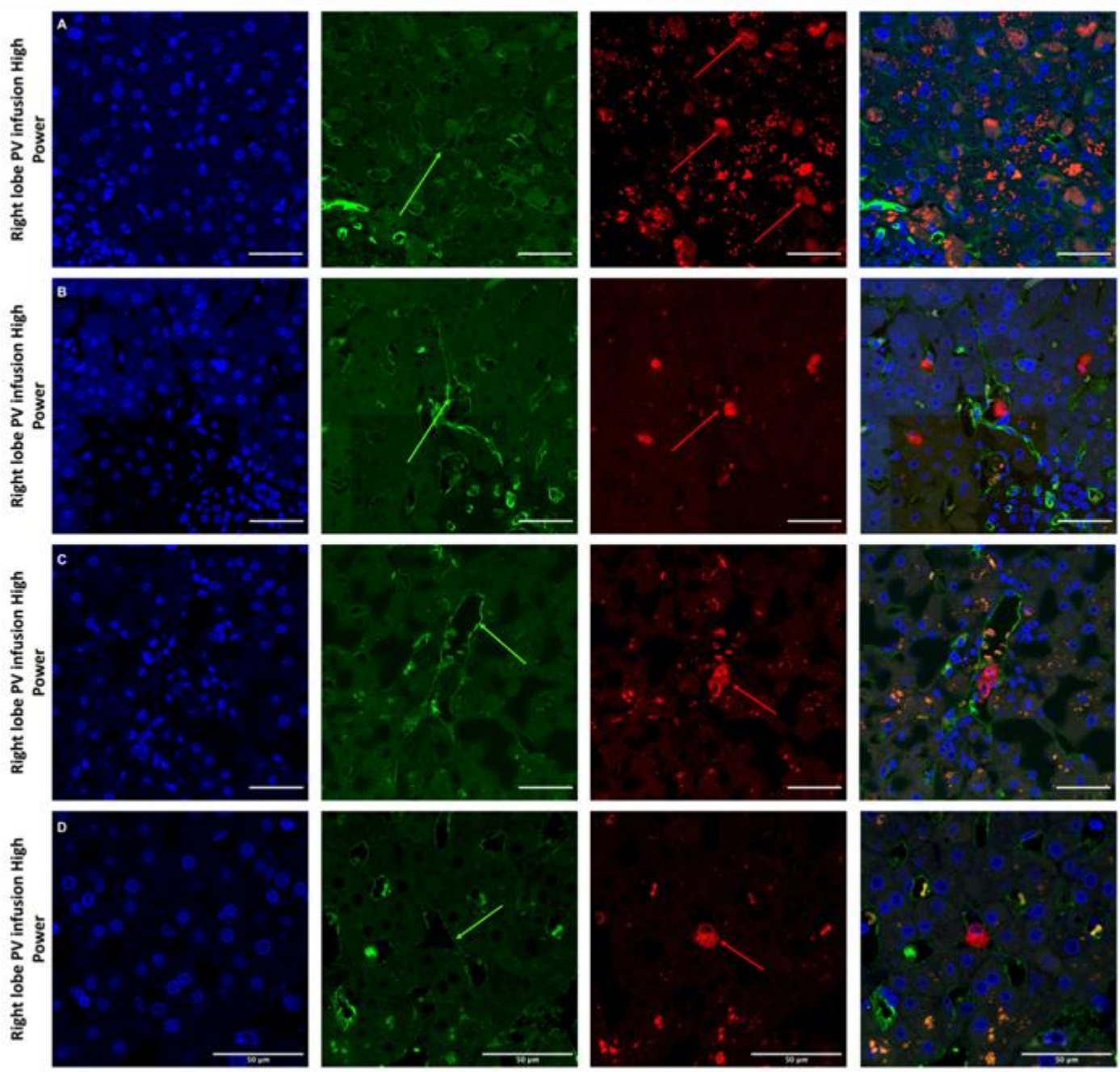


Figure 8.7 High power confocal images following PV MAPC cell infusion

Four confocal microscopy panels (blue channel, green channel, red channel and composite) of representative images of the right lobes of 3 livers infused with MAPC cells via the right portal vein after 1 hour (A – high power) and 4 hours (B-D – high power). In this series, the green arrows again demonstrate the vascular endothelium using the CD31 stain but here the MAPC cells are barely visible in the FITC channel and are clearly fluorescing in the red channel suggesting that that are yet to undergo the changes seen in Figure 6. A-D x40 objective, scale = 25 μm.

8.8.4.3 Cytokine and chemokine analysis of perfusate using Luminex

From the 34-plex multiplex analysis, the concentrations of 13 out of 34 targets were shown to increase over the course of the perfusion: IL-1RA, IL-1beta, IL4, 5, 6, 8, 10, 18, IFN-gamma, TNF-alpha, MCP-1, GM-CSF, SDF-1 alpha. The results are displayed in Figure 8.8 (median values with range), with the six livers split into two groups – group 1 (n=2) cells infused after 5 hours and group 2 (n=4) cells infused after 1 hour. A transplanted control which underwent perfusion was also analysed at 2 time points (0 hours and 6 hours). The changes in concentration of nine targets (Figure 8.8A) – IL4, 5, 6, 8, 10, MCP-1, SDF-1 alpha, IL-1 beta and GM-CSF appeared related to the presence of MAPC cells, as they were only detected after their infusion. The levels of the remaining four targets (Figure 8.8B) TNF-alpha, IFN-gamma, IL-18 and IL-1RA appeared unrelated to the presence of MAPC cells.

