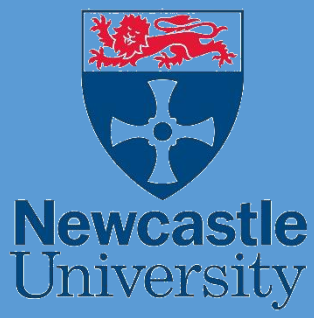
Donor Liver Optimisation and Real-Time Pre-Implant Assessment

PhD Thesis



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Abstract

The number of livers offered for cadaveric donation currently exceeds demand in the United Kingdom. However, the quality of these organs means that nearly a third are rejected for transplantation. In particular utilisation of fatty (steatotic) and organs from donors after circulatory death (DCD) are poor. The aims of this project were to develop novel cost effective ex-situ perfusion viability and reconditioning techniques and explore accurate methods of steatosis quantification.

Ex situ perfusion was introduced using our previous experience in kidney perfusion, taking advantage of the inherent safety benefits of hypothermic perfusion before progressing to normothermic perfusion. A novel perfusion setup was utilised for Hypothermic Oxygenated Perfusion of livers (HOPE), with a custom-designed circuit and adapting existing experience of ex-vivo kidney perfusion using Medtronic perfusion equipment.

A total of 10 livers were transplanted following HOPE, all with significant donor and recipient risk factors, with very promising results. All recipients are alive with a minimum of 20-months follow-up and none have developed clinically-significant ischaemic cholangiopathy. This compares favourably with a historical local cohort that would have met current inclusion criteria for perfusion. Only 1 recipient has had a retransplant, due to rejection.

A number of modalities for assessing livers during perfusion have been explored. We have demonstrated that lactate clearance in normothermic machine perfusion (NMP) cannot be safely relied upon, as livers that had sustained damage well beyond the scope of transplantation still cleared lactate. Bile production has proved to be of some importance in our series, but not all published evidence supports this. Extensive analysis of perfusate biomarkers has identified a potential scoring system that could differentiate between transplantable and untransplantable livers in our series, but needs further validation. We have described an objective method of analysing liver microcirculation, and have demonstrated that NMP improved the arterial microcirculation, but that with extended ischaemia the benefit of NMP is lost.

We have demonstrated in rodent cell lines that steatosis adversely affects viability in hypoxia. In addition we have developed a preliminary point-of-care steatosis assessment method, which could prove beneficial with further calibration and validation.

Utilising ex-situ perfusion to modulate and treat organs is an exciting potential to further improve marginal organs. We selected Pregnane-X Receptor (PXR) activation as our treatment target for this based on rodent experiments demonstrating its benefit in reducing ischaemia-reperfusion injury. These experiments demonstrate upregulation of the end-targets of PXR during human NMP, proving

its potential as a platform for drug delivery. We were unable to demonstrate significant benefits to the livers within the limited timeframe of perfusion, as it is likely that any potential advantages of PXR activation would only become apparent in a much longer time period.

Dedication

This thesis is dedicated to my family, without whose love and support it would not have been possible.

Acknowledgements

Firstly, I would like to thank my wife Sarah who has been alongside me throughout and has provided unwavering support and understanding in the many hours of absence during the past few years that has been dedicated to this thesis. And to our sons Luciano and Emilio, who have provided much love and happiness in our lives. And also my parents, my brother and his family, for encouragement, love and support throughout.

I would also like to thank my supervisors; Mr Colin Wilson and Professor Matthew Wright, who have always provided leadership, guidance, and support. Have always been available when needed and helped me keep my research on track. Without their wisdom, hard work and experience, this would not have been possible.

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Incredible support was offered to us by Dr Andrew McNeill and Ben Stenberg from our radiology department, being available at unsociable hours to perform contrast-enhanced ultrasound on our livers, for which I am very grateful. Dr John Brain provided very useful histopathological advice and support as well as grading countless slides, and Dr Kim Pearce very kindly analysed my statistical methods and gave useful insights.

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Presentations and Publications

Figueiredo RS, Sewpaul A, Bates L, Ibrahim I, Thompson E, Talbot D, Sen G, French JJ, Manas DM, White SA, Wilson CH. **End-Ischaemic Hypothermic Liver Perfusion is Safe and Effective Method of Reconditioning High-Risk Livers**. Oral Presentation and Runner up to Calne-Williams Medal at British Transplant Society meeting March 2019

Figueiredo RS, Sewpaul A, Leitch A, Bates L, Ibrahim I, Thompson E, Wright MC, Wilson CH. **Viability assessment during D-HOPE liver perfusion**. Oral Presentation at British Transplant Society meeting March 2019

Figueiredo RS, Sewpaul A, Bates L, Ibrahim I, Thompson E, Talbot D, Sen G, French JJ, Manas DM, White SA, Wilson CH. **Clinical experience of hypothermic oxygenated liver perfusion with standard UW**. Oral Presentation at Society of Academic & Research Surgery January 2018

Figueiredo RS, Sewpaul A, Stenberg B, McNeill A, Manas DM, White SA, Wilson CH. **Contrast-Enhanced Ultrasound for Assessment of Livers Undergoing Ex-Vivo Perfusion**. Oral Presentation at Alpine Liver and Pancreatic Surgery meeting February 2017

Figueiredo RS, Sewpaul A, Manas DM, White SA, Wilson CH. **A single centre experience of establishing ex-vivo liver perfusion**. Oral Presentation at Alpine Liver and Pancreatic Surgery meeting February 2017

Figueiredo RS, Sewpaul A, Stenberg B, McNeill A, Manas DM, White SA, Wilson CH. **Normothermic Ex-situ Perfusion Improves Microcirculatory Flow on Contrast-Enhanced Ultrasound.** Poster Presentation at British Transplant Society meeting March 2017

Figueiredo RS, Sewpaul A, Bates L, Ibrahim I, Thompson E, Talbot D, Sen G, French JJ, Manas DM, White SA, Wilson CH. . **Clinical experience of hypothermic oxygenated liver perfusion.** Poster Presentation at British Transplant Society meeting March 2017

Figueiredo RS, Sewpaul A, Stenberg B, McNeill A, Manas DM, White SA, Wilson CH. **Normothermic Ex-situ Perfusion Improves Microcirculatory Flow on Contrast-Enhanced Ultrasound.** Poster Presentation at European Society for Organ Transplantation meeting September 2017

Figueiredo RS, Sewpaul A, Bates L, Ibrahim I, Thompson E, Talbot D, Sen G, French JJ, Manas DM, White SA, Wilson CH. **Clinical experience of hypothermic oxygenated liver perfusion.** Poster Presentation at European Society for Organ Transplantation meeting September 2017

Manuscript in submission on clinical outcomes of hypothermic liver perfusion

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Equations

List of Abbreviations

µg	microgram
µL	microlitres
ABG	Arterial Blood Gas
AF2	Activation Function 2
AhR	Aryl hydrocarbon receptors
AKI	Acute kidney injury
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
Apo	Apolipoproteins
AST	Aspartate Aminotransferase
AT	Anaerobic Threshold
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
BD	Bile Duct
bFGF	basic fibroblast growth factor
BNF	beta-naphthoflavone
C	Centigrade
Ca	Calcium
CAR	Constitutive Androstane Receptor
CBD	Common Bile Duct
CCL	Chemokine Ligand
CD	Control Diet
CEUS	Contrast-Enhanced Ultrasound
CIT	Cold Ischaemic Time
cm	Centimetres
CO2	Carbon Dioxide
COPE	Consortium for Organ Preservation in Europe
CRP	C-Reactive Protein
CT	Computerised Tomography
CYP	Cytochrome
DAMPs	Danger-Associated Molecular Patterns
dAUC	Difference in Area Under the Curve
DBD	Deceased after Brainstem Death
DCD	Deceased after Circulatory Death
DGF	Delayed Graft Function
dH2O	Distilled water
D-HOPE	Dual Hypothermic Oxygenated Perfusion
DM	Diabetes Mellitus
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DRI	Donor Risk Index
EAD	Early Allograft Dysfunction

EBSS	Earle's Balanced Salt Solution
ECMO	extracorporeal membrane oxygenation
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(oxyethylenenitrilo)tetraacetic acid
ERCP	Endoscopic Retrograde Cholangiopancreatography
FA	Fatty Acid
Flt1	Vascular endotherlial growth factor receptor 1
Fr	French
g	Grams
GapDH	Glyceraldehyde 3-phosphate dehydrogenase
GMCSF	Granulocyte-macrophage colony-stimulating factor
HA	Hepatic Artery
HAT	Hepatic Artery Thrombosis
HCl	Hydrochloric acid
HFD	High-Fat Diet
HMP	Hypothermic Machine Perfusion (Alternative name for HOPE)
HNF-4α	Hepatic Nuclear Factor-4α
HOPE	Hypothermic Oxygenated Perfusion
hr	Hour
ICAM	Intercellular Adhesion Molecule 1
ICH	Intra-cranial Haemorrhage
ICU	Intensive Care Unit
IFN-GAMMA	Interferon Gamma
IL	Interleukin
IoT	Institute of Transplantation (Freeman Hospital, Newcastle)
IP-10	Interferon gamma-induced protein 10
IRI	Ischaemia-Reperfusion Injury
ITBL	Ischaemic-type biliary lesions
IVC	Inferior Vena Cava
Kg	Kilogram
KO	Knock-Out
kPa	kilopascal
L	Litres
LIQu	Liver Image Quality
M	Moles
MA	Maine
MAPC	Multipotent Adult Progenitor Cells
MCP-1	Monocyte chemoattractant protein 1
MD (state)	Maryland
MDC	Macrophage-derived chemokine
MELD	model for end-stage liver disease
Mg	Magnesium
mg	milligrams
MI	Michigan
min	Minute
MIP	Macrophage Inflammatory Protein

ml	millilitres
mmHg	milimetres of mercury
MMLV	Moloney Murine Leukemia Virus Reverse Transcriptase
mmol	milimoles
MPS	Machine Perfusion Solution
MPT	Mitochondrial Permeability Transition
MRCP	Magnetic Resonance Cholangiopancreatography
MRSA	Methicillin-Resistant Staphylococcus Aureus
M-W U	Mann-Whitney U
n	number
NAC	N-acetyl cysteine
NaCl	Sodium chloride
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHSBT	National Health Service Blood and Transplant
NJ	New Jersey
nm	nanometres
nmol	nanomols
NMP	Normothermic Machine Perfusion
NP-40	nonyl phenoxypolyethoxylethanol
NPV	Negative Predictive Value
NR	Nuclear Receptor
NRP	Normothermic Regional Perfusion
NTOT	Novel Technologies in Organ Transplantation
ODT	Organ Donation and Transplantation
OH	Ohio
OLT	Orthotopic Liver Transplantation
OOH	Out of hospital
PBS	Phosphate Buffered Saline
PCN	Pregnenolone-16α-carbonitrile
PGC-1α	Peroxisome proliferator-activated receptor-gamma coactivator-1α
PNF	Primary Non-Function
PPARα	proliferator-activated receptor-α
PPL	Personal Project Licence
PPV	Positive Predictive Value
PTC	Percutaneous Transhepatic Cholangiography
PV	Portal Vein
PXR	Pregnane-X Receptor
r2	Coefficient of determination
RET	Reverse Electron Transfer
RNA	ribonucleic acid
ROC	Receiver Operator Characteristics
ROI	Regions of Interest
ROS	Reactive Oxygen Species
rpm	revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction

RT-qPCR	Quantitative reverse transcription PCR
RXR	Retinoid-X Receptor
s	Seconds
SAA	Serum Amyloid A
SCS	Static Cold Storage
SRC-1	Steroid Receptor Coactivator-1
TAE	Tris, acetic acid and EDTA buffer
TARC	Thymus- and activation-regulated chemokine
Tie-2	Tyrosine kinase with immunoglobulin-like and EGF-like domains 2
TNF	Tumor necrosis factor
u	units
UKELD	United Kingdom Model for End-Stage Liver Disease
USA	United States of America
UW	University of Wisconsin (solution)
VCAM	vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
WME	Williams' Medium E
WT	Wild Type

Chapter 1. Introduction

The first human liver transplant was performed by Starzl over 50 years ago. Many aspects of liver transplantation have changed over that time, of the first 5 patients to receive a liver transplant, none survived longer than 23 days[1]. Over the decades that followed, great advancements were made to overcome the immunological barriers to successful transplantation. The obstacle that followed was the development of suitable organ preservation techniques. Static Cold Storage (SCS) works on the principle of reducing metabolic rates by reducing the temperature of the organ. The rationale for this is that most enzymatic reactions slow down by 50% for every 10°C reduction in temperature (known as van't Hoff's rule), therefore at 4°C the enzymatic rate is approximately 10% of that at 37°C. Although the metabolic demand reduces significantly, some metabolic reactions continue, even at 4°C. Adenosine Triphosphate (ATP) levels deplete as anaerobic metabolism cannot generate ATP at a sufficient rate to counter ATP consumption. In addition anaerobic glycolysis produces lactic acid as its end product, leading to tissue acidosis.

Whilst cold anti-coagulated blood can preserve organs for a limited amount of time, thrombosis at reperfusion limited its use[2, 3]. Replacing this with an alternative preservation solution was key to prolonging the preservation time. The first clinically adopted preservation solution was Eurocollins Solution[4], which remained in standard usage for 20 years until Belzer produced the University of Wisconsin (UW) solution[5]. Eurocollins solution permitted safe preservation of livers for up to 6 hours, beyond which deleterious effects became common and primary non-function was almost universal beyond 12 hours[6], UW solution allowed a prolongation of preservation times.

Static Cold Storage has been an excellent preservation modality but in recent years with the deteriorating quality of organs the incidence of early allograft dysfunction and primary non-function has been increasing. It is likely that more recent pressures require organ preservation techniques beyond the suitability of SCS.

Improvements in outcomes from liver transplantation over the years have transformed it from an experimental procedure, to almost routine. The average 5-year survival post liver transplantation is now 80%[7]. These improvements have led to an exponential growth in patients being added to the waiting list for liver transplantation, but organ donation has been unable to keep up with the rising demand. As a result, the waiting list for liver transplantation in the UK peaked in 2015, at almost ten times what it was in 1992[8], with a significant mortality rate whilst on the waiting list [9], which is similar to other regions [10, 11]. The waiting list peaked in 2015/16, and there has been a decline since then from 611 to 359 in 2018 (Figure 1)[9].