A

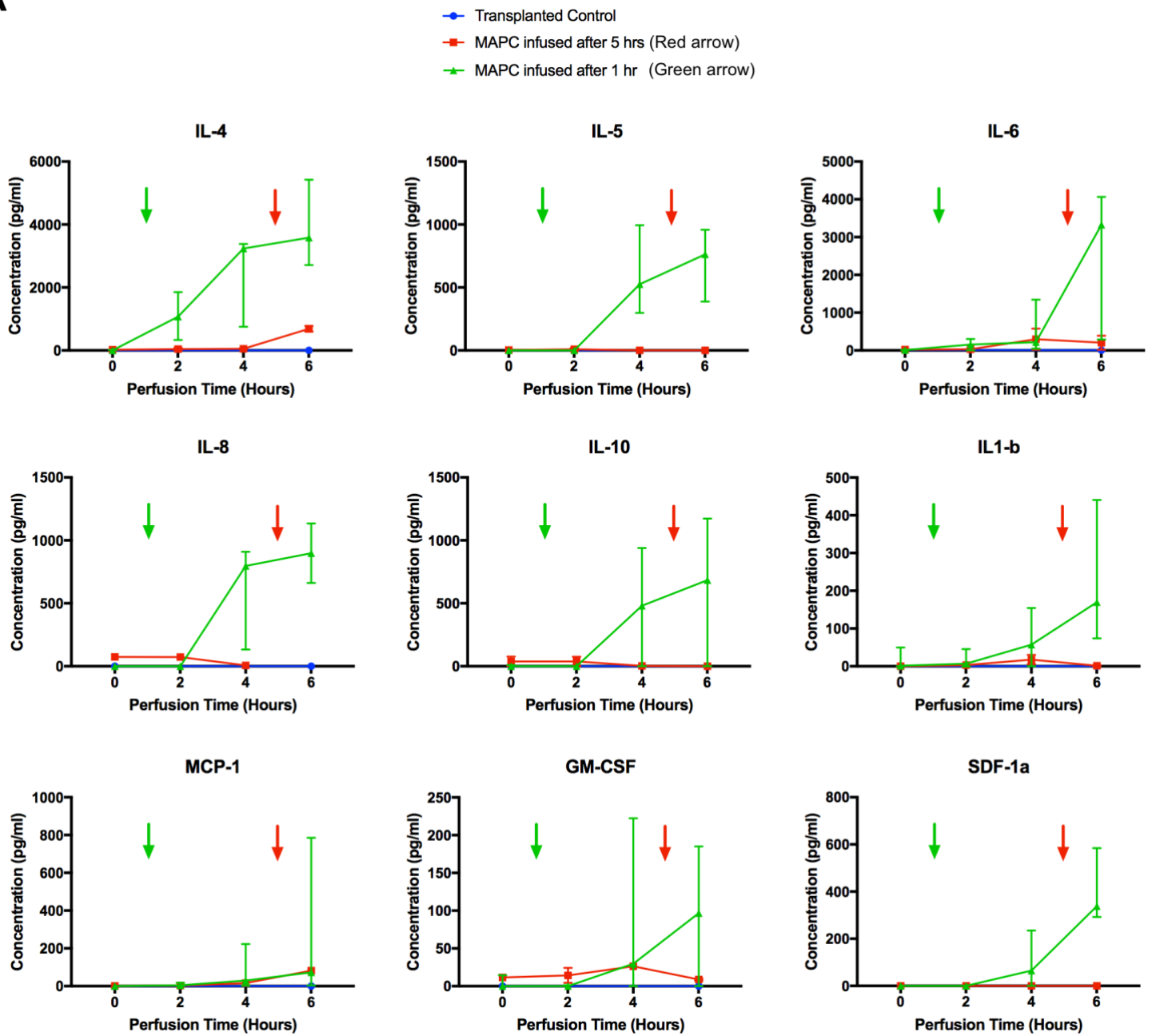

Transplanted Control
MAPC infused after 5 hrs (Red arrow)
MAPC infused after 1 hr (Green arrow)
IL-4
IL-5
IL-6
IL-8
IL-10
IL1-b
MCP-1
GM-CSF
SDF-1a
Concentration (pg/ml)
Perfusion Time (Hours)

Figure 8.8 Perfusate analysis using Luminex platform (Panel A and B).

Perfusate analysis using Luminex platform. Pattern of nine targets appear related to cellular perfusion (Panel A), with concentrations increasing in perfusates of livers after the infusion of MAPC cells. Four targets (B) appear unrelated to the infusion of MAPC cells. Panel A shows nine targets that had an apparent increase in concentration within the perfusate samples following MAPC cell administration. IL4 appeared to increase shortly after cell administration – increasing in the final hour of the perfusion after MAPC administration – whereas the remaining targets required longer to increase. As can be seen in the legend the green arrow denotes the administration of MAPC cells at 1 hour and the red arrow at 5 hours.

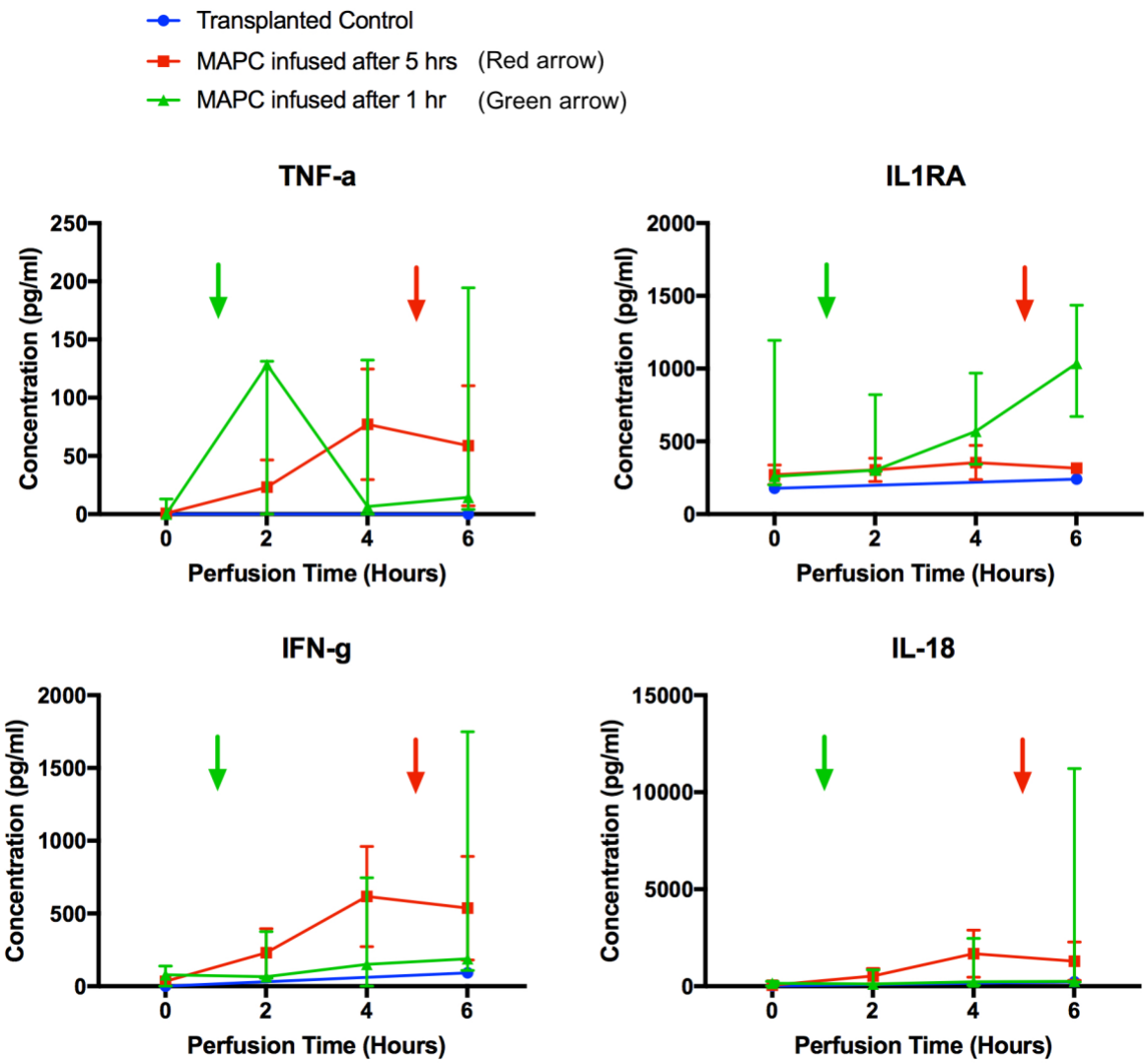


Figure 8.8 Panel B shows four targets (TFN-alpha, IL1RA, Interferon-gamma and IL-18) that increased their concentrations during the perfusion and appear unrelated to MAPC cell administration but more likely linked with levels of inflammation within the marginal livers.

8.8.4.4 Proteomic analysis of perfusates

Analysis of perfusates from the 6 donor livers identified a total of 1300 unique proteins of which 48 were present in every sample. Of interest these included alcohol dehydrogenase Ib and 4, superoxide dismutase 1, aldehyde dehydrogenase, complement component 3, apolipoproteins A-II, B and H, glyceraldehyde-3-phosphate dehydrogenase, serpin peptidase inhibitor clade G member 1, kininogen 1 and inter-alpha-trypsin inhibitor heavy chain family, member 4. When the results from these perfusions were compared to a group of 8 contemporaneous perfusions with similar demographics and characteristics that had not received therapeutic intervention, 295 unique proteins were identified in the perfusate from time-points following the infusion of cellular therapy (i.e. after 5 hours for HA1 and PV1 and after 1 hour in HA2 and 3 and PV2 and 3). The network edges were set to high confidence (>0.700 interaction score) which yielded a PPI enrichment p-value of 1.05e-05 showing that it was highly likely that this group of proteins were biologically connected. Unconnected nodes were removed and 191 proteins were imported to Cytoscape for further functional enrichment and network analyses. These proteins (Figure 8.9), through functional enrichment analysis, were shown to be involved with 549 gene ontology processes (GO:Processes) (false discovery rate [FDR] <0.05). These are grouped and depicted in Figure 8.10. Seventeen of these proteins were also identified as having strong links to MAPC cells and MSC in the literature (Figure 8.11) – with 14 of 17 in the top 50 most connected proteins in terms of "node degree" or PPI (Supplementary Table S8.1). Many of these had strong tissue associations with the bone marrow and the liver (Supplementary Table S8.3). The descriptions of these proteins can be found in Table 1 whilst the functional enrichment data can be seen in Supplementary Tables S8.1, S8.2 and S8.3.

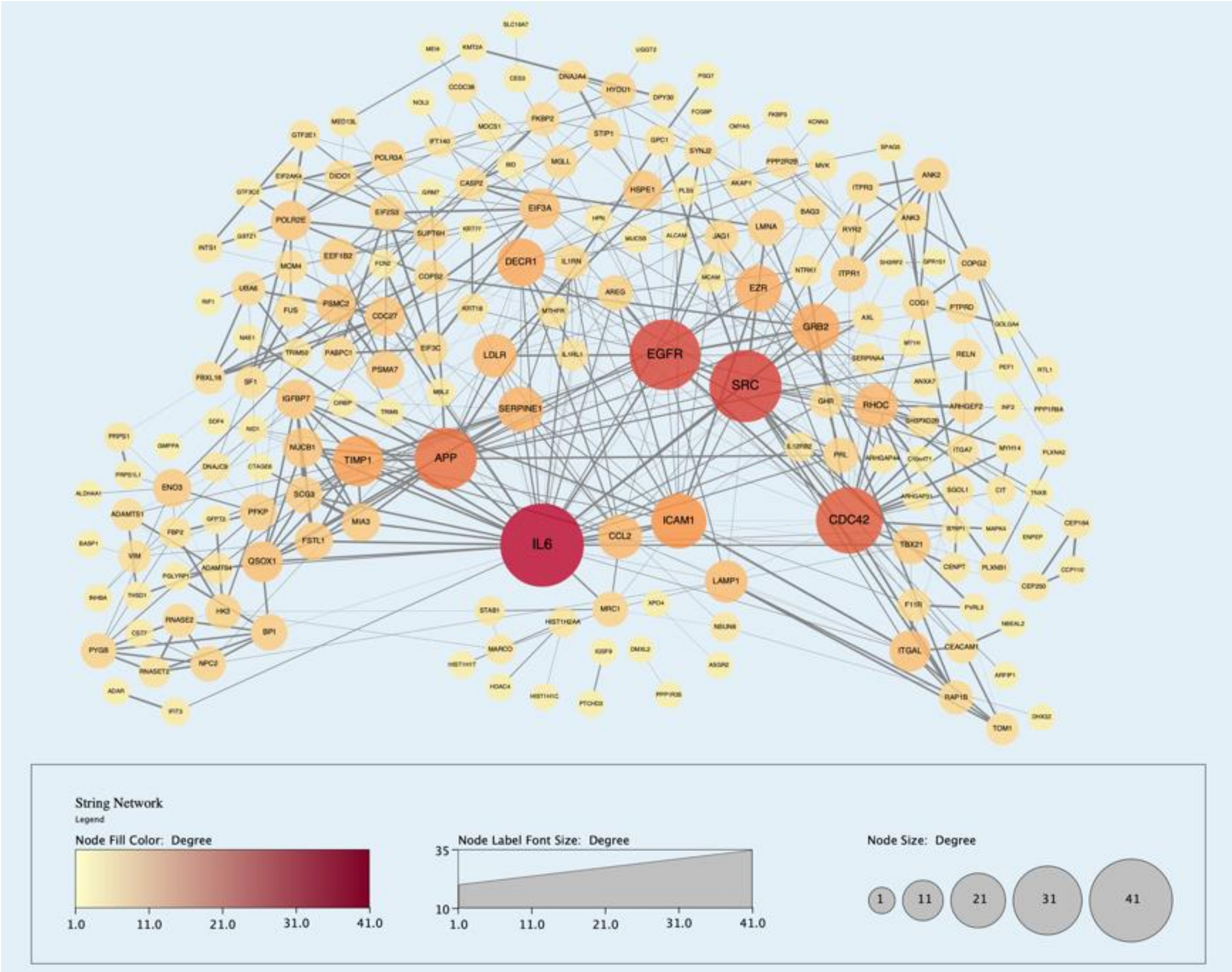
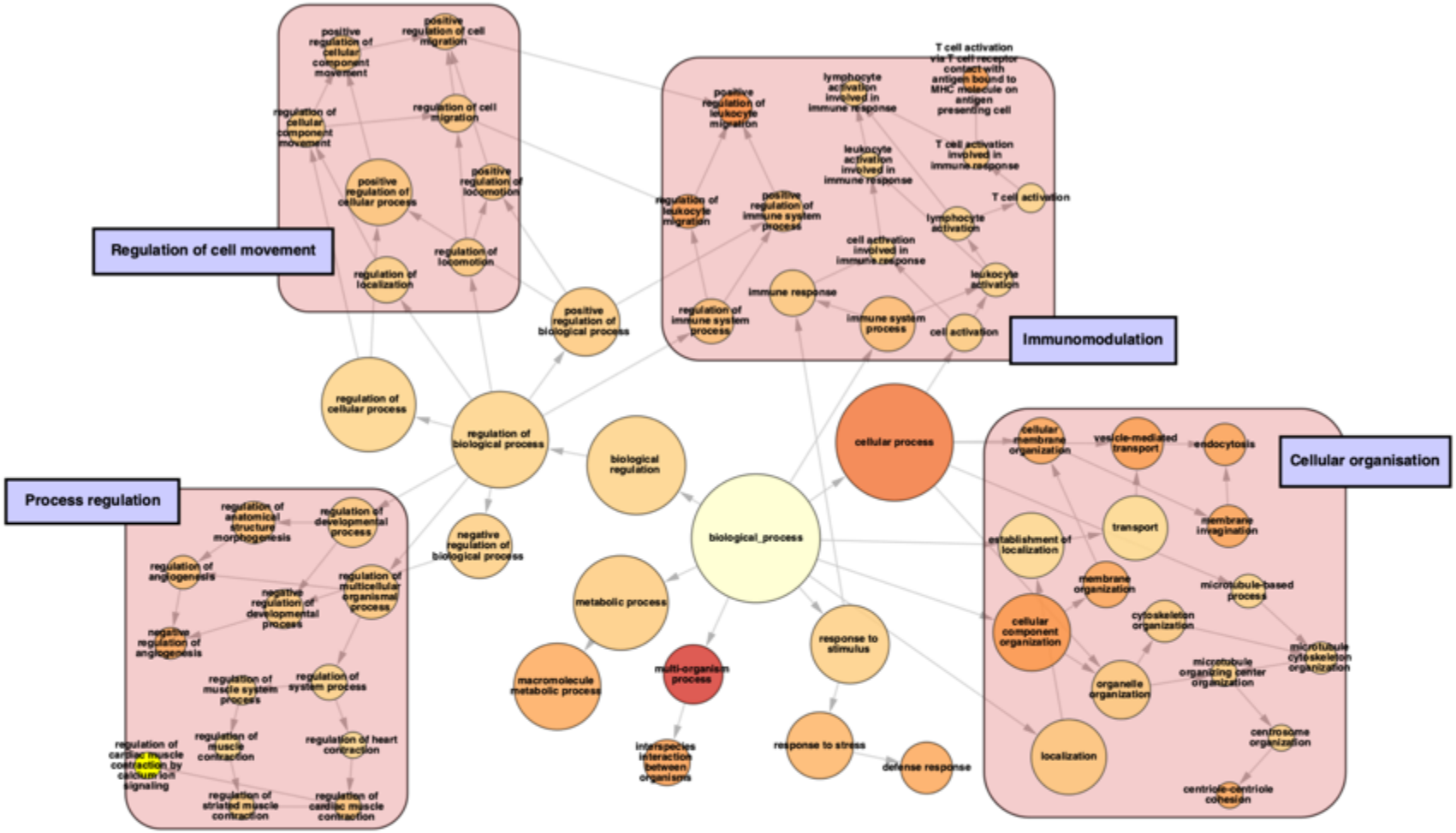