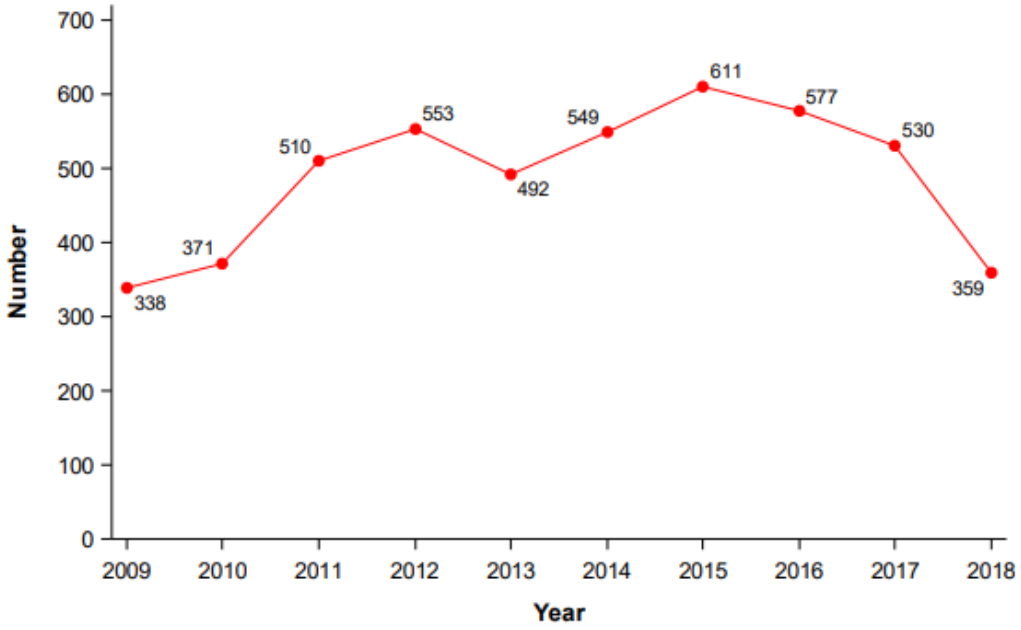


Figure 1 - Patients on active liver transplant waiting list [9]

The increasing demand for liver transplantation has led to the use of increasingly marginal organs. In particular, there has been increasing utilisation of livers from donors after cardiac death (DCD), as demonstrated in Figure 2. This is one possible explanation for the decline in the waiting list observed over the last 3 years.

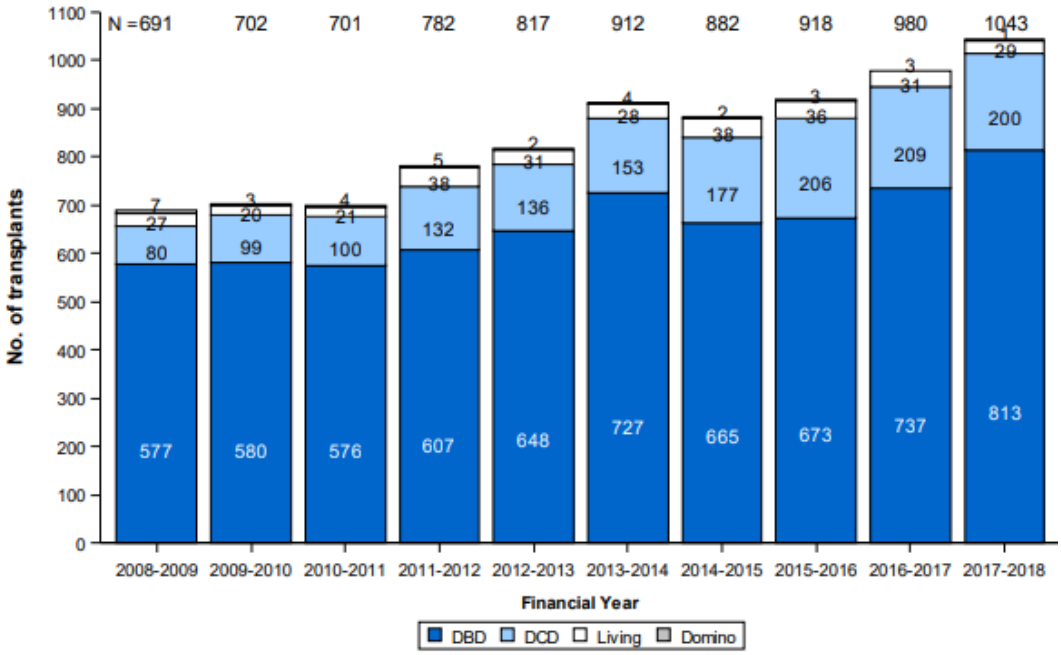


Figure 2 - Total number of liver transplants in the UK by donor type, 1 April 2007 - 31 March 2017, NHSBT [9]

However, increased use of DCD livers without changes to preservation methods would most likely result in inferior outcomes to those transplanted. There is evidence to demonstrate the inferiority of DCD livers when

compared with livers from donors after brain death (DBD). DCD livers have higher rates of early allograft dysfunction (EAD), biliary complications, acute kidney injury and worse survival compared with DBD livers [12-14]. Although some centres have demonstrated that with very judicious donor and recipient selection, adequate outcomes can be achieved [15, 16], there is a growing perception that SCS is inadequate preservation for these organs. For sustained widespread safe utilisation of DCD livers, novel methods of organ preservation need to be developed.

Although organ machine perfusion is not a new technique, there has been a resurgence in interest in this modality. In fact, it is almost a century since Carrel and Lindbergh developed a device for automatic *ex-vivo* organ perfusion[17]. Experience in kidney allograft machine perfusion has demonstrated a reduction in delayed graft function (DGF) and improved 1-year survival [18]. It has been demonstrated that machine perfusion increases levels of adenosine triphosphate, which is thought to be an important mechanism for reducing ischaemia-reperfusion injury (IRI) [19], in effect "recharging the batteries". There is more limited experience in *ex-vivo* liver perfusion, but promising initial results.

Another major benefit to *ex-vivo* perfusion is the possibility to provide an opportunity for real-time assessment of graft function and viability prior to implantation; thereby providing a powerful tool for predicting the risk to the recipient from receiving a particular liver.

In addition to DCD utilisation, another major barrier to organ utilisation is donor liver steatosis. Increasing steatosis in the donor allograft leads to poorer recipient outcomes [12, 20]. However, currently steatosis assessment is visual and is largely down to retrieval surgeon experience, and therefore can be very subjective. A rapid, point-of-care, objective assessment of liver steatosis would therefore have a significant role in real-time liver assessment.

Further potential work to follow on from this, is to use *ex-vivo* machine perfusion as a platform for drug delivery for further improvement of the liver during the perfusion period.

Alternative strategies to increase the donor pool include live-donor liver transplants. Although good outcomes have been reported with relatively low donor morbidity and mortality[21], there remains a reluctance to subject healthy donors to the risks inherent with a live donor hepatectomy particularly in the United Kingdom.

1.1 Oxygenated Machine Perfusion

Although there is general acceptance that the future of liver preservation will involve some form of machine perfusion, there is no consensus over the preferred method, site, duration or temperature. Options include intervention at the donor hospital, in transit, or at the recipient hospital, or a combination of these. Modalities currently under investigation are Normothermic Regional Perfusion (NRP) at the donor site, Normothermic Machine Perfusion (NMP) either in transit or at the recipient transplant centre, or Hypothermic Oxygenated PErfusion (HOPE) at the recipient centre. Each have their advantages and drawbacks on a clinical, logistical or financial basis.

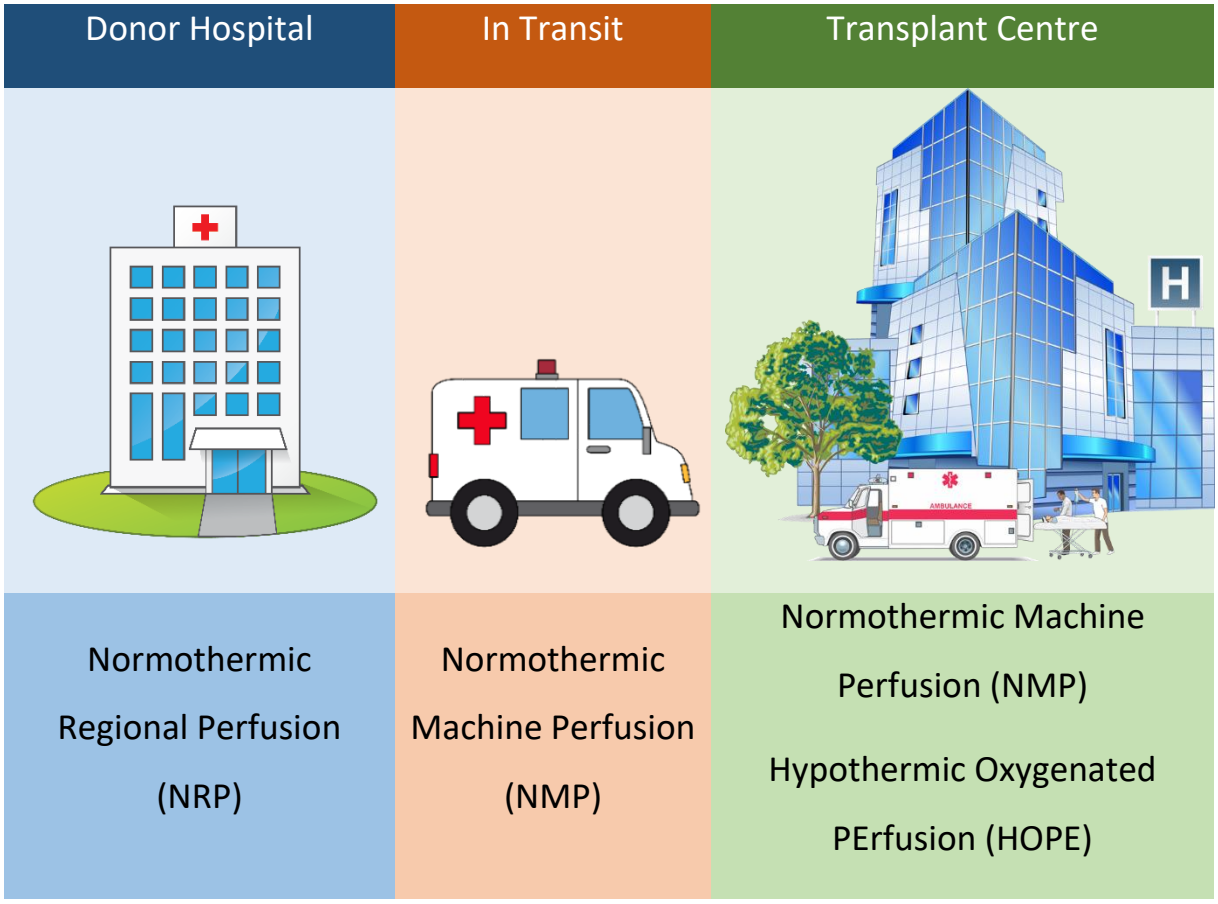


Figure 3 - Diagrammatic representation of types of organ perfusion

1.1.1 Hypothermic Oxygenated PErfusion (HOPE)

HOPE aims to recondition livers prior to implantation, to reverse some of the deleterious effects of SCS, and subsequently reduce IRI. As previously stated, the liver is retrieved and transported as standard to the transplanting centre, where it is prepared for transplantation ('back-benched') and placed on perfusion whilst the recipient is being anaesthetised and the explant hepatectomy is being performed.

There are multiple proposed beneficial effects of performing HOPE. Firstly, there is a physical washout benefit that helps clear microcirculation in the liver, including diluting waste products and blood remnants.

Furthermore, experience from the Porte group has also demonstrated that HOPE increases ATP content more than 15-fold, which remain elevated after reperfusion (in discarded organs)[22, 23]. There also appears to be a reduction in expression of pro-inflammatory cytokines[24], downregulation of Kupffer cell activity [25], and reducing vascular resistance[26, 27]. Oxygenation at low temperatures also has the potential benefit of not generating Reactive Oxygen Species (ROS), which has been implicated as a deleterious effect of normothermic perfusion[28]. Porte *et al*. and Schlegel *et al*. have both also demonstrated in a rodent model that HOPE reduces markers of biliary epithelial injury [29, 30], which is likely to be of critical importance to increased use of DCD livers.

To date, three centres internationally have published initial clinical experience with HOPE – the Guarrera group in New York[31], the Dutkowski group in Zurich [32, 33] and the Porte group in Groningen[23, 34].

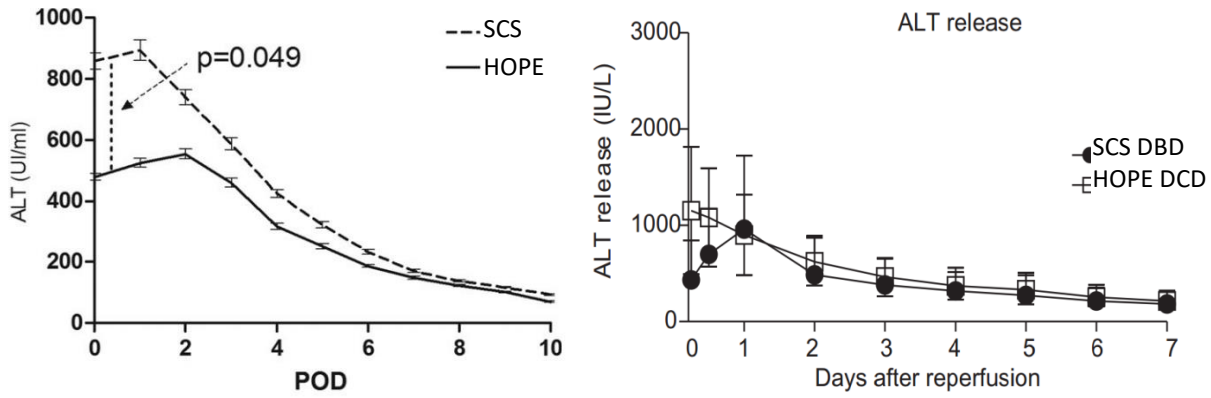
There is still some debate over optimum procedures for HOPE, including whether hepatic arterial perfusion is necessary, or whether portal venous perfusion is sufficient. Although, arterial and venous circulations mix in the liver sinusoids, we feel that including arterial circulation in the perfusion will be beneficial at least for physical clearance of debris and blood remnants in the vessels; in particular there is theoretically better supply of oxygen to the peribiliary vascular plexus[27, 35, 36]. Jomaa *et al* published no significant difference in endothelial injury, flow or vascular resistance between single or dual perfusion [37]. Good clinical results have been published using both portal-only perfusion (Zurich group) [32, 33], and dual perfusion (New York and Groningen) [23, 25, 31, 34, 38].

Relatively low perfusion pressures have been demonstrated to be sufficient, particularly as higher pressures at low temperatures induce endothelial shear injury[23, 39]. A portal perfusion pressure of 3mmHg is sufficient to deliver perfusate to the liver circulation without endothelial injury[23, 39]. Similarly, an arterial pressure of 25mmHg is sufficient[23, 31]. There has previously been concern that exposure of hepatocytes to oxygen at low temperatures will lead to oxidative stress and subsequent free-radical mediated cell injury[40]. This concern was based on isolated hepatocytes, but this has not materialised in significant numbers of liver[39, 41, 42] and kidney experiments[43].

1.1.1.1 Clinical Experience from Other Centres

The groups from Zurich and New York have led the evaluation of hypothermic liver perfusion, and have demonstrated that DCD livers that undergo HOPE become equivalent to matched DBD livers in many parameters. Dutkowski *et al*. analysed peri-operative markers including blood loss, transfusion rates, duration of surgery, and cardiopulmonary instability during reperfusion, and found similar rates when comparing DCD HOPE vs matched DBD recipients[32].

In terms of post-operative outcomes, Dutkowski *et al*. demonstrated equivalence to DBD livers and Guarrera *et al*. demonstrated superiority compared to SCS livers. Measures of peak ALT (Figure 2) and AST were equivalent to DBD[32] and superior to DCD[31]. Equally, hospital length of stay was lower than SCS group[31] and equivalent to DBD group[32].



A B

Figure 4 - Clinically measures serum ALT in recipient compared with DCD SCS (A)[31] and DBD (B) [32] matched recipients

Subsequent to the experience of these 2 groups, Porte *et al.* have developed their technique of D-HOPE, with perfusion of arterial and portal venous circulations[23]. They have demonstrated safety and efficacy with an apparent reduction in retransplantation for non-anastomotic biliary strictures when compared with their historical DCD cohort[23].

A more recent study from France found that HOPE improved clinical outcomes and reduced length of ICU stay and total hospital stay in extended criteria DBD donors. The reduction in length of stay more than offset the cost of HOPE, thereby concluding that the introduction of HOPE was cost-neutral in their setting[44]. In a further Swiss/French study, a retrospective analysis of HOPE versus NRP in DCD donors found no significant difference in recipient and graft survivals [45].

An interesting development from the Zurich group has shown particular benefits of utilising HOPE for liver recipients with HCC. They found a significant reduction in tumour recurrence in recipients of livers that had undergone HOPE compared with their controls, which included a local DBD cohort as well as a DCD and DBD cohort in an external centre [46]. They postulate that this may be due to a reduction in ischaemia-reperfusion injury and subsequent inflammatory response. The cascade of IRI induces injury to hepatic sinusoids, which leads to a dysfunction of the hepatic microcirculatory barrier and activates cell signals related to invasion and migration [46].

Although, there is agreement that the highest risk livers stand to benefit most from HOPE, there is no consensus on selection criteria. Higher average donor risk in Europe compared with North America means a variable definition of 'extended criteria'[47]. Schlegel *et al*. recently postulated an inclusion criteria for HOPE including DBD grafts with donor age >80 years, cold ischaemia >10 hours or macrosteatosis >20%. They also included DCD grafts with donor age >60 years, functional warm ischaemia >20 mins, cold ischaemia >6 hours, or macrosteatosis >5%[47].

1.1.1.2 Advantages and Disadvantages over SCS and NMP

Retrievals and organ transportation continue as normal, and all interventions occur at the transplanting centre, where equipment and expertise already exist, therefore implementation is logistically fairly uncomplicated.

Hypothermia has the added advantage of being a lower risk option than normothermic perfusion, such that if perfusion were to fail for technical reasons, the liver is still kept cool, and therefore no worse than SCS.

There is potential for the perfusion period to allow for viability testing and other forms of organ assessment, and perfusion parameters may prove to have a predictive value for organ outcome, although significantly more experience is required to validate this. However, measures of liver function in hypothermic conditions may prove difficult to measure or less representative of physiological function than at normothermia. Evidence supporting the use of perfusion for viability assessment is starting to emerge, but so far only in normothermic perfusion[48].

Ex-vivo perfusion in general has potential as a platform for drug delivery, for example drugs that may be protective against ischaemia-reperfusion injury, or drugs to treat steatosis; however it is likely that any pharmacoactive interventions are far less likely to be effective at low temperatures due to the reduced metabolic rate.

Furthermore reperfusion of livers with hypothermic oxygenated perfusate triggers significantly less oxidative damage in livers and subsequently downregulation of downstream inflammatory pathways [30, 49]. During SCS, metabolic transitions shift electrons to succinate, which acts as a store of electrons in hypoxic conditions [50]. If a liver is then subsequently reperfused with normothermic oxygenated blood, there is reverse electron transfer (RET) fuelled by increased succinate accumulation during hypoxia [51]. The first few minutes of reperfusion are critical in the formation of ischaemia-reperfusion injury (IRI) as mitochondrial reactive oxygen species (ROS) are produced by mitochondria [52, 53]. These ROS subsequently disrupt ATP production, open mitochondrial permeability transition (MPT) pores and release danger-associated molecular patterns (DAMPs) [52-54]. The aim of HOPE is to restore mitochondria from ischaemia to replete stores of ATP without triggering the same oxidative damage experienced with normothermic IRI [30, 49]. This is probably related to low proton motive forces below 15°C [47].

1.1.2 Normothermic Machine Perfusion (NMP)

Similar to HOPE, NMP aims to recondition organs prior to implantation, however it aims to achieve this by replicating physiological conditions as closely as possible. Mimicking physiological conditions is achieved by providing the liver with oxygen and nutrition at physiological temperatures. It is possible to perform NMP in transit, or as an end-ischaemic setup. NMP in transit is logistically much more challenging to implement as well as having substantially higher costs. However, performing NMP in transit eliminates the need to cool the liver, and it can remain metabolically active throughout. Whereas, end-ischaemic NMP still relies on SCS for transportation, and aims to recondition the organ only when already in the transplanting centre, prior to implantation. As well as potentially improving the quality of the liver prior to transplant, it may also eventually have the advantage of being able to delay transplantation either to allow for stabilisation of the recipient or to allow surgery to be performed in daylight hours.

There are a number of setups currently in use internationally, all consisting of the same primary components – a reservoir, pump, heat-exchanged and oxygenator. Contrary to hypothermic setups, they all perfuse both

arterial and portal circulations. The commercially-available setups currently available in clinical use are: OrganOx Metra (OrganOx Ltd., Oxford, UK), LiverAssist (Organ Assist, Groningen, Netherlands), TransMedics OCS™ (TransMedics, Andover, MA, USA) and the Cleveland NMP device (Cleveland Clinic, Cleveland OH, USA). There are variations in their designs. The OrganOx device uses a closed IVC setup, whereas other allow open drainage of the IVC. LiverAssist and TransMedics devices deliver pulsatile flow to and continuous flow to the portal vein, whereas OrganOx and Cleveland devices provide continuous flow to both. OrganOx and TransMedics devices offer automated pressure control. The LiverAssist device is the only one that allows fully hypothermic and normothermic utilisation, but is the only one that is not transportable whilst a liver is undergoing perfusion.

The principle of NMP in transit is to eliminate the hypoxic period during SCS. As described previously, although cooling an organ during SCS significantly reduced the metabolic rate, anaerobic metabolism continues resulting in depletion of ATP stores and the accumulation of waste products of metabolism. Also, by reducing succinate accumulation there should be reduced IRI through RET.

NMP is typically performed using a red-cell-based perfusate to allow adequate oxygen transport, although there are reports of alternative perfusates including using polymerised bovine haemoglobin HBOC-201[55] or Hemopure®[56]. NMP is also typically performed using arterial and portal venous circulation.

1.1.2.1 Clinical Experience of NMP

The first phase I study in clinical use of NMP was published by Ravikumar *et al*.[38] using the OrganOx Metra device. This was a non-randomised prospective trial of 20 consecutive NMP livers, which were then compared to matched controls. There was a mixture of DBD and DCD livers, with 80% being DBD In this study, the safety and feasibility of the device was established, together with gaining some experience of perfusion parameters. There was stable haemodynamic, synthetic and metabolic function in all livers, with maintenance of pH between 7.2 and 7.4 without correction. Bile production commenced within the first hour in all livers. Their primary outcome was 30-day survival, which was 100% in the study group and 97.5% in their control group ($p = 1.00$). However, as one of their secondary outcomes, they identified a significantly improved peak AST in first 7 days post-transplant (417 vs 902 IU/L, $p = 0.034$). There is evidence to suggest that peak AST is indicative of graft and patient survival, EAD and PNF[57, 58].

Subsequently, 2 Canadian centres have published their experiences of clinical NMP also using the OrganOx Metra device [59, 60]. The Toronto group [59] published their series of 10 cases in a single-arm non-blinded pilot study, which they compared to a matched SCS control group. In contrast to Ravikumar *et al*, they used Steen solution in addition to 3 units of packed red blood cells instead of a standard colloid solution. Although they perfused 12 organs, 2 were not transplanted – one due to concerns over the arterial reconstruction of a replaced right hepatic artery and the other due to persistently raised lactate and poor bile production. Similar to Ravikumar *et al*. they demonstrated lower AST levels post-transplant although were not able to reach significance. Therefore they also confirmed safety of the procedure but were unable to demonstrate significant benefit against their control cohort. The Edmonton group also performed a non-randomised study with the aim of assessing safety[60]. Similarly, they compared their outcomes to a case-matched cohort who

had received transplants after SCS. In contrast to the Toronto group, they used Gelofusine in their perfusate. They perfused 10 livers, but did not transplant 1 due to a twist in the portal vein preventing perfusion. They had a higher proportion of DCD grafts at 40%. For their final 3 livers, an iliac artery extension graft was used to minimise desiccation of the hepatic artery patch. In contrast to Ravikumar *et al*. they stated that most perfusions required supplementation of sodium bicarbonate to maintain physiological pH. Although they did demonstrate safety, there was similarly no difference in their primary outcome of 30-day survival as both groups were 100%. Interestingly, they observed a higher incidence of EAD in the perfused group compared with controls (55.5% vs 29.6%, $p = 0.23$), and a significantly longer median hospital stay in the perfused group (45 vs 25 days, $p = 0.01$). In contrast with the previous 2 studies, they did not demonstrate a reduction in peak AST. These outcome differences may reflect a higher proportion of DCD utilisation than other reported studies.

These preliminary non-randomised studies led to initiation of a large randomised control study comparing the efficacy of NMP rather than merely safety. This recently-completed study by the Consortium for Organ Preservation in Europe (COPE), published in Nature[61], resulted in the transplantation of 222 livers (121 NMP, 101 SCS)[62]. This was a randomised study of adult DBD and DCD standard criteria livers, being transplanted into patients receiving a liver-only transplant, excluding fulminant hepatic failure. They had one case of being unable to perform NMP due to anatomical variant (accessory left hepatic artery arising from aorta). There was one reported graft loss in the arm due to device malfunction. They demonstrated a 49.4% reduction in peak AST (their primary outcome) in the NMP arm compared with SCS ($p < 0.001$). Subgroup analysis demonstrated that the reduction in peak AST was greater in DCD organs. They also demonstrated a significant reduction in EAD (74% reduction, $p = 0.002$). Their protocol required an MRCP at 6-months follow-up, which was performed in 155 of the 222 transplanted patients; this did not demonstrate any significant difference in rates of non-anastomotic strictures between the two arms of the trial. There was also no difference in ICU or total hospital stay. Importantly, as with previous studies, it has failed to demonstrate a significant improvement in patient or graft survival or rates of biliary complications. It should also be noted that they enrolled 272 livers to the trial (137 NMP and 135 SCS), but a significant number that were randomised to SCS were not transplanted. This most likely represents faith the participating surgeons have in the technology. However if any bias results from this, it is most likely to favour the SCS group.

The above 3 publications were based on standard criteria donors that would otherwise have been transplanted anyway. The first study assessing NMP in declined livers was by Mergental *et al*. [63], in which perfusion was performed at their transplanting hospital rather than in transit and a combination of LiverAssist device and OrganOx Metra were used. They utilised experience they had acquired on discarded livers to establish a protocol for viability testing, and on this basis discarded 1 of the 6 livers they perfused. Interestingly, 2 of the livers they transplanted produced no bile during perfusion, and these 2 recipients had the highest serum bilirubin during follow-up. The liver that was discarded following perfusion had an accessory right hepatic artery that was not reconstructed prior to perfusion, and only after 90 minutes of perfusion demonstrated a noticeable colour difference in perfusion was reconstruction performed. After implantation of the accessory right hepatic artery, the lactate levels rose and did not fall again.

The Cambridge group have also published their initial series of 12 transplants following NMP using the LiverAssist device[28]. Similarly to Mergental *et al*. they aimed to perfuse livers where there was uncertainty about the liver, such that recipient hepatectomy could not be started until the liver was visualised. NMP therefore allowed these livers to be transplanted without prolonging cold ischaemic times. The livers were accepted through a mixture of the standard offering process, fast-track offers and research offers of discarded livers. In contrast with other studies, they commenced perfusion at 20°C and rewarmed the liver gradually over 20-30 minutes. They also had a change to their protocol halfway – from supplying an 95% Oxygen / 5% CO_2 mixture to medical air with occasional oxygen supplementation. Supplying medical air provided a HA pO_2 of 20 kPa, although the portal saturation was significantly lower due to increased flow through the oxygenator. This reduced IRI and also reduced post-reperfusion syndrome. They also added sodium bicarbonate to maintain a pH > 7.2 rather than allowing the liver to correct the acidosis, although used the requirement for sodium bicarbonate in their viability assessment. They had one death from PNF despite an urgent retransplant. Of note the PNF liver had markedly elevated ALT during perfusion and higher requirement for supplementation of sodium bicarbonate to maintain pH, but did clear lactate and produce bile.

There are a few ongoing multicentre trials, most notably 2 large trials in the USA – the Liver PROTECT trial aiming to recruit 300 livers to use the TransMedics OCS device and the WP01 trial aiming to recruit 266 to use the OrganOx Metra device. Both these trials randomise to either NMP or SCS.

1.1.2.2 Advantages and Disadvantages over SCS and HOPE

Normothermic perfusion closely replicates physiological conditions, and thereby maintains liver function. Initiation of perfusion at the donor site significantly minimises any ischaemic period, thereby reducing ATP depletion and succinate accumulation. It also allows for pharmacological manipulation of the organ, and may facilitate a reduction in hepatic steatosis[64].

Perhaps its greatest advantage over SCS and HOPE is the potential for viability assessment, particularly of previously-declined organs. This has significant potential to expand the donor pool by more objectively pushing the boundaries of transplantability, however more information is required on the parameters of value.

NMP also has the potential to preserve livers for an extended period, with transplantation after a total preservation time of 26 hours having been performed[65]. There is also a report of successfully preserving a liver for 86 hours [66], although this was not transplanted.

Disadvantages over hypothermic perfusion include increased cost and complexity, particularly if it is performed in transit. This has significant implications for personnel required and transportation logistics. The requirement for packed red cells in the perfusate also adds cost and results in utilisation of banked blood that may otherwise have been avoided.

Furthermore, any technical failure during perfusion as was observed by Bral *et al*. [60] would require emergency repair or switch to SCS, but is likely to result in organ loss as warm ischaemia will immediately ensue.

1.1.3 Real-time assessment

As well as the potential to optimise the quality of organs, ultimately, one of the main goals of ex-vivo perfusion in any form is the potential to test the function of an organ prior to implantation. There is a relatively high proportion of organs currently retrieved that do not go on to be transplanted. The principal reason for discard of a liver that has been retrieved with the intention of being transplanted is fear that the liver will not function adequately after transplantation, usually in the setting of steatosis, prolonged warm ischemia, adverse hemodynamic characteristics during the DCD withdrawal phase, or prolonged cold ischemia[28]. Although the liver may have functioned well in the donor, warm and cold ischaemia impose an unpredictable injury on the liver that may manifest only after reperfusion in the recipient. The ability to objectively predict future function in the recipient is the ultimate aim of real-time viability assessment.