IL6
EGFR
SRC
CDC42
APP
ICAM1
TIMP1
DECR1
SERPINE1
LDLR
EZR
GRB2
RHOC
CCL2
LAMP1
ITGAL
TBX21
QSOX1
FSTL1
MIA3
SCG3
NUCB1
IGFBP7
PFKP
ENO3
PSMC2
PSMA7
CDC27
EEF1B2
POLR2E
EIF3A
HSPE1
LMNA
ITPR1
ANK2
COPG2
String Network
Legend
Node Fill Color: Degree
1.0
11.0
21.0
31.0
41.0
Node Label Font Size: Degree
35
10
1.0
11.0
21.0
31.0
41.0
Node Size: Degree
1
11
21
31
41

Figure 8.9 Protein-protein interaction (PPI) network demonstrating unique MAPC perfusate proteome

Protein-protein interaction (PPI) network demonstrating 191 unique proteins (nodes) identified in the perfusate of livers infused with MAPC cells during NMP-L. Node size and colour is proportional to the number of Interactions associated with said protein. “Edges” or Interactions are based on high confidence of interaction (String database confidence score >0.700).



Network cluster demonstrating the pathways involved following functional enrichment analysis

Legend

Node Size: Number of proteins associated with pathway/process

Node Fill Color: Proportional to p-value after Function Enrichment

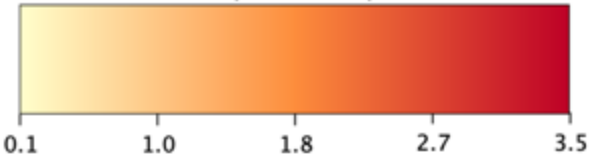


Figure 8.10 Network cluster demonstrating categories of gene ontology processes

Network cluster demonstrating categories of gene ontology processes that the proteins are involved with following function enrichment analysis. Proteins are grossly involved with regulation of a range of biological a cellular processes, immunomodulation, cellular movement and compartment organisation. Node size is proportional to the number of proteins involved with said process.

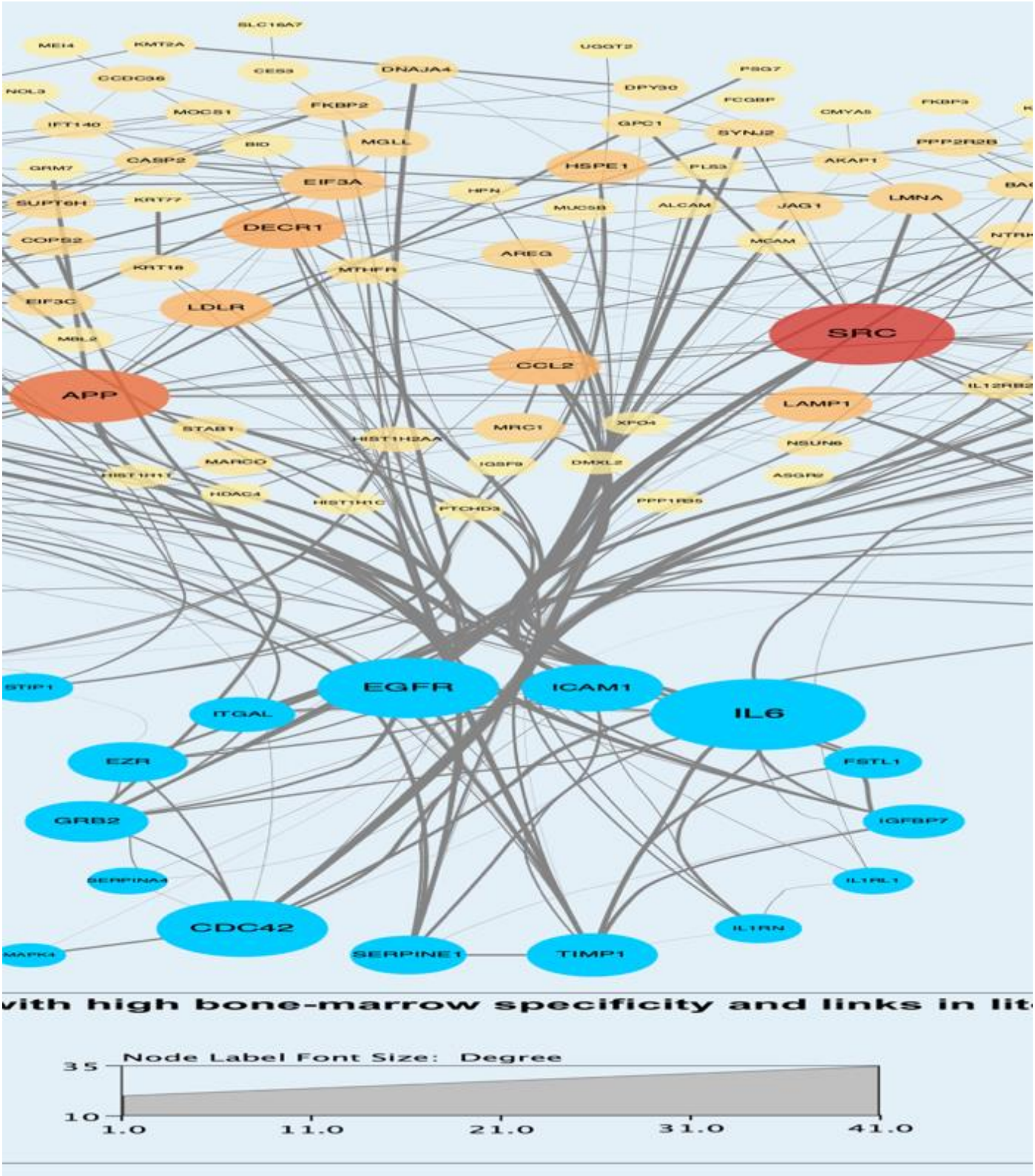


Figure 8.11 Protein-protein interaction (PPI) network highlighting proteins with evidential links to MAPC cells. Protein-protein interaction (PPI) network highlighting proteins with evidential links to MAPC cells and MSC in the literature (blue nodes). Of note, these proteins are some of those with the largest number of interactions and roles in biological processes.