There are many factors that contribute to the potential transplant risk associated with any given liver. Some of these are included in the Donor Risk Index (DRI) published by Feng *et al*.[67] and include age, cause of death, race, whether DCD or DBD, whether split or not, height, organ location and cold ischaemic time. Whilst this is a useful tool, there are many more individual factors that it can't account for. For example, liver steatosis is known to adversely affect transplant outcome [12, 20], but is subjectively assessed and not accounted for in the DRI. At present the decision on whether to accept a particular liver for transplant is complex and requires the transplanting surgeon to weigh up multiple factors from both the donor and recipient. Further information attained by objectively assessing the viability of a liver prior to implantation has the potential to make the process more reliable and repeatable.

In addition to enhancing the objectivity of liver assessment prior to transplantation, by doing so one may be able to expand the donor pool more safely provided livers that will have poor outcomes in the recipient display different characteristics during perfusion. Mergental *et al*.[63] and Watson *et al*.[28] have both utilised NMP to assess livers that had been declined and subsequently transplanted them.

Arguably the main advantage of normothermic perfusion over hypothermic is increased opportunities to assess liver function, as in normothermia the liver's metabolic function should be similar to physiological conditions. However, a consensus on the criteria for assessment of livers has yet to be established. Table 1 summarises reported parameters used for assessment of livers during NMP.

Reference	Model	Hepatocellular function	Cholangiocyte function	Notes
Op den Dries [68]	Discarded human (n=4)	*Function:* Bile output, perfusate lactate, glucose, urea, bilirubin, bicarbonate *Damage:* Perfusate ALT, GGT, Potassium	Bile bilirubin and bicarbonate concentrations Bile enzyme concentrations: GGT and LDH	Non-transplant model
Sutton[69]	Discarded human (n=12)	*Function (assessed at 6hrs):* Bile output >20g; perfusate lactate, (glucose), albumin, sO_2, Hepatic ATP content, Requirement for bicarbonate replacement	Bile bilirubin and bicarbonate concentrations and pH	Extension of Op den Dries study Recommended 2.5 hours perfusion to fully assess

		Damage: Perfusate ALT, ALP, GGT, LDH, Potassium		
Reiling *et al*. [70]	Discarded human (n=4)	*Function:* Bile production, lactate, urea, glucose *Damage:* ALT	None	
Mergental *et al*. [63]	Clinical (n=6, 5 transplanted)	*Function:* Lactate <2.5 mmol/L; pH >7.3, Bile production *Damage:* Hepatic and portal flows, Clinical appearance	None	Recommended assessment at 3 hrs. Declined one liver with rising lactate. All 5 recipients well at median follow up 7 months
Ravikumar *et al*. [38]	Clinical (n=20)	*Monitored parameters:* Perfusate pO_2, pCO_2, pH, glucose, flow, bile production	None	Decision to use organ left to transplanting surgeon
Selzner *et al*. [59]	Clinical (n=12, 10 transplanted)	*Function:* Lactate, pO_2, pCO_2, pH, bilirubin *Damage:* AST, ALT	None	No criteria stated to determine viability, but 1 graft declined due to raised lactate (level not specified)
Watson *et al*. [28]	Clinical (n=12)	*Function:* Lactate, Glucose, Transaminases, requirement for sodium bicarbonate	Bile production and pH	Extended criteria donors, including discard livers
Westerkamp *et al*. [50]	Discarded human	*Function:* Bile production, Glucose, lactate *Damage:* HA and PV resistance, ALT, AST, LDH, GGT	Bile bilirubin, bicarbonate and pH	Good function is bile production >2 ml/kg/h during period of 1.5 to 2.5 hrs after start of NMP and >5 ml/kg/h after 2.5 hrs

Table 1 - Summary of reported parameters for assessment of human livers during NMP. Adapted from Watson et al [45]

The ability of the liver to reduce lactate in the perfusate is the most widely accepted marker of viability during NMP[71]

Mergental *et al*.[63] published a proposed acceptance criteria of:

Required:	Lactate < 2.5 mmol/L	**Or**	Bile Production
AND 2 out following 3 criteria			
Perfusate pH >7.3	Stable HA Flow > 150 ml/min & PV flow > 500ml/min		Homogenous graft perfusion with soft parenchymal consistency

Table 2 - Mergental et al proposed NMP acceptance criteria

In the clinical series by Watson *et al*.[28] there was one death following transplantation, and that case both cleared lactate adequately and produced bile, but interestingly had a significantly elevated ALT during perfusion and a higher requirement for sodium bicarbonate supplementation to maintain a pH > 7.2. The authors have not commented on homogenous perfusion or parenchymal consistency, but assuming these were acceptable, the liver that suffered PNF would have met Mergental's criteria. In Watson's series, the poorest bile producer has adequate function and no evidence of ischaemic cholangiopathy, whereas 2 of the cases that have developed cholangiopathy were amongst the best bile producers.

In the COPE trial, the only liver undergoing NMP that developed PNF was persistently acidotic with a lactate > 4 mmol/L, and produced minimal or no bile. However, there were 17 other livers in the trial that also produced no/minimal bile, but all functioned after transplant [61].

The VITTAL trial has now closed and has been presented at international meetings[72], although publication is still awaited. The study aimed to provide further information on the useful parameters for real-time viability assessment. They recruited discarded livers that were retrieved but deemed untransplantable and performed NMP at the transplant hospital after a period of SCS. Their criteria for transplantation was as follows[73]:

Required:	• Lactate <= 2.5 mmol/L
AND 2 or more of the following:	• Bile production • pH >= 7.30 • Metabolism of glucose • HA flow >= 150 ml/min and PV flow >= 500 ml/min • Homogenous perfusion

Table 3 - VITTAL trial criteria for transplantation after NMP

They included 31 livers in their trial, and 22 livers met viability criteria and were transplanted. They have reported 100% patient and graft survival at 90-days[72].

More advanced assessment of livers including real-time metabolomics have also been postulated in the absence of NMP as a predictor of future graft function [74], and the authors propose this may be of further value during perfusion in future studies.

It is therefore clear to see there remains a lack of consensus on acceptable perfusion parameters that determine transplantability of a liver.

1.2 Steatosis

Steatosis in the donor liver adversely affects the recipient outcomes [12, 20, 75], and is therefore a frequent reason for declining livers. The rates of early allograft dysfunction (EAD) and primary non-function (PNF) in these livers is also increased [76], and it is widely accepted that this is due to poor tolerance of steatotic livers to ischaemia-reperfusion[77, 78]. Increased IRI is secondary to increased ROS generation, pro-inflammatory immune system activation, impaired mitochondrial ATP production and microcirculatory dysfunction, resulting in hepatocyte necrosis and subsequent graft failure [79, 80]. The incidence of fatty liver disease is increasing, with 40-60% of donor livers having fatty infiltration [75]. The limits of acceptable graft steatosis have not yet been established [81]. At present assessment of steatosis relies on surgeon visual assessment. A more objective quantitative assessment of liver steatosis, may allow more livers to be transplanted by more accurately stratifying risk in mild-moderately steatotic livers.

There is some early evidence to suggest that machine perfusion can be utilised as a platform to 'de-fat' steatotic livers prior to implantation[82]. This can be augmented by stimulating lipid metabolism [82], which has been effective in rat livers [83]. In hepatocyte culture, a defatting protocol reverses the elevated sensitivity to IRI seen in macrosteatotic hepatocytes[79]. Some studies have suggested performing a biopsy on the liver prior to implantation to assess levels of steatosis [84].

Kron *et al*. studied the effect of HOPE on severely steatotic rat livers prior to transplantation. As the main mechanism for worsened outcomes in steatotic liver transplantation is increased ROS production and subsequent IRI, they postulated HOPE may attenuate mitochondrial oxidative stress and subsequently improve outcomes. They found that although HOPE has no effect on the level of steatosis, it reduces the inflammatory injury and subsequent fibrosis [85]. They also identified improved outcomes in their clinical series of HOPE in steatotic livers when compared with SCS controls [85].

We aim to compare alternative methods of steatosis quantification with the aim of identifying a reliable, reproducible test that can be adapted to be performed at the point-of-care (either at the time of retrieval or during perfusion). This includes non-invasive methods such as photographic assessment of the macroscopic liver, which has not previously been validated.

1.3 AR42J-B13 Cell-Line

Cultured hepatocytes provide a means to test the response of hepatocytes to external insults such as hypoxia. In addition, the ability to modify cells, such as levels of steatosis, provides an opportunity to measure the deleterious effects of this. However, human hepatocytes are of variable quality, in short supply and rapidly de-differentiate in culture[86].

The Wright research group has extensively investigated the utility of B13 cells as a cost-effective donor-free hepatocyte source [86-90]. The B13 cell line is a male rat progenitor-like pancreatic cell line that is readily expandable on simple plastic and culture media with the capacity to generate an unlimited supply of functional hepatocyte-like cells (termed B13/H) *in-vitro*. B13 cells undergo differentiation to a non-replicative

hepatocytes-like cell expressing near normal levels of many cytochrome P450 enzymes [87, 91, 92], in response to elevated glucocorticoid levels[86], this has also been demonstrated *in-vivo* [93]. A major advantage over primary hepatocytes is that B13 cells remain differentiated for several weeks [87]. This will allow for experimentation of different levels of micro- and macro-steatosis, and their effects when exposed to varying durations of warm ischaemia.

Of particular interest to this study, a cell-line hepatocyte model allows for experimentation of reproducible, measurable levels of steatosis and their effects on viability following a hypoxic insult.

1.4 Potential Therapy for Protection against Ischaemia-Reperfusion Injury

Perhaps the most exciting potential advantages of *ex-vivo* liver perfusion is possibility to use it as a platform for drug delivery to the liver prior to implantation. During NMP, the liver maintains normal acid-base balance and electrolyte homeostasis, requirements for most physiological processes including cellular transport, protein synthesis and aerobic metabolism [94]. Due to near-physiological conditions, pharmacological interventions should have similar function to *in-vivo*. As well as potentially utilising pharmacological interventions already clinically available, as there are no concerns regarding non-liver systemic toxicity, further opportunities may exist. This has several potential benefits, including reducing the effects of ischaemia-reperfusion.

Although pharmacological intervention in liver grafts prior to transplantation is not a new concept, the delivery of this to a metabolically active organ has greater potential. Previous studies have investigated the potential for pharmacomodulation of grafts during SCS, but benefits demonstrated in animal models have largely failed to translate to clinical practice [95-97].

The Pregnane-X Receptor (PXR) is a promising target for anti-inflammatory treatment of liver disease[98]. The PXR is a member of the nuclear receptor gene superfamily of ligand-activated transcription factors [99]. Nuclear receptors have the ability to activate target gene transcription when bound to ligands. Unbound nuclear receptors can either be in the cytoplasm or nucleus, but cytoplasmic nuclear receptors migrate to the nucleus following activation. Activation of the nuclear receptors by a ligand leads to dissociation of co-repressor molecules and changes to the activation unction 2 (AF2) region of the nuclear receptor that allows interaction with various coactivator molecules. Activation of the nuclear receptor also leads to changes to the DNA binding domain region that subsequently allows binding to sequence-specific hormone-response elements on the target DNA thereby leading to gene transcription. Some nuclear receptors actually suppress gene transcription when inactivated[100]. Unbound PXR nuclear receptors are located in the cytoplasm, but translocate to the cytoplasm once ligand-activated[101].

When activated and translocated to the nucleus, PXR forms a heterodimer with the Retinoid X receptor (RXR). This heterodimer is stabilised by 3 coactivators – Steroid receptor coactivator 1 (SRC-1), Hepatocyte nuclear factor 4α (HNF4α), and peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α). The

PXR/RXR/coactivator complex then promotes the transcription of CYP3A4 by binding to the xenobiotic responsive enhancer molecule (XREM/PXRE). This process is illustrated in Figure 5.

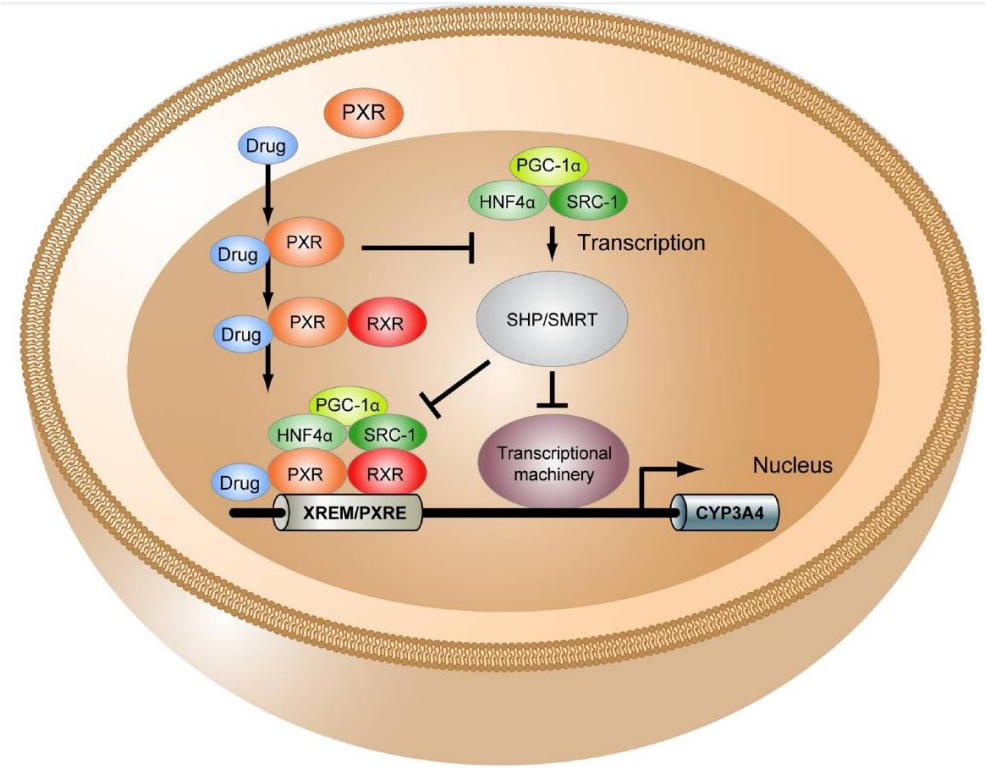


Figure 5 - Ligand-mediated PXR activation and CYP3A4 induction [102]

The PXR receptor has a number of roles, predominantly in the regulation of xenobiotic metabolism, whereby PXR induces the expression of a number of enzymes and transporters that metabolise and excrete these drugs and xenobiotics. In addition to this, PXR has a role in the regulation of bile acid metabolism, glucose metabolism, lipid and cholesterol metabolism and other hormone synthesis roles.

There is good evidence supporting the anti-inflammatory effects of PXR activation, particularly in the liver[103]. There also appears to be an inverse relationship with NF-κB signalling, suggesting that the anti-inflammatory effect of PXR may be due to inhibition of NF-κB[103].

Our group has previously shown that PXR agonists significantly reduce NF-κB-induced peri-portal inflammation and fibrosis [103]. More recently, it has been shown that PXR plays a pivotal role in inhibiting and resolving the inflammatory response in cultured hepatocytes [104].

To demonstrate that the previously-observed reduction in peri-portal inflammation is PXR-dependant, the effects on rodents with the PXR gene knocked out will be investigated. Pregnenolone-16α-carbonitrile (PCN) is a rodent-specific PXR activator available from Sigma Aldrich (St. Louis, MI, USA). We intend to investigate whether PXR activation by PCN protects wild-type mice against whole-liver ischaemia reperfusion, and whether this protection is lost in PXR knock-out mice.

Ultimately the aim of investigating the protective effects of PXR, is the potential to translate to clinical practice. If by exposing ex-vivo livers to PXR, the same protective effects are demonstrated, this holds great potential in the field of graft modulation. Our rodent experiments will therefore be followed by introduction of a PXR activator into livers undergoing normothermic machine perfusion. Our primary objective is to demonstrate target activation and thereby prove the therapeutic potential of pharmacological interventions during ex-vivo perfusion. We will also assess the effects on how the liver performs during perfusion and the perfusate characteristics.

Chapter 2. Aims

There are two overarching aims to this project

1. Donor liver optimisation by oxygenated machine perfusion
2. Real-time donor liver assessment of viability and steatosis

Chapter 3. Project Outline

In line with the above aims, we have investigated methods of liver machine perfusion including HOPE and NMP. Methods of steatosis quantification were also investigated both *in-vitro* and *in-vivo* as well as investigating means of viability and functional assessment of the liver whilst on perfusion. The effects of steatosis on viability in hypoxic settings have been analysed in a rodent hepatocyte cell line, together with assessing oxidative stress. This has been correlated with samples taken from perfused human livers. Further to this, a potential target for drug therapy to minimise the effects of ischaemia-reperfusion injury has been assessed in a mouse model and subsequently translated to perfused livers.

3.1 Perfusion

3.1.1 Hypothermic Oxygenated Perfusion

A preliminary series of 6 discarded livers were perfused to establish safety, followed by 10 clinical liver perfusions

3.1.2 Normothermic Machine Perfusion

Following clinical translation of HOPE, discarded livers were utilised to establish our NMP protocol. 10 livers underwent NMP under our standard protocol, followed by 5 livers that were subjected to extended ischaemia. These livers were then perfused to assess what characteristics are able to distinguish a liver that has suffered extreme ischaemia that renders it unequivocally untransplantable. A further 5 livers then were given an active treatment (PXR) and the effects analysed (see section 3.4)

3.2 Real-Time Pre-Implant Liver Assessment

3.2.1 Perfusate Analysis

Perfusate samples were taken at regular intervals from all livers perfused and stored at -80°C for later analysis. A multiplex array was performed on perfusate samples from all livers to assess for any biomarkers that could differentiate between different groups of livers.

Lactate and ALT levels on all perfusate samples were also analysed to also assess for differences between groups.

3.2.2 Contrast-Enhanced Ultrasound

The ability to accurately quantify tissue perfusion, blood flow standardised for the volume or weight of the perfused tissue ($cm^3/s/cm^3$ or grams), is essential for the assessment of the physiological functionality and viability of a tissue [105]. In my second year, we investigated CEUS as a measure of global perfusion in the

liver. We have observed that the anatomical peripheries of livers appear to improve during the perfusion period. We believe this may be an important measure of liver 'quality'.

3.2.3 Steatosis

We have collected an image bank of livers *in-vivo* and during perfusion. Images are taken with a calibration slide that allows the software to correct for lighting and camera variations. We have worked in collaboration with Aedstem Ltd to develop an 'app' that analyses these images in real-time and provides a numerical predictive value of steatosis based on hue and granularity of the liver surface.

In parallel various methods of lab-based steatosis quantification have been performed. Initially, this has been performed on rodent specimens that have been fed either a control or high-fat diet. Methods analysed to date include positive-pixel image analysis, oil-red colorimetry and triglyceride quantification.

3.3 Cell Line and Hepatocyte Isolation

A hepatocyte-like cell line of rodent origin has been established. This cell line has been used to model effects of steatosis on viability when exposed to hypoxia. Viability studies have been performed at different levels of micro- and macro-steatosis.

Hepatocytes have also been successfully isolated from livers following ex-vivo perfusion, and these have been used to assess the effects of targeted drug therapies.

3.4 Pregnane-X Receptor

Previously published work has demonstrated the anti-inflammatory effect of activating the pregnane-X receptor, and it has also been demonstrated to reduce levels of isoprostanes following ischaemia reperfusion in a rat model [106].

Mice with PXR target gene knocked out have been used to assess the effects of PXR activation on ischaemia-reperfusion. Mice were divided into 4 groups – wild-type or knock-out, and treated with PXR or placebo. All mice had a laparotomy under anaesthesia, and clamping of the portal vein and hepatic artery for 30 minutes. Survival to 7 days has been analysed.

PXR has then been added to the perfusate of 5 discarded livers. The effects on perfusion dynamics, blood gases and perfusate analysis were assessed for any differences against the control group to establish safety. Liver biopsy samples were used to measure downstream activation of PXR targets (CYP3A) to determine the ability to use ex-vivo perfusion as a platform for drug delivery.

Chapter 4. Materials and Methods

4.1 Hypothermic Oxygenated Perfusion (HOPE)

Discarded livers offered for research and suitable livers that will be transplanted were perfused in accordance with the protocol in Appendix 2.

Figures below demonstrate the set up in our perfusion room, and a liver being perfused.

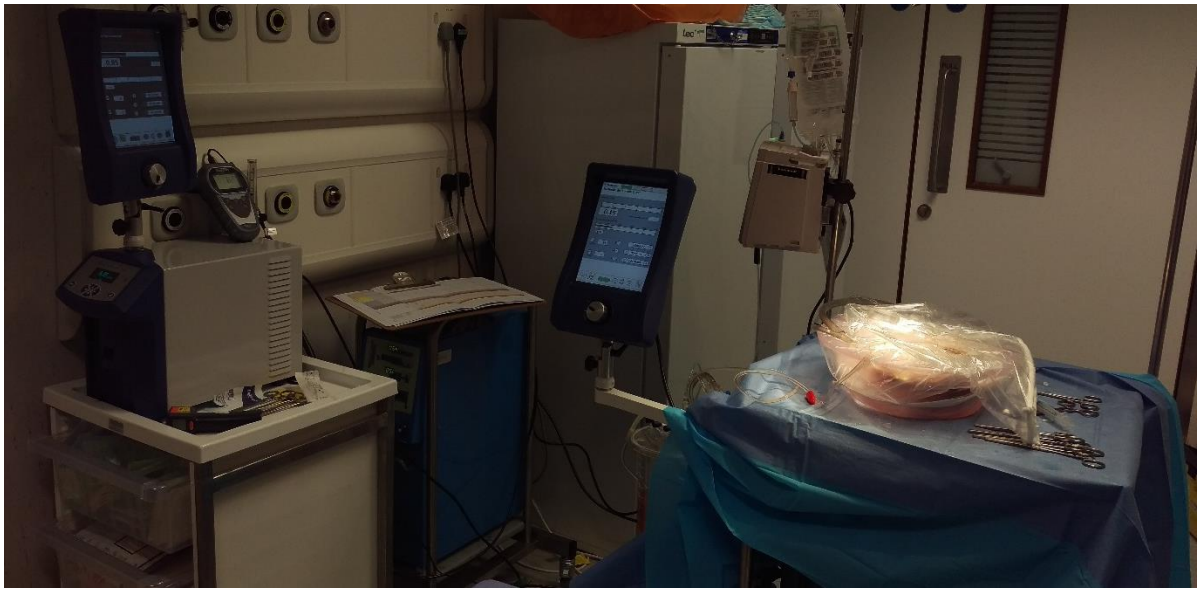


Figure 6 - The perfusion room set up with liver being perfused

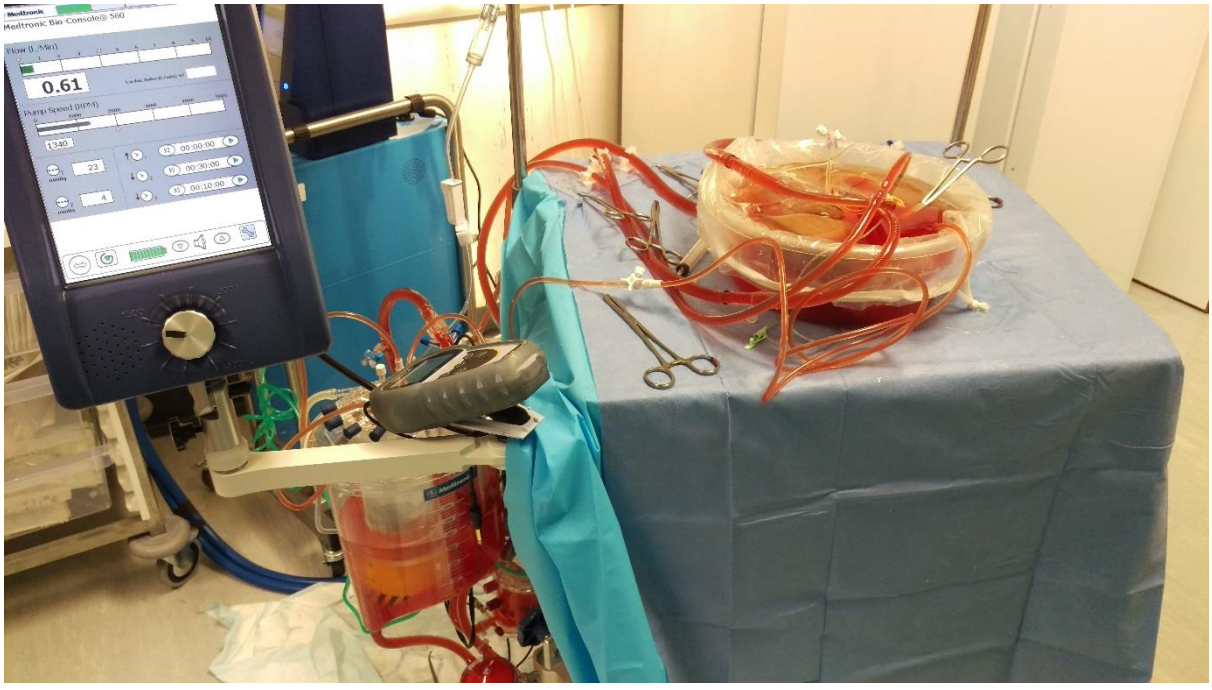


Figure 7 - The equipment set up with liver being perfused. Pump is displaying portal venous flow (0.61L/min), as well as portal pressure (4mmHg) and arterial pressure (23mmHg).

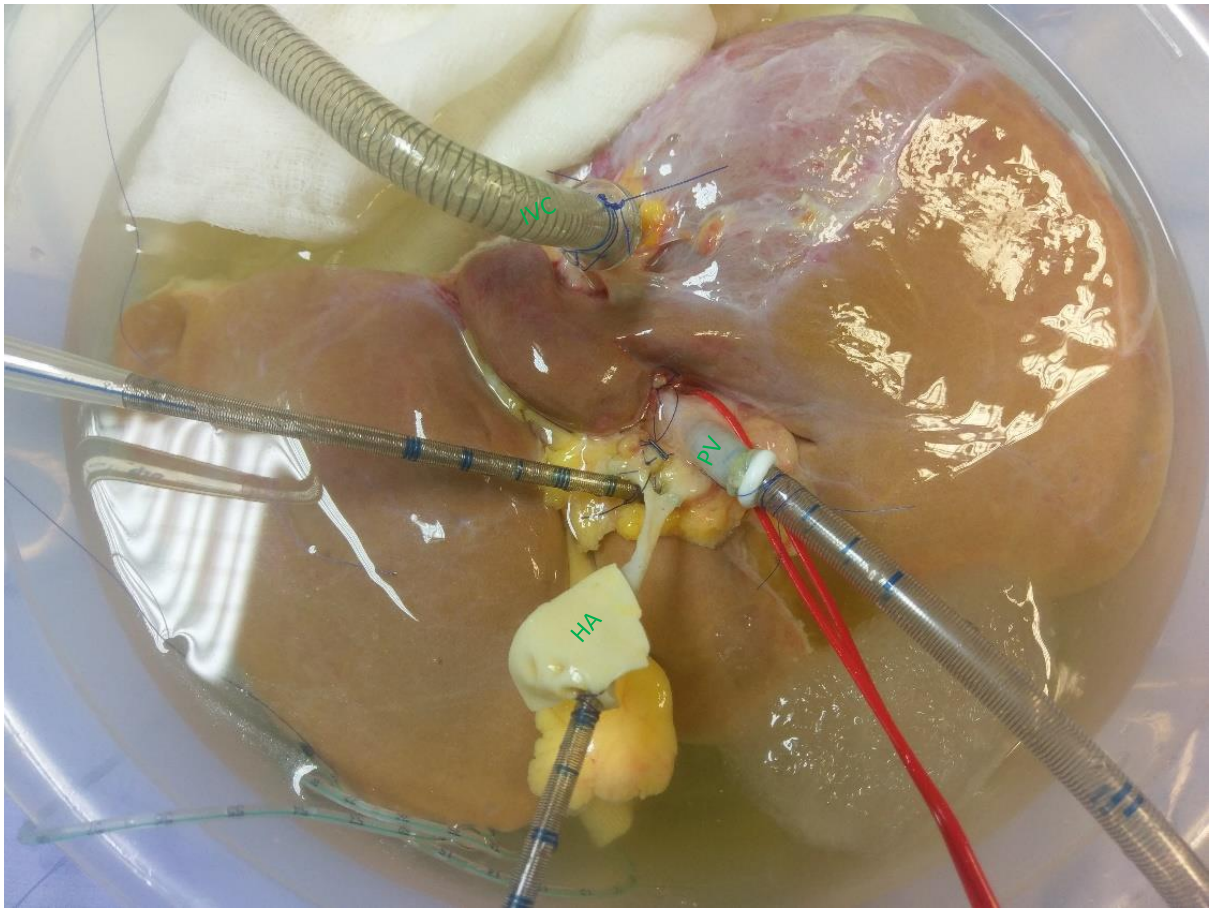


Figure 8 - A liver being perfused. HA: Hepatic Artery, PV: Portal Vein, IVC: Inferior Vena Cava

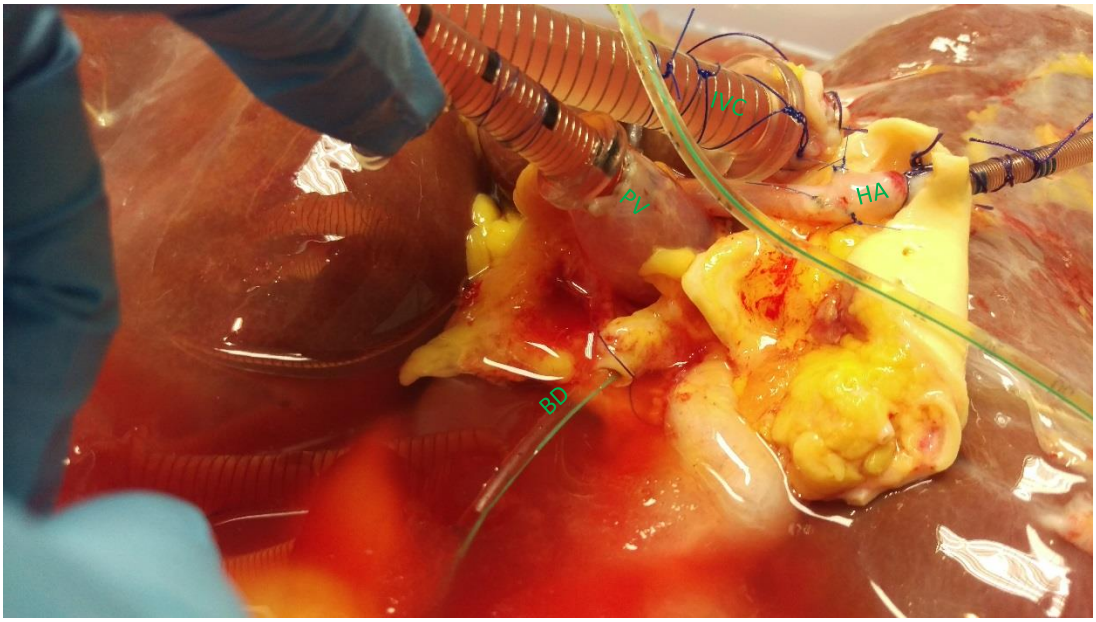


Figure 9 - A close-up view of porta-hepatis. HA: Hepatic Artery, PV: Portal Vein, IVC: Inferior Vena Cava, BD: Bile Duct

4.1.1 Circuit Design

The circuit is manufactured to our design and specifications by Medtronic Ltd (Minneapolis, MN) with a heparin coating (Carmeda BioActive® surface). Although the heparin coating is arguably not required in hypothermic perfusion due to the lack of blood-based perfusion, it was felt that utilising the same circuit for all perfusions would simplify our supply chain and minimise the risk of circuits expiring whilst on our shelves. The added cost was felt to be worthwhile. The feeding line splits to form hepatic artery and portal venous lines. The flows in the two limbs are measured independently, and pinch valves in the lines can control differential flow.