Table 8.1 Descriptions of proteins identified unique to perfusate following MAPC cells administration with links in the literature to MAPC cell and MSC activity

Protein	Description
IL6	B-cell stimulatory factor 2; Cytokine with a wide variety of biological functions. It is a potent inducer of the acute phase response.
EGFR	Receptor tyrosine kinase binding ligands of the EGF family and activating several signalling cascades to convert extracellular cues into appropriate cellular responses.
CDC42	Cell division control protein 42 homolog; Plasma membrane-associated small GTPase which cycles between an active GTP-bound and an inactive GDP-bound state.
ICAM1	Intercellular adhesion molecule 1; ICAM proteins are ligands for the leukocyte adhesion protein LFA-1 (integrin alpha-L/beta-2).
TIMP1	Tissue inhibitor of metalloproteinases 1; Metalloproteinase inhibitor that functions by forming one to one complexes with target metalloproteinases.
GRB2	Growth factor receptor-bound protein 2; Adapter protein that provides a critical link between cell surface growth factor receptors and the Ras signalling pathway.
EZR	Cytovillin; Probably involved in connections of major cytoskeletal structures to the plasma membrane.
SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1; Serine protease inhibitor. This inhibitor acts as 'bait' for tissue plasminogen activator, urokinase, protein C and matriptase-3/TMPRSS7.
ITGAL	Leukocyte function-associated molecule 1 alpha chain; Integrin alpha-L/beta-2 is a receptor for ICAM1, ICAM2, ICAM3 and ICAM4.
IGFBP7	Insulin-like growth factor binding protein 7; Binds IGF-I and IGF-II with a relatively low affinity. Stimulates prostacyclin (PGI2) production. Stimulates cell adhesion.
FSTL1	Follistatin-related protein 1; May modulate the action of some growth factors on cell proliferation and differentiation.
HYOU1	Hypoxia up-regulated 1. Has a pivotal role in cytoprotective cellular mechanisms triggered by oxygen deprivation. May play a role as a molecular chaperone and participate in protein folding.
IL1RN	Interleukin-1 receptor antagonist protein; Inhibits the activity of interleukin-1 by binding to receptor IL1R1 and preventing its association with the coreceptor IL1RAP for signalling.
STIP1	Transformation-sensitive protein IEF SSP 3521; Acts as a co-chaperone for HSP90AA1. Mediates the association of the molecular chaperones HSPA8/HSC70 and HSP90.
IL1RL1	Interleukin 1 receptor-like 1; Receptor for interleukin-33 (IL-33). Its stimulation recruits MYD88, IRAK1, IRAK4, and TRAF6, followed by phosphorylation of MAPK3/ERK1 and/or MAPK1/ERK2, MAPK14, and MAPK8.
SERPINA4	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 4; Inhibits human amidolytic and kininogenase activities of tissue kallikrein.
MAPK4	Extracellular signal-regulated kinase 4; Atypical MAPK protein. Phosphorylates microtubule- associated protein 2 (MAP2) and MAPKAPK5. May promote entry in the cell cycle.

8.8.5 Discussion

This is the first study to demonstrate the feasibility and potential advantages of using NMP-L to deliver stem cell therapy to marginal human donor livers. Our data demonstrate that delivery of MAPC cells to human donor livers is feasible, has no detrimental effect on flow or resistance, cells infused via the artery appear to undergo transendothelial migration and there is evidence of beneficial biological activity.

MAPC cells are a distinct bone-marrow derived cellular population that share properties associated with MSC. Unlike standard MSC culture conditions however, they prefer hypoxic conditions in media supplemented with epidermal growth factor and platelet-derived growth factor. MAPC cells have been shown to be non-immunogenic and exert strong immunosuppressive effects on T-cells in vitro and may also supress an ongoing immune response (12, 42). These findings paved the way for the use of MAPC cells in models of graft-versus host disease and as an anti-inflammatory therapeutic treatment in models of transplantation. MAPC cells were chosen for this study because they share many of the positive properties of MSC, and a clinical grade version of MAPC cells, MultiStem® cells, have been evaluated in several clinical trials and are easily scalable for use in future NMP-L clinical trials (43-45).

Most animal studies using stem cells in models of liver transplantation deliver the cells either systemically intravenously (where most cells are trapped in the lungs) or via the portal vein – a route that was used for a safety and feasibility study in human subjects (16). Indeed, the portal venous route is also the preferred route for islet cell infusion for the treatment of Type-I diabetes although increased portal venous resistance has been demonstrated (46). The argument for systemic infusion is that the cells appear to exert effects through paracrine mechanisms and

soluble mediators (47). Despite this, their effects appear to be strengthened when cell–cell contact is present (42). This points to the presence of cell contact-dependent suppressive activity or suggests that the interaction of immune cells to MAPC cells upregulates their suppressive function through other soluble factors. The process of machine perfusion provides a valuable window of opportunity to deliver cellular therapy directly into the target donor organ, ensuring the presence of the anti-inflammatory therapy before the onset of the immune response during organ reperfusion at clinical transplantation. In this study, there was no evidence of increased resistance or reduced flows when cells were infused via either vascular route. The transient increases in arterial flow are addressed later in the discussion.

Cells were easily identified using fluorescence microscopy, although cells never appeared in the left lobe suggesting that cells became trapped in the disposable circuit if they did not engraft on the first pass. There appeared to be a difference in MAPC homing depending on route of infusion with cells infused via the portal vein "arresting" within the sinusoidal channels (localisation) whereas arterially-infused cells transmigrated across the vascular endothelium (homing) (Figures 8.5, 8.6 and 8.7). These cells also appeared to undergo some form of conformational change possibly through "inside-out" signalling or changes in integrin conformation (48). They fluoresce in the green channel as well as the red after crossing the vascular endothelium to reside within the parenchyma (Figure 8.7). This observation is similar to that seen in flow assays when migrated cells go from phase light to phase dark and may well influence fluorescent spectral overlap during confocal microscopy.

Hepatic sinusoidal endothelium differs from vascular endothelium in terms of structure and adhesion molecule expression. Despite hepatic sinusoidal endothelium having increased expression of intercellular adhesion molecule-1 (ICAM-1), the absence of cell-cell junctions

and reduction in p- and e-selectin expression may reduce the chances of MAPC transmigration across sinusoidal endothelium when infused via the portal route. Cells infused via the artery must pass through a narrow pre- or inter-sinusoidal confluence which may improve their changes of retention within the tissue. The arterial system also supplies the bile ducts and presence of cells near the bile ducts may help ameliorate the bile duct endothelial damage that can occur at reperfusion.