A major advantage of our setup when compared with alternative liver perfusion setups is cost-effectiveness. By using mostly generic components in our custom-designed circuit and standard cannulae and cardiopulomonary bypass equipment we were able to keep our consumables cost significantly lower than proprietary setups such as OrganOx. Our complete custom-designed circuit, including the oxygenator/heat-exchanger, centrifugal pump head, flow probes, pressure lines, reservoir and tubing was £525 including delivery. In addition there was a cost of £92 for a full set of cannulas for each perfusion. In hypothermic perfusion, the cost of UW solution was not factored as that would have been used on the back-table for perfusion of the liver regardless of machine perfusion. For normothermic perfusion, there was a supply cost of £126 per unit of red blood cells used for research but our blood bank are happy to supply red blood cells for clinical perfusion without cost.

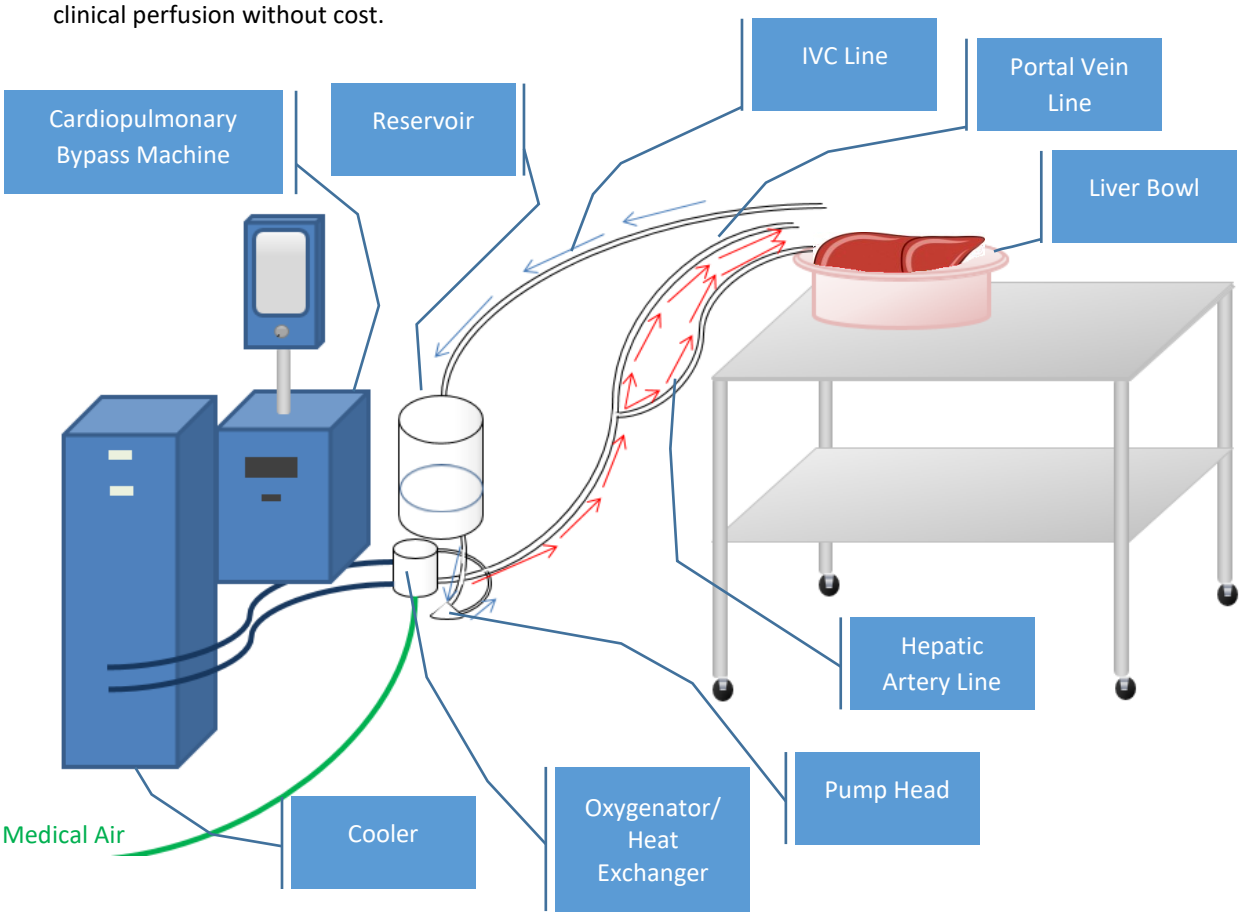


Figure 10 - Diagrammatic representation of HOPE circuit, with cardiopulmonary bypass machine and cooler

4.1.2 HOPE Protocol

All HOPE perfusions were performed following our protocol in Appendix 2. Minor modifications to the protocol were performed over the course of the first 6 discarded liver perfusions and are detailed in Section 5.3.1 Discard Series. All perfusions were carried out in a clean theatre perfusion room within the transplant theatre complex at the Freeman Hospital, Newcastle upon Tyne.

The protocol consists of dual arterial and portal-venous perfusion, using a fixed-pressure variable-flow algorithm. The arterial perfusion is non-pulsatile and a fully cannulated closed circuit was utilised.

The primary perfusate was Belzer University of Wisconsin solution with the addition of N-acetylcysteine, dexamethasone and antibiotics. The heater-cooler was set at 5°C, achieving a perfusate temperature of approximately 8°C at the point of entry to the liver.

4.1.3 Sample Handling

Biopsies at 0, 60 and 120 minute time-points were obtained from discarded organs, and perfusate samples obtained at 20-minute intervals from all livers. Biopsies have been taken of the bile duct and portal vein at the end of perfusion. All biopsies were split into 3 for formalin, RNAlater and snap-frozen fresh. All perfusate and biopsy samples (excluding formalin) were stored at -80°C. Arterial and venous blood gases were performed at 20-minute intervals and recorded. Perfusion dynamics and temperature were also charted at 20-minute intervals.

4.1.4 Comparisons to published hypothermic perfusion methods

There are various differences between the setups described by Guarrera, Dutkowski, Porte and ours, as detailed in Table 4.

	Guarrera *et al*[25, 31, 107]	Dutkowski *et al*[32, 33]	Porte *et al*[23, 34]	Newcastle
Type of Perfusion	D-HOPE	HOPE	D-HOPE	D-HOPE
Arterial Perfusion	Non-pulsatile perfusion	No perfusion	Pulsatile perfusion	Non-pulsatile perfusion
IVC	Open	Open	Open	Cannulated
Duration of Perfusion	3-7 hours	1-2 hours	>2 hours	80-150 mins
Oxygenation	No active oxygenation	40-60 kPa	>60 kPa	20-30 kPa
Device	Medtronic	LiverAssist	LiverAssist	Medtronic
Temperature	4-8°C	8-12°C	8-12°C	8-10°C
Flow	Fixed to 0.667ml/g liver/min	Variable on pressure	Variable on pressure	Variable on pressure
Pressure	Variable on flow	3 mmHg (PV)	5 mmHg (PV) 25 mmHg (HA)	3 mmHg (PV) 25 mmHg (HA)
Perfusate	Vasosol	Belzer MPS	Belzer MPS	Belzer UW
Additives	Antioxidants, metabolic substrates and vasodilators		Glutathione	Antioxidant and vasodilator
Perfusate volume	3 L	3 L	4 L	3 L

Table 4 - Comparison of hypothermic perfusion methods

4.2 Normothermic Machine Perfusion

NMP was performed with the same equipment and circuit set-up as HOPE, but at a higher pressure and a change to the perfusate. HA pressure was targeted to 75 mmHg and PV pressure was 4-5 mmHg. We retained a fixed-pressure, variable flow setup.

4.2.1 NMP Protocol

The NMP protocol was an adaptation of our established HOPE protocol (see Appendix 3), and all perfusions carried out in the same clean theatre perfusion room. As such, dual arterial and portal-venous perfusion was utilised, with non-pulsatile arterial flow. Contrary to some published designs, a fully cannulated closed circuit was used.

The main difference compared with the HOPE protocol is temperature and perfusate. A red-cell based perfusate with 3 units of clinical-grade human donated banked type-specific blood was used. Additives were included to attempt to achieve and maintain a near-physiological environment for the liver. These included amino acids and multivitamins, as well as heparin, antibiotics, and a prostaglandin vasodilator. The heater-cooler was set to 39°C, achieving perfusate temperature of 37°C at the point of entry into the liver.

4.2.2 Sample Handling

Biopsies at 0, 60 and 360 minute time-points were obtained from discarded organs, and perfusate samples obtained at 20-minute intervals for the first 2 hours, followed by 150 minutes, and hourly thereafter from all livers. Biopsies were taken of the bile duct and portal vein at the end of perfusion. All biopsies were split into 3 for formalin, RNAlater and snap-frozen fresh. All perfusate and biopsy samples (excluding formalin) were stored at -80°C. Arterial and venous blood gases were performed at 20-minute intervals and recorded. Perfusion dynamics and temperature were charted at 20-minute intervals.

4.2.3 Comparisons to published normothermic perfusion methods

	OrganOx Metra	LiverAssist	TransMedics OCS	Cleveland NMP Device	Newcastle
Type of Perfusion	HA & PV	HA & PV	HA & PV	HA & PV	HA & PV
Arterial Perfusion	Non-pulsatile perfusion	Pulsatile Perfusion	Pulsatile perfusion	Non-Pulsatile perfusion	Non-pulsatile perfusion
IVC	Cannulated	Open	Open	Open	Cannulated
Duration of Perfusion	24 hours	24 hours	24 hours	18 hours	6 hours
Oxygenation	12-20 kPa[28]	20 kPa[28]	NP	NP	20kPa
Temperature	37°C	37°C	37°C	37°C	37°C
Pressure	60-75/gravity	60/9	NP	NP	75/5
Transportable	Yes	No	Yes	Yes	No

Table 5 - Comparison of normothermic perfusion methods (NP = Not Published)

4.3 Real-Time Pre-Implant Liver Assessment

4.3.1 Methods

37 livers underwent ex-vivo perfusion by one of 2 methods – either hypothermic or normothermic. There remains ongoing debate over which method provides most benefit to the liver, but establishing a comparison between hypothermic and normothermic was not within the remit of this study.

4.3.1.1 Hypothermic perfusion (n=16)

Hypothermic livers were divided into 2 groups – ones that were deemed transplantable on clinical grounds, and those that weren't (and therefore were perfused purely for research purposes). Livers that were deemed untransplantable were declined by all transplant centres in the UK, these are termed 'discard' livers (n=6). Clinical livers were classed as transplantable purely on clinical grounds, but were all high-risk livers that had been declined by at least one centre, and hence felt that ex-vivo perfusion could potentially provide benefit. Livers were allocated into groups sequentially, 6 discards were perfused followed by 10 clinical livers. All clinical livers were transplanted with good immediate clinical outcomes.

4.3.1.2 Normothermic perfusion (n=21)

By contrast, all livers that underwent normothermic perfusion were 'discard', and hence none were transplanted. Nevertheless, we divided them into 3 groups. The control group (n=11) underwent standard normothermic perfusion with as short a cold-ischaemic time as was feasible. One of these was initially accepted on clinical grounds but declined due to performance on perfusion, and was subsequently declined by all other centres also. The second group of livers (n=5) was subjected to extended cold ischaemia followed by 1 hour of warm ischaemia at 37°C, this would establish an extreme scenario of an untransplantable liver. The third group of livers was perfused as per the control group, but with the addition of a Pregnane-X Receptor (PXR) activator drug (Avasimibe). PXR upregulates the CYP3A subfamily of cytochrome P450, and in rodent models has been shown to be protective against liver ischaemia-reperfusion injury

Samples of perfusate from all liver perfusions was collected at regular intervals and stored at -80°C until the day of analysis.

4.3.2 *In-Vivo* Non-Invasive Steatosis Estimation (LIQu)

An image and biopsy bank is currently being developed to allow calibration of the software and validation against histological analysis of the biopsies. Approval was obtained from the Newcastle Hepatobiliary Biobank for collection, storage and analysis of liver samples obtained from patients undergoing liver resection.

Any patient undergoing liver resection at the Freeman Hospital was approached for consent to participation in this, which included consent for medical photography. Provided this was obtained, a photograph of the liver with overlying calibration slide was taken in theatre prior to resection (Figure 9).

In addition, all livers undergoing perfusion also had similar photographs taken, and biopsies taken for assessment of steatosis. These samples have been stored under licence from the Newcastle Transplant Biobank.

The image was then analysed by the software (Figure 9) and a sample of liver was taken from the resected specimen for histological analysis of steatosis. This has been performed by a qualified histopathologist, but has also been compared to laboratory-based methods of steatosis quantification described in Section 5.4.

Figure 11 - A photograph taken of a liver in-situ with overlying calibration slide and the image analysis in progress

Once this initial calibration phase is complete, we aim to start a pilot study of image analysis at the point of retrieval. This will allow for more objective description of the liver to the implanting surgeon who is usually at another location.

4.3.3 Contrast-Enhanced Ultrasound

During *ex-vivo* liver perfusion, global perfusion has been assessed by contrast-enhanced ultrasound (CEUS). A microbubble contrast agent was injected into the circuit whilst scanning the liver parenchyma. Backscatter from the microbubble contrast agent was processed to provide a value that is representative of instantaneous *in-situ* concentration of the contrast agent. The values for each liver have been collected and correlated to findings in other methods of liver assessment.

4.4 Steatosis Quantification

A mouse model was used for steatosis quantification. 18 male C57BL/6J mice were purchased from a licenced supplier and maintained under standard animal house conditions in filtered cages with 12-hour light/dark cycles. To establish steatosis, 4-week old mice were maintained on either a high-fat diet or control diet. The high-fat diet contained 45%kcal fat (D12451, Research Diets Inc., New Brunswick, NJ, USA), and the control diet was 10% kcal fat (D01060501, Research Diets Inc., New Brunswick, NJ, USA). This diet was maintained for 6 weeks, the livers were then retrieved, snap-frozen in liquid nitrogen and stored at -80°C. These livers were then used for a variety of methods of steatosis quantification described below.

4.4.1 Oil-Red O Staining and Positive Pixel Analysis

10µm sections were cut using a cryostat and mounted on superfrost plus slides. Sections were air-dried at room temperature, then fixed in 10% formalin for 5 minutes. The slides were then rinsed in dH_2O, then 60% isopropanol. The slides were stained in Oil-Red O (ORO) stain for 15 minutes then washed in isopropanol 60% for 1 minute then rinsed in dH_2O. They were then stained in haematoxylin and washed in dH_2O again. The slides were mounted with 20% glycerol and photographed.

These images were analysed using Leica Slidepath Image IA software (Leica Biosystems, Wetzlar, Germany). The software was trained to identify pixels determined to be ORO stain, therefore giving a quantification of ORO staining for an area of the slide. This was repeated 3 times for each liver sample and an average obtained.

4.4.2 Oil-Red Colorimetry

A simpler and faster method of quantifying oil red staining is potentially to use colorimetry to quantify absorbance at 515nm.

The same liver samples as above were used. A specimen of each liver weighing approximately 20mg was taken and weighed individually. They were fixed in 3.7% formalin for 20 minutes then washed twice in PBS. Isopropanol was then added to each sample and left for 10 minutes. 150µL was then removed from each sample and added to a well on 96-well plate. This was read in colorimeter at 515nm, including a blank. The reading was corrected for sample weight and blank subtracted.

4.4.3 Triglyceride Determination

Further samples of these mice livers weighing approximately 10mg each were taken and weighed individually. 100µL of 20mM Tris + KCl was added to each sample and sonicated. 10x volume (i.e. 1ml) of 2:1 chloroform/methanol mix was added and the sample vortexed. Each sample was then centrifuged at 13000rpm for 90 seconds. The bottom layer was extracted into fresh eppendorfs and placed onto heat block to evaporate.

20µL of 5:1 mix of free glycerol/triglyceride reagent was added to each well at 37°C, then 20µL of sample was added to each well and mixed. The plate was then read in a colorimeter.

4.5 AR42J-B13 Cell-Line

4.5.1 Cell Culture

B13 cells were cultured in Dulbecco's Modified Eagle Medium – low glucose (DMEM with 1000mg/L glucose) with 10% foetal calf serum, penicillin 80 units/ml, streptomycin 80 µg/ml, and l-glutamine 80 µg/ml. Cells were incubated in a humidified incubator at 37°C with 5% CO_2 in air. Cell passage was performed every 3-4 days.

4.5.2 Cell Passage

1. Pre warm culture media to 37°C in water bath
2. Defrost 1x Trypsin EDTA (stored as 10x stock at -20°C, make 1x using sterile PBS, also stored at -20°C)
3. Pre warm 1x Phosphate Buffered Saline (PBS) to 37°C
4. Remove culture media from flask
5. Wash with 10mls of 1xPBS to remove remaining media
6. Add 5mls (for typical 75cm^2 flask) 1x Trypsin EDTA
7. Incubate cells until detached (2-5 mins, tap flask to encourage detachment)
8. Add 5mls media to trypsinised cells (stops actions of the trypsin)
9. Collect media/cell mix and pipette into sterile falcon tube
10. Centrifuge at 20000rpm for 4 minutes
11. Remove media and leave pelleted cells
12. Resuspend cells in 8mls fresh media
13. Add 2mls of cells to each of 5 75cm^2 flasks
14. Add a further 8mls of fresh media to cells
15. Incubate at 37°C in 5% CO_2

4.5.3 Differentiation to B13/H

Cell media as above, with the addition of dexamethasone to a final concentration of 10nM was used. The media was changed every 3-4 days, and differentiation to B13/H occurs after 10-14 days.

4.5.4 Isolation and Quantification of RNA

4.5.4.1 RNA Purification

1. Remove cell media from wells
2. Wash in PBS
3. Add Trizol to wells, then remove 1ml of cells into Eppendorf tubes
4. Add 200µL chloroform and mix thoroughly
5. Centrifuge at 12000rpm for 15 minutes

6. Transfer upper aqueous layer to fresh Eppendorf tube and add 500µL isopropanol
7. Incubate on ice for 10 minutes
8. Centrifuge at 12000rpm for 10 minutes at 4°C
9. Discard supernatant
10. Dislodge pellet with 500µL 70% ethanol
11. Centrifuge at 12000rpm for 10 minutes at 4°C
12. Discard supernatant and air dry
13. Resuspend in 20 µL sterile dH_2O

4.5.4.2 RNA Quantification

A nanodrop was used to measure absorbance at 260nm and 280nm to give the concentration of RNA in ng/µL. RNA purity was verified with the 260/280 ratio, aiming for a value of 2.0.

4.5.5 Reverse Transcription-Polymerase Chain Reaction

RNA was diluted to a final concentration of 200ng/µL and incubated with 50ng/µL random primers at 95°C for 3 minutes. Each sample was then mixed with reverse transcriptase buffer, dNTPs, water and Moloney Murine Leukaemia Virus (MMLV) and incubated at 42°C for 1 hour.

Into each mini-eppendorf add 10µL go-Taq master mix (Taq DNA polymerase, 1.6mM dNTPs, 3mM $MgCl_2$ and reaction buffers), 2 µL upstream and downstream primers, 1ml cDNA (from step above) and 7µL sterile H_2O.

4.5.6 Agarose Gel Electrophoresis

The gel solution was made by diluting 1-2% agarose powder in 1x Tris-Acetate EDTA (TAE), and heated. Once mixed, the gel was allowed to cool at room temperature until set. The tank was filled with 1x TAE and RT-PCR samples from above were added to wells. 80 V was run through gels for approximately 45 minutes, and the gels were read under ultraviolet light.

4.6 Effect of Steatosis on Viability in Hypoxic Conditions

B13 cells underwent standard treatment as above for 10-14 days to allow differentiation into hepatocytes.

Cells were treated with 1mM solution of oleic and linoleic acid for a period of 24 hours. Following this the viability was assessed to ensure no significant detrimental effect of fatty acid media prior to the ischaemic insult. The cell media was changed to standard media, and cells incubated for a further 24 hours in a hypoxic incubator (O_2 = 1%). At this point cells were stained with trypan blue and slides photographed for analysis. A viability cell count was performed using ImageJ software (Open Source, University of Wisconsin). This was repeated 6 times.

4.7 Effect of Pregnane-X Receptor Genotype PCN-Dependent Protection against Ischaemia Reperfusion Injury

Approximately 30 male wild-type and 30 male PXR knock-out mice were bred in the Centre for Comparative Biology at Newcastle University.

The mice were divided into 8 groups as shown in Table 6.

	Genotype	Dosage (i.p. pre-operative / s.c. post-operative)	Operative procedure (30 mins whole liver clamp of portal vein/artery + bile duct)	Indicative n/group
Group 1	PXR+/+	PCN(50mg/kg bw)	IRI	12
Group 2	PXR+/+	DMSO vehicle	IRI	12
Group 3	PXR+/+	PCN (50mg/kg bw)	sham	2
Group 4	PXR+/+	DMSO vehicle	sham	2
Group 5	PXR-/-	PCN (50mg/kg bw)	IRI	12
Group 6	PXR-/-	DMSO vehicle	IRI	12
Group 7	PXR-/-	PCN (50mg/kg bw)	sham	2
Group 8	PXR-/-	DMSO vehicle	sham	2

Table 6 - Mice genotype and treatment subgroups

PCN was administered for 2 days prior to insult, then continued daily until the endpoint, DMSO was used as a vehicle control.

Ischaemia-Reperfusion Injury was induced in mice by performing a midline laparotomy under suitable anaesthesia, then a 'Pringle manoeuvre' (clamp hepatic artery and portal vein) was performed for 30 minutes, then released. The laparotomy was then closed and the mice recovered in a suitable manner. A sham laparotomy was used as surgical control. They will be observed for 7 days then humanely killed by schedule 1 method and their liver retrieved (as shown in Figure 10).

4.7.1 Study Plan and Endpoints

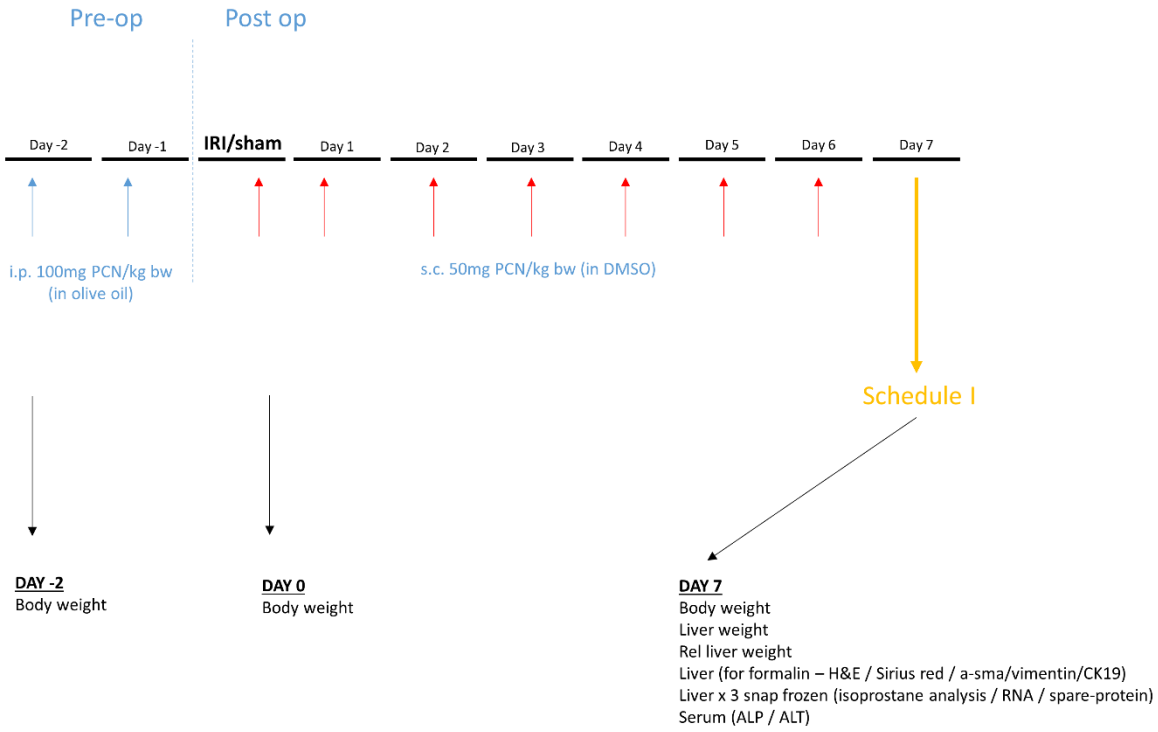


Figure 12 – Mouse PXR study plan and endpoints

4.8 Regulatory Requirements

Research Ethics Committee approval for HOPE and NMP on discarded organs were granted on 22nd June 2015 (REC: 15/EM/0285).

The Newcastle upon Tyne NHS Hospitals Foundation Trust Clinical Governance and Quality Committee approved HOPE as a New Procedure on 29th July 2015.

The NHSBT/ODT Research Approval Committee have approved this project and approved providing discarded organs for this project.

The NHSBT Research Innovation and Novel Technologies (RINTAG) group have also approved these procedures.

The Newcastle Transplant Biobank have approved taking, storing and using samples for research from HOPE.

The Newcastle Hepatobiliary Biobank have approved taking, storing and using samples from hepatobiliary resection patients for the purpose of steatosis research.

Rodent studies were performed under project licence (PPL) no. 60/4534 at the Comparative Biology Centre in Newcastle University, which holds an Establishment Licence no. 50/2507

Chapter 5. Establishing Hypothermic and Normothermic Liver Perfusion

5.1 Introduction

There is growing evidence that ex-vivo perfusion in some form is beneficial to liver grafts. There is still no definite consensus on the preferred modality to be used. This includes hypothermic versus normothermic and perfusion in transit versus in the recipient centre. Post-ischaemic reconditioning in the form of perfusion for a defined period of time once the liver has arrived at the recipient centre has advantages in terms of ease of setup, logistics and cost. Furthermore, hypothermic perfusion has inherently lower risks than normothermic perfusion in terms of technical failure. In the event of technical failure during hypothermic perfusion, the liver would automatically revert to static cold storage as it would already be cold and filled with preservation solution.

When initiating an ex-vivo liver perfusion service, the safety profile and reduced complexity of hypothermic perfusion make it a logical starting point. Safety, efficacy, equipment testing and verification of protocols was performed with a cohort of 6 discarded human livers prior to translating to clinical perfusion for transplantation. These 6 discarded livers would then form a comparison group to establish differences between the clinical and discarded livers.

Generic cardiopulmonary / extra-corporeal membrane oxygenation (ECMO) equipment provided the opportunity to use lower cost consumables compared with specifically-designed liver perfusion machines. This provides longevity and sustainability to the programme, facilitates its future adoption into NHS practice, and provides value for money to our research funders.

5.2 Hypothermic Oxygenated Perfusion

We developed our HOPE protocol mostly based on published experience from 2 centres – Zurich[39] and New York[31]. We opted for dual hepatic arterial and portal venous perfusion because, although it adds a further element of complexity, we felt it provided the best opportunity for optimal perfusion of the whole organ. A circuit was designed such that a single pump head motor could drive both circulations by splitting the tubing distal to the oxygenator. A pinch-valve on the portal venous limb would then variably restrict flow to the PV to maintain preferential HA flow and therefore achieve the differential pressures desired. A pressure-controlled, variable flow setup was used with target pressures of 25mmHg (HA) and 3mmHg (PV). Pressure readings were taken from a venting port immediately proximal to the respective cannulae. Perfusate temperature was maintained with an external heater-cooler connected to the oxygenator/heat-exchanger. Perfusate temperature readings were taken from a non-contact thermal probe within the exit limb of the oxygenator.

The liver surface temperature was verified with an infra-red thermometer. A heater-cooler setting of 5°C would achieve a liver surface temperature of 8-10°C.

5.2.1 Liver Selection Criteria

The initial pilot cohort of 6 livers was obtained through offers for research from NHSBT. Livers that were declined by all centres, and from whom the donor families had consented for research, were offered to NHSBT-approved research projects. We accepted 6 consecutive liver research offers when staff were available to perfuse. Learning points from each of these were recorded and minor adjustments to the protocol were performed accordingly.

When initiating the liver perfusion service, an acceptance criteria for perfusion were established based on livers that were higher risk and therefore stood to benefit most from perfusion. As such, any DCD liver was eligible, and DBD livers that had been declined by at least one other centre due to adverse donor factors.