When looking for evidence of MAPC functional activity, Luminex analysis of perfusates from different time points yielded some interesting results. Of the 35 intended targets, 13 were detectable in the perfusate. Four of these appeared to be related to graft quality and not the presence of cells although TNF-a, IFN-gamma and IL1-RA have been shown to upregulate the immunomodulatory effects of stem cells. TNF-a and IFN-gamma, which drive inflammatory and immune mediated responses via activation of macrophages and induction of MHC-II molecules, increased over the course of the perfusion. In combination, they have been shown to increase the immunosuppressive effects of MAPC cells through indoleamine 2,3 dioxygenase activation (49, 50). IL-1RA has also been shown to be an effective anti-inflammatory mediator when used in combination with MSC in models of acute liver failure (51). As mentioned, the concentrations of 9 targets appeared to be related to the timing of MAPC cell infusion. IL4 has been shown to suppresses liver TNF-a mRNA expression, neutrophil accumulation and liver injury (52) whilst IL-10 has been shown to protect against hepatic ischemia-reperfusion injury by suppressing NFkB activation and subsequent expression of pro-inflammatory mediators (53) and importantly both have been shown to be upregulated following MAPC administration (54, 55). MCP-1 (CCL2) expression appeared to correlate with cell infusion and has been shown to be secreted by MAPC cells (56). Stimulation of MAPC cells using TNF-a and IFN-g increases expression of chemokine receptor type 2 and

promotes migration of the cells to areas of inflammation where MCP-1 (CCL2) is being secreted. This stimulation also increases transcription of iNOS and cyclooxygenase-2 mRNA which leads to production of NO and PGE which are involved mechanistically in the suppression of T-cell proliferation (57, 58). The presence of NO in the MAPC cells-containing media may explain the transient increase in arterial flow and decreased vascular resistance when cells were infused into the right lobe, which subsided within 10 minutes of the infusion stopping(59). The precise mechanistic relevance of these observations are not clear at present and remain the subject of ongoing research in our group. However, a potential explanation is that the anti-inflammatory response may be liver centric and attempting to reduce the extent of parenchymal injury whilst the increase in inflammatory markers is allowing the potential influx of immune cells that are required for later liver injury resolution (60, 61).

To determine the presence of potentially unique MAPC-associated proteins, proteomic analysis of the individual perfusate samples taken after cell infusion was compared to those samples pre-infusion and to eight similar livers that did not receive cellular therapy. The analysis as described in the results section would suggest that MAPC cells, in the presence of a pro-inflammatory environment as confirmed by multiplex analysis, secrete molecules that regulate the biological activity of the extracellular matrix as well as chemokines, cytokines, and molecules that participate in and regulate a variety of biological pathways (Figure 8.10 and Table 8.1). Many of these proteins have previously been described in the secretome of MAPC cells and could play an important role in a pro-inflammatory environment, during for example, ischemia-reperfusion (62). The expression of HYOU1 suggests that MAPC cells may be involved in the enhancing the cytoprotective mechanisms within the liver during NMP-L (63). In addition, MAPC cells increase the expression of known cell cycle proteins such as GRB2, MAPK4 and the growth factor EGFR. Furthermore, proteins involved in tissue injury

resolution such as TIMP-1 and STIP1 are also upregulated suggesting that MAPC cells may regulate this part of the IRI process too (64). The expression of ITGAL and ICAM-1 suggests a potential immuno-modulatory role for MAPC cells although this needs further experimental clarification (65).

We are aware of several limitations in this study, in particular the number of livers included, the timings of the infusions and the different routes of delivery, all of which in combination impact upon the statistical power of the study. We spent a long time considering how best to carry out this research in a cohort of organs that are scarce and generally very heterogenous in nature. Importantly it is precisely such organs that may benefit from this type of therapeutic approach in future. In terms of research, livers they obviously differ to kidneys in terms of blood supply and the number in the body. The use of discarded kidneys affords the researcher the opportunity to use one for the intervention and one as a control. Nor is there the need to consider the blood supply to use for delivery of the therapy. In contrast in livers, we must consider the optimal route for delivery and also try to create some form of internal control as discarded human livers are too heterogenous to be able to draw robust statistical conclusions given the limited numbers offered for scientific research. We were also unable to comment on the effect of MAPC cell delivery on overall organ "viability" or the ability of the MAPC cells to "rescue" an organ currently deemed untransplantable. In this regard, multiple factors are at play in terms of overall organ viability. It is likely that the mechanisms at play may not significantly impact upon gross organ viability but are more likely to attenuate the inflammatory and immune responses at a cellular level and this would hopefully translate into improved outcomes following in-situ reperfusion.

This research, as stated in the aims was a pilot study that set out to a) develop a technique for infusion and demonstrate the feasibility of NMP-L to deliver cellular therapy to extended criteria human donor livers; b) determine the best vascular route for delivery and confirm the presence of cellular engraftment and c) determine parameters that may reflect biologically functional activity imparted by the presence of the therapeutically administered MAPC cells. Whilst we recognise that the comparatively small n-numbers and differences of timing of infusion of the cells were potential limitations to our study, we nevertheless believe that the techniques and the data obtained are sufficiently robust to permit cautious but valid analysis and conclusions.

8.8.6 Conclusion

This is the first study to investigate the feasibility of using machine perfusion to deliver cellular therapy to human donor livers. We have demonstrated that cells can be delivered directly to the target organ without compromising the perfusion. This not only overcomes the disadvantages associated with systemic infusion, but ensures the cells are present before ingress of the recipient immune cell population. The arterial route of infusion appears to result in more effective cellular engraftment. MAPC cells secrete a host of soluble factors that are known to have anti-inflammatory and immunomodulatory effects that would be especially beneficial for extended criteria donor livers.

8.8.7 Conflict of Interest

Anthony Ting is an employee of Athersys Inc. Samantha Stubblefield was an employee of Athersys at the time the work was completed. Valerie Roobrouck is employed by ReGenesys BVBA. 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.

8.8.8 Author's contributions

Richard W Laing: Conception and design, Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing, Final approval of manuscript,

Samantha Stubblefield: Conception and design, Administrative support, Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing

Lorraine Wallace: Collection and/or assembly of data, Data analysis and interpretation

Valerie D Roobrouck: Provision of study material or patients, Data analysis and interpretation, Manuscript writing

Ricky H Bhogal: Data analysis and interpretation, Manuscript writing,

Andrea Schlegel: Data analysis and interpretation, Manuscript writing,

Yuri L Boteon: Collection and/or assembly of data,

Gary M Reynolds: Collection and/or assembly of data, Data analysis and interpretation,

Anthony E Ting: Conception and design, Manuscript writing, Final approval of manuscript,

Darius F Mirza: Conception and design, Manuscript writing,

Philip N Newsome: Conception and design, Manuscript writing,

Hynek Mergental: Collection and/or assembly of data, Provision of study material or patients,

Simon C Afford: Conception and design, Manuscript writing, Final approval of manuscript,

8.8.9 Acknowledgments

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Bridget Gunson for her assistance in obtaining the regulatory approval for the study. Finally, we would like to thank our organ donors, their families and the NHSBT network for allowing us to perform this work. This paper presents independent research supported by the NIHR Birmingham Liver Biomedical Research Unit and the views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

8.8.10 Supplementary Material

Table S8.1. Analysis of 17 proteins with links to MAPC cells and MSC in the literature using Cytoscape.

Protein	Interaction Rank	Betweenness Centrality	Closeness Centrality	Clustering Coefficient	Degree	Neighbourhood Connectivity	Accession Number
IL6	1	0.241	0.463	0.148	41	10.39	P05231
EGFR	3	0.189	0.458	0.165	32	11.66	Q9H2C9
CDC42	4	0.163	0.427	0.128	29	9.69	P60953
ICAM1	6	0.051	0.409	0.252	21	13.38	P05362
TIMP1	7	0.023	0.376	0.373	18	13.44	P01033
GRB2	8	0.054	0.412	0.258	16	14.44	P62993
EZR	10	0.064	0.390	0.219	15	13.27	P15311
SERPINE1	11	0.016	0.384	0.473	14	17.43	P05121
ITGAL	16	0.009	0.349	0.382	11	14.00	P20701
IGFBP7	19	0.007	0.338	0.711	10	14.80	Q16270
FSTL1	25	0.002	0.336	0.806	9	15.33	Q12841
HYOU1	36	0.016	0.292	0.190	7	5.14	Q9Y4L1
IL1RN	46	0.002	0.330	0.467	6	16.67	P18510
STIP1	47	0.007	0.326	0.400	6	9.00	P31948
IL1RL1	82	9.44E-05	0.320	0.500	4	15.75	Q01638
SERPINA4	83	6.31E-05	0.336	0.833	4	24.75	P29622
MAPK4	122	4.23E-04	0.303	0.000	2	17.00	P31152

Table S8.2. Analysis association with tissue types of 17 proteins identified, with links to MAPC cells and MSC in the literature using Cytoscape. The analysis shows the high affinity of some of the proteins for liver and bone marrow tissue.

	Tissue Association						
Protein	**Blood**	**Bone marrow**	**Intestine**	**Kidney**	**Liver**	**Lung**	**Spleen**
IL6	3.918653	3.707035	3.74526	3.165083	3.650198	4.847484	3.552847
EGFR	3.107672	2.458089	3.563364	3.261474	4.720948	3.374401	2.973813
CDC42	4.863896	3.196989	3.647546	4.279603	3.263513	4.30881	3.330003
ICAM1	4.737942	4.679953	3.26731	4.60969	4.691104	4.959892	3.788965
TIMP1	4.692988	4.617691	3.790703	3.386743	3.687787	4.853358	3.464487
GRB2	4.657075	3.150647	3.148853	3.156573	3.319363	4.865175	3.031142
EZR	3.024185	2.792432	4.969846	4.81621	3.44159	4.420051	3.301009
SERPINE1	3.547812	2.039812	3.167064	3.13832	4.324948	4.830076	2.521216
ITGAL	3.40197	3.010623	2.826971	2.152066	2.310327	2.582356	3.337133
IGFBP7	2.347871	2.696723	3.946526	3.646674	3.184157	3.302853	3.127803
FSTL1	2.419277	4.358165	2.774837	4.775193	2.714848	3.660654	3.017779
HYOU1	4.515013	4.423618	3.726329	4.020683	4.803292	3.948119	2.567119
IL1RN	3.484812	4.664049	2.953125	2.249941	3.062847	2.741958	2.559191
STIP1	3.115767	2.036527	4.161274	2.649974	4.665295	4.709531	2.188149
IL1RL1	4.463048	4.381139	2.319659	2.361319	2.240113	3.18001	2.439147
SERPINA4	2.459454	4.224	4.442	2.701973	3.569866	1.841511	1.329396
MAPK4	1.733858	1.412989	2.402393	2.309261	2.427458	2.231179	2.146079

Table S8.3. Analysis association with cellular compartment of 17 proteins identified, with links to MAPC cells and MSC in the literature using Cytoscape.

	Compartment association										
Protein	**Cytoskeleton**	**Cytosol**	**Endoplasmic reticulum**	**Endosome**	**Extracellular**	**Golgi apparatus**	**Lysosome**	**Mitochondrion**	**Nucleus**	**Peroxisome**	**Plasma membrane**
IL6	3.354946	3.659442	4.611653	2.931142	5	2.31369	3.06277	3.428203	3.932258	2.669886	4.722855
EGFR	3.35739	4.117444	4.096257	5	5	4.025284	3.138361	2.935897	5	2.122962	5
CDC42	5	4.764543	4.466275	2.728715	4.591196	4.004077	2.395977	2.609172	3.439988	1.37444	5
ICAM1	2.988354	2.938765	2.407166	2.411702	5	1.848586	2.474571	2.701092	3.286699	2.017282	4.892737
TIMP1	2.748153	2.429379	4.423712	1.699162	5	2.717778	2.041388	2.415094	2.844126	1.758408	2.783159
GRB2	2.719478	4.800379	2.43886	5	4.542171	3.747823	2.221659	2.163797	5	1.251704	4.613605
EZR	5	5	1.974761	5	4.516862	1.89956	1.982185	1.962349	4.70477	1.40228	5
SERPINE1	2.655127	3.471297	2.129191	1.643996	5	1.446422	2.200859	2.444159	2.884933	2.087754	4.565001
ITGAL	2.222182	1.935194	1.875613	1.617751	4.526557	1.386448	1.841888	1.805171	2.130471	0.28125	5
IGFBP7	2.014915	1.870236	4.284489	1.383464	5	2.277696	1.552975	1.896298	2.189255	0.956855	2.650884
FSTL1	1.788215	2.859218	4.286644	1.660466	4.780764	2.022792	1.36825	1.478438	1.95462		1.908227
HYOU1	1.595914	2.019739	5	1.533618	4.47633	2.341553	1.647339	1.984172	1.819646	1.1072	1.641448
IL1RN	2.052677	3.682477	1.811694	1.611266	5	1.027892	1.939878	2.092753	3.033866	1.302627	4.537062
STIP1	1.898959	4.746566	1.990733	1.244352	1.685461	4.243705	1.364771	1.889113	5	1.180357	2.756355
IL1RL1	1.652793	4.36579	1.272179	1.233713	5	0.951325	1.187238	1.364088	2.263667	1.421838	5
SERPINA4	2.385003	2.441871	2.953946	2.557145	5	2.741868	2.128396	1.991044	2.233325	1.485331	3.128373
MAPK4	1.549089	4.542494	1.047963	0.937485	1.485496	0.890861	0.651552	1.479566	4.51324	0.853315	1.455112

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PART IV – CONCLUDING REMARKS

CHAPTER 9 - OVERVIEW OF THESIS

9.1 GENERAL DISCUSSION AND CONCLUSION INCLUDING LIMITATIONS AND FUTURE WORK

9.9.1 Summary

The projections for the prevalence of liver disease worldwide are sobering. Despite advances in the treatment and prevention of hepatitis B and C, they are still the main cause of cirrhosis globally, especially in low-income countries(1). As healthcare infrastructure improves, hepatitis B and C are expected to be overtaken by NASH as the biggest cause of cirrhosis in the near future and we also know that the reported burden of NAFLD and NASH appears to be under-reported(2). In an absence of effective pharmacological treatments for end-stage liver disease and NAFLD, an ongoing problem with alcohol-related liver disease and the inability to achieve eradication of hepatitis B and C, the end result is inevitably an increase in the number of patients requiring transplantation. This, in an era when people are living longer with an increasing number of co-morbidities, means that we become reliant on the use of donor livers which we know are associated with inferior outcomes. Chapter 1 summarises these challenges and Chapter 2 highlights some of the issues that are associated with marginal DBDs and extended criteria DCDs. Despite being able to achieve comparable outcomes in our high volume centre, these are not necessarily translatable to smaller centres with less experienced surgeons who are unfamiliar with the assessment of such grafts and where graft loss, or worse patient loss, could have wider reaching implications for a low-volume transplant programme.

Normothermic machine perfusion of the liver (NMP-L) offers the surgeon the opportunity to observe the liver in near-physiological conditions and assess metabolic and biliary function. This objective assessment is far superior to the subjective assessment of donor and recipient factors and also allows for extension of the preservation time and improved transplant logistics and Chapter 5 highlights some of the past and current research in the field. Chapters 3, 4, 5 and

6 show how we firstly developed our viability criteria for use during normothermic machine perfusion and then how we used them in a clinical pilot study to transplant 5 patients with livers discarded due to a perceived high-risk of use. The VITTAL Trial (Viability testing and transplantation of discarded livers) was funded by the Wellcome Trust and was designed to safely validate our viability criteria and assess the ability of NMP-L to salvage discarded liver grafts. The trial met its co-primary endpoints with 22/31 livers used and 22 patients meeting 90-day patient survival. The trial has undoubtedly demonstrated the promise of NMP-L in being able to improve the lives of transplant recipients.

Part III of this thesis shows ways in which the process of NMP-L can be manipulated to improve certain aspects of perfusion and how the technology can be used to introduce therapeutics. Chapter 7 describes how Hemopure, a haemoglobin-based oxygen carrier, can be used in place of packed red cells in the perfusion fluid. This off-the-shelf product has excellent oxygen carrying capacity and low immunogenicity. The low viscosity and oxygen dissociation characteristics also mean it can be used across a range of temperatures. Chapter 8 shows how NMP-L can be used to deliver cellular therapy to donor livers prior to transplantation. Multipotent adult progenitor cells (MAPC cells, Athersys, Cleveland, Ohio) were infused into discarded donor livers and were shown to engraft when infused via the hepatic artery and have a beneficial unique secretory proteome.

9.9.2 Evolution of the research

I joined the lab at a time when machine perfusion was really starting to garner interest in the world of liver transplantation. As a result, the field progressed quickly and to a degree has been a victim of its own success. I am sure many would agree that the determination for groups to advance the field and publish meant that methodological rigour was not always at the forefront

of the research. Small numbers of cases, single centres, non-randomised trials focussing on a single modality, matched retrospective case series' (heavily open to bias)…and yet, it is now 6 years since I started my period pf research, during which time an international multicentre randomised control trial comparing different perfusion modalities could be well on the way. There is now an acceptance that the field requires high quality RCT's in order to answer important questions, which up until now have been debating using a degree of 'eminence-based medicine'. The COPE trial and the VITTAL trial set the standard for others as they attempted to use both novel trial design and higher levels of evidence to bring robust data to the literature. Both trials however struggled to develop primary end points that were broadly accepted amongst competing units. Considering what is entailed – taking an organ from a dying patient and placing it in another humans failing body - outcomes in transplantation are already exceptional. There is however a stark disparity in one area – organ utilisation. Both trials demonstrated an improvement in organ utilisation when compared to cold storage which is of course still the gold standard for organ preservation. It may be that going forward, organ utilisation is employed as a primary endpoint for machine perfusion studies.

The detailed limitations of the studies were addressed in the publications and we have always been very open and realistic about our results and conclusions. The debate surrounding normothermic machine perfusion versus other modalities such as normothermic regional perfusion in DCDs, hypothermic, subnormothermic or controlled oxygen rewarming continue to rage on and unless we find a way as a community to collaborate, it may never be conclusively settled. Each has its own advantages and disadvantages and, in the future, I suspect as a community we will conclude that certain techniques are suitable for different grafts. Work carried out by my colleague Yuri Boteon for example has shown that controlled oxygenated rewarming was beneficial for steatotic marginal grafts and it may be that steatotic grafts are

better preserved normothermically from the point of retrieval to avoid cold storage all together(3). The results from the HOPE and DHOPE trials will be important in determining the place of hypothermic machine perfusion in the future.

Graft "viability", we now know, is controlled by a huge number of variables, circumstances (both donor and recipient) and metabolic pathways. At the beginning of this research, the simplistic view of 'lactate metabolism = graft viability' was borne from the observing a so-called 'transplantable' liver on the device. The viability criteria were built around this concept and the projects progressed. The importance of assessing cholangiocyte health during the perfusion process was less well known and we focussed more on the hepatocyte metabolic capacity of the grafts when developing our criteria. We now know that cholangiocyte health, certainly in our extended criteria DCD cohort, is very important and despite our criteria being sensitive and specific for immediate graft function, we had three grafts in the VITTAL trial that were lost due to ischemic type biliary lesions. For the majority of organs we test, true "viability" i.e. the likelihood of developing primary non-function, is less of a concern. In fact what is more of a concern is the degree of immune-mediated injury at a cellular level and this is the direction in which the field is moving. Cellular therapy holds some promise here but again, the methodology that was used in Chapter 8 (although novel and of some importance) was not ideal and these points were highlighted. Animal models provide the researcher the opportunity to theoretically deliver reproducible results from a homogenous group in greater numbers. It is my belief that for early mechanistic evidence, animal models are an important adjunct to further the field (something that Schlegel et al have been able to do so well with HMP and Friend et al achieved with the early NMP work as referenced through this thesis). Prior to starting work on cellular therapy in the human liver MP model, a body of animal work could have unlocked several of the questions regarding homing, immunomodulation, dose

escalation etc. The small number of included grafts in some of the proof-of concept work has been highlighted and we appreciate that this is suboptimal. These human livers that are offered for research purposes are an incredibly valuable, yet scarce, resource and we believe that despite the small numbers used in some of the experiments, the models still generate important results that the transplant community can gain vital information from.

9.9.3 Future direction

There are several interesting avenue for future research work. The first is to analyse different viability criteria in order to take into account factors such as graft size and correlate these with 'transplantability' and patient outcomes. Some of these criteria such as tissue oxygen extraction were explored in Chapter 7, but others such as rate of change of lactate adjusted for liver weight, perfusate enzymes, flow rates adjusted for liver size etc, may well hold the key to more accurate stratification of grafts during machine perfusion. At the same time, further analysis of the VITTAL cohort examining biliary health and bile biochemistry will also be looked at to see if this is able to in fact predict the development of biliary complications. The use of artificial oxygen carriers continues to garner interest and haemoglobin based oxygen carriers address issues surrounding immunogenicity, logistics and rheology across a range of temperatures.

Chapter 8 highlighted the huge potential of this technology to intervene in the transplant process with therapeutics. The use of stem cells in a transplant model is of great interest and hopefully this publication is a starting point for the clinical application of stem cell therapy in liver transplantation. Multipotent adult progenitor cells, mesenchymal stem cells and extracellular vesicles, delivered pre-transplantation, continue to attract attention and phase I clinical trials should be the next step to take forward the animal work that has already been performed. The challenge with such trials will be again deciding on trial end-points, modality

used and supporting the clinical trials with a robust and well-designed scientific work package that can report on important mechanistic effects.

9.9.4 Conclusion

The development of machine perfusion has brought about the dawn of a new golden age in transplantation. It has been an incredible honour to be involved with this exciting field of research which has already seen patients benefit from the technology. Irrespective of which path it takes, this important work will always form part of the backbone upon which improvements can and will undoubtedly be made.

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