5.2.2 Recipient Consent

All potential recipients were consented for HOPE the time of placement on the waiting list. At the same time, a full consent for orthotopic liver transplantation (OLT) was obtained and specific consent for ex-vivo perfusion and biobanking of samples as well.

5.3 Materials and Methods

All ex-vivo perfusion was performed in a designated 'perfusion room' within the transplant theatre complex of the Freeman Hospital, Newcastle. Equipment consisted of a Medtronic Bio-Console 560 Speed Controller to drive the 560A centrifugal external drive motor. Medtronic flow probes were used to measure individual flow in HA and PV limbs of the circuit (TX50 for PV and TX50P for HA). A Hirtz Hico-Variotherm 555 heater-cooler with an operating range of 5°-40°C was used and connected to the heat exchanger via an insulated hose. A custom circuit was designed in conjunction with the product design department at Medtronic and manufactured by them to our specification. A technical drawing of the circuit is attached in Appendix 4. In summary, the cannulated IVC drains through 3/8" tubing into an Affinity Fusion® hard-shell reservoir with an integrated 30 micron filter. The reservoir drains into an Affinity CP (AP40) centrifugal blood pump, before being pumped into an Affinity Fusion® oxygenator/heat-exchanger. Distal to the oxygenator/heat-exchanger, the circuit splits through a Y-connector into 2 limbs, one remaining 3/8" (PV) and the other 1/4" diameter (HA). Differential diameters were used to help attain the desired pressure/flow difference between the two limbs and also because paediatric cannulae most appropriate for HA cannulation are mostly available with 1/4" connectors. All circuit components were coated in CARMEDA® BioActive Surface, a long-acting heparin-based coating which was later rebranded Cortiva™. The circuit contained two non-contact temperature sensing ports, one on oxygenator/heat-exchanger outlet and one on the return (IVC) limb. An Oakton Temp-14 (Cole-Parmer) thermistor thermometer connected to a Medtronic ATP210 temperature probe with a 400-series phono connectorwas also in the circuit. The hepatic artery was cannulated with a 12Fr Medtronic DLP

paediatric vented arterial cannula (77012) and the portal vein with a 24Fr Medtronic EOPA™ vented arterial cannula (77424). The IVC was cannulated with a 32Fr Edwards LifeSciences Thin-Flex angled venous cannula (TF032L90). The bile duct was cannulated with the same type of paediatric arterial cannula (77012), which contains a 90° bend, thus facilitating the layout of cannulae once attached to the liver. Lastly, an overflow line was used for any accumulation of perfusate within the bowl, a Medtronic DLP pericardial sump (12010) was used for this.

5.3.1 Discard Series

To ascertain safety and efficacy and refine our protocol, a series of 6 discarded human livers were obtained from NHSBT via the research organ offering system. There were minor alterations to the protocol in accordance with learning points. These are recorded in Table 7. Biopsies and perfusate samples were obtained from these livers. After 6 discarded livers, it was felt that the protocol could be finalised and clinical perfusion commenced.

Discard Perfusion Number	Date	Learning Points
1	06/10/2015	Heater-cooler setting of 5°C required to achieve liver surface temperature of 8-10°C
2	10/12/2015	Simultaneous venous blood gas sample required for oxygen consumption calculation – sampling protocol altered Bile duct cannula perhaps too narrow for viscosity of bile, so changed from paediatric feeding tube to paediatric arterial cannula.
3	08/01/2016	Over-stretching diaphragm in pressure line affected reliability of reading
4	18/02/2016	Placing flow probes in close proximity causes interference resulting in fluctuating flow readings – protocol altered and fed back to Medtronic
5	18/02/2016	Pinch valve not required on HA limb, as preferential flow to PV
6	16/03/2016	No changes to protocol

Table 7 - Learning points from preliminary series of hypothermic perfusions

Perfusion dynamics for the discard cohort were collected and it was established that with the desired pressure limits, adequate flow was achieved. There was significant variability between livers, particularly with regard to portal venous flow, which was further analysed in conjunction with the clinical group of livers.

	Time	Start	20	40	60	80	100	120
Discard Liver 1	HA Pressure	26	26	26	24	26	26	25
	PV Pressure	6	4	4	3	6	5	4
	Temp	8.8	7.6	7.7	7.7	7.8	7.8	7.9
	PV Flow	800	670	660	650	760	780	790
	HA Flow	40	60	65	80	90	90	100
Discard Liver 2	HA Pressure	26	26	27	26	26	26	25
	PV Pressure	6	6	6	5	5	5	5
	Temp	8.2	8.2	8.3	8.4	8.4	8.4	8.4
	PV Flow	340	160	150	160	120	110	110
	HA Flow	60	50	50	60	60	60	60
Discard Liver 3	HA Pressure	26	26	25	26	25	25	25
	PV Pressure	4	3	3	3	4	3	3
	Temp	8.9	8.1	8	8.1	8.3	8.3	8.4
	PV Flow	300	240	200	220	330	340	380
	HA Flow	70	80	80	100	110	110	120
Discard Liver 4	HA Pressure	25	25	25	23	24	25	25
	PV Pressure	3	3	2	2	3	2	2
	Temp	10.7	10.3	9.9	9.4	9.1	8.7	8.5
	PV Flow	480	600	660	720	720	700	730
	HA Flow	60	100	120	140	130	140	150
Discard Liver 5	HA Pressure	25	26	26	26	27	25	25
	PV Pressure	3	2	2	2	2	3	3
	Temp	8.6	7.9	7.9	8	7.7	7.8	7.8
	PV Flow	610	590	580	570	580	620	600
	HA Flow	90	90	100	110	80	120	110
Discard Liver 6	HA Pressure	24	25	24	25	24	24	24
	PV Pressure	2	1	1	1	1	1	1
	Temp	8.5	8	7.7	7.7	7.7	7.7	7.6
	PV Flow	850	820	820	900	910	930	930
	HA Flow	150	160	170	180	180	180	190

Table 8 - Perfusion flow dynamics inpreliminary series of discarded livers

5.3.2 Clinical translation

Following successful refinement of the protocol during the discard series, required regulatory approvals were obtained and clinical application of the technology commenced. Communication channels with transplanting surgeons and transplant coordinators were established to ensure inclusion into the programme where appropriate. A minimum of one perfusion-trained surgeon and one perfusion technician / scientist was required to safely perform ex-vivo perfusion. It was decided that a series of 10, would be perfused prior to full analysis. The first clinical perfusion was performed on 19/02/2016 and it took 15 months to recruit 10 livers.

5.4 Results

5.4.1 Transplants to Date

Date	Recipient Age	Donor Age	Donor BMI	Donor Type	Cause of Death	Donor Hospital	Donor Risk Factors	Recipient Risk Factors
19/02/2016	55	25	18.3	DCD	ICH	Nottingham	DCD, out of area	3rd transplant, super-urgent for HAT
24/02/2016	63	61	26.5	DBD	Hypoxic brain injury	Nottingham	Out of area, in-hospital cardiac arrest x2 plus >30 functional ischaemia. Elevated ALT (387)	
02/05/2016	71	52	27.4	DCD	ICH	Newcastle RVI	DCD, poor in-situ perfusion	
04/05/2016	36	71	21.5	DBD	ICH	Bury St. Edmonds	Donor age Out of area (11hrs CIT)	2nd transplant for late HAT
15/08/2016	63	54	25.6	DBD	ICH	Coventry	Out of area	
07/09/2016	63	19	21.3	DCD	Suicide	Liverpool	DCD, Out of area (14.5hrs CIT)	
01/11/2016	58	52	38.7	DCD	Hypoxic brain injury	Newcastle RVI	DCD, Moderate steatosis	Top band, UKELD 71, Failed to reach AT
04/11/2016	73	41	17.1	DCD	Hypoxic brain injury	Leeds	DCD, Out of area, OOH Cardiac arrest, anorexic BMI 17	Age, DM
01/05/2017	45	52	24.5	DCD	ICH	Middlesbrough	DCD	
20/05/2017	65	51	25.0	DCD	Trauma	Nottingham	DCD, out of area, OOH cardiac arrest, major trauma (motorcycle RTA 120 mph)	

Table 9 - Donor and recipient characteristics of transplants after hypothermic perfusion

5.4.2 Perfusion Dynamics

	Time	Start	20	40	60	80	100	120	150	180
Clinical Liver 1	HA Pressure	24	25	25	24	25	25	24		
	PV Pressure	3	4	4	5	4	4	4		
	Temp	10.9	10.1	9.6	9.4	9.2	9.1	9.1		
	PV Flow	640	590	600	600	610	640	660		
	HA Flow	90	100	120	130	140	140	140		
Clinical Liver 2	HA Pressure	26	27	26	26	23	24	24		
	PV Pressure	2	3	3	3	2	1	1		
	Temp	9.3	8.8	8.1	8	8.2	8.3	8.4		
	PV Flow	410	400	360	550	510	530	510		
	HA Flow	80	110	110	110	170	170	160		
Clinical Liver 3	HA Pressure	26	27	26	26	26	25	26		
	PV Pressure	2	1	2	1	2	2	2		
	Temp	8.4	7.9	7.9	7.9	7.9	8	8.1		
	PV Flow	510	500	510	480	480	480	480		
	HA Flow	30	80	100	110	120	130	100		
Clinical Liver 4	HA Pressure	25	25	25	25	25	25	25		
	PV Pressure	2	2	2	2	2	2	1		
	Temp	7.9	7.7	7.7	7.6	7.6	7.6	7.6		
	PV Flow	420	450	460	470	480	490	600		
	HA Flow	90	100	100	100	100	110	100		
Clinical Liver 5	HA Pressure	25	25	25	24	24				
	PV Pressure	2	1	1	1	2				
	Temp	9.1	9	8.8	8.8	8.7				
	PV Flow	310	330	380	390	400				
	HA Flow	70	80	80	90	110				
Clinical Liver 6	HA Pressure	23	25	24	23	24	25	25	24	
	PV Pressure	3	1	1	2	1	1	2	1	
	Temp	9.7	9.6	9.5	9.4	9.3	9.3	9.4	9.3	
	PV Flow	120	180	280	290	300	310	310	310	
	HA Flow	150	160	160	90	120	180	130	180	
Clinical Liver 7	HA Pressure	23	24	25	25	25	25	25	24	25
	PV Pressure	2	3	2	2	2	3	2	2	2
	Temp	8.8	8.7	8.7	8.6	8.5	8.5	8.4	8.3	8.2
	PV Flow	280	310	310	310	300	310	320	330	310
	HA Flow	40	60	80	120	120	130	180	130	220
Clinical Liver 8	HA Pressure	25	24	23	25	25	24			
	PV Pressure	4	3	3	4	3	3			
	Temp	9.2	9.1	9	8.9	8.8	8.7			
	PV Flow	80	90	100	110	130	130			

	HA Flow	160	160	160	170	190	200			
Clinical Liver 9	HA Pressure	20	21	20	25	20	21	22		
	PV Pressure	4	5	4	5	5	5	5		
	Temp	8.7	8.8	8.6	8.6	8.6	8.6	8.6		
	PV Flow	300	320	390	460	450	460	450		
	HA Flow	180	150	190	220	230	250	260		
Clinical Liver 10	HA Pressure	21	22	22	23	23	22	23		
	PV Pressure	2	3	3	3	3	3	2		
	Temp	9.1	9	8.9	8.8	8.8	8.7	8.7		
	PV Flow	120	120	130	130	150	150	170		
	HA Flow	80	80	70	80	90	90	100		

Table 10 - Perfusion flow dynamics in 10 transplanted livers after hypothermic perfusion

5.4.3 Perfusate Gas Analysis

	Time	Start	20	40	60	80	100	120	150
Clinical Liver 1	pH	7.09	7.11	7.16	7.19	7.2	7.22	7.25	
	pO2	53.4	51.6	56	56.4	56.5	56.9	56.7	
	pCO2	1.57	1.52	1.48	1.46	1.43	1.42	1.43	
	BE			-30.4					
	Glu	18.1	18.3	18.4	18.8	19.1	19.3	19.4	
	Lac	7.52	7.98	6.3	8.23	8.88	9.1	7.9	
Clinical Liver 2	pH	7.565	7.559	7.578	7.579	7.575	7.563	7.544	
	pO2	17.75	16.89	18.57	19	19.61	19.68	20.11	
	pCO2	1.13	0.74	0	0	0	0	0.73	
	BE	-21.5	-22.6					-22.9	
	Glu	0	1.1	1.1	0	0	0	0	
	Lac	2.22	2.48	2.55	2.39	2.08	2.04	2.2	
Clinical Liver 3	pH	7.475	7.418	7.437	7.465	7.46	7.465	7.462	
	pO2	16.39	17.14	18.21	19.45	18.53	19.81	20.45	
	pCO2	1.27	0.97	0.83	0	0.72	0.8	0.85	
	BE	-23.5	-25.3	-25.1		-24.9	-24.6	-24.6	
	Glu	2.4	2.5	2.9	3	3	3.2	3.3	
	Lac	2.29	3.08	3.4	4.19	3.75	3.72	3.72	
Clinical Liver 4	pH	7.358	7.39	7.451	7.499	7.45	7.445	7.456	
	pO2	17.08	18.32	20.38	18.15	18.62	20.8	18.99	
	pCO2	1.26	1.05	0.96	0.8	1.03	0.93	0.88	
	BE	-26.3	-25.9	-24.6	-23.8	-24.5	-24.8	-24.7	
	Glu	4.2	5.8	4.4	4.6	5.2	5.8	6.1	
	Lac	3.62	4.46	3.41	2.63	3.03	3.74	3.58	
Clinical Liver 5	pH	7.599	7.556	7.51	7.508				
	pO2	15.22	17.77	18.3	18.24				
	pCO2	0.79	0.82	0.83	0.82				
	BE	-21.5	-22.5	-23.5	-23.6				
	Glu	0	2.7	3.4	3.9				

	Lac	1.62	2.6	2.91	2.9				
Clinical Liver 6	pH	7.712		7.459	7.432	7.428	7.462	7.478	7.458
	pO2	14.9		19.6	21.3	25.55	16.64	15.15	16.37
	pCO2	0.87		0.99	1	0.87	0.72	0.74	0.82
	BE	-17.2		-24.1	-25.8	-25.3	-24.8	-24.4	-24.7
	Glu	1.3		5	7.2	8.3	9.3	9.2	10.1
	Lac	0.79		4.41	5.26	4.72	5.27	4.93	5.56
Clinical Liver 7	pH	7.312	7.313	7.377	7.408	7.403	7.412	7.438	7.431
	pO2	14.2	18.4	18.77	18.66	20.32	20.8	26.69	29.96
	pCO2	1.78	0.99	0.8	0	0	0	0	0
	BE	-26.9	-26.7	-26.6	-26.9	-26.7	-26.8	-26.3	-26.2
	Glu	1.9	3.6	4.9	5.9	6.3	6.7	6.9	7.5
	Lac	3.31	5.27	5.33	5.5	5.77	5.96	5.89	6.36
Clinical Liver 8	pH	7.05	7.616	7.515	7.516	7.498			
	pO2	16.8	16.26	17.26	17.27	34.74			
	pCO2	0	0	0	0	0.74			
	BE	-31.9	-27	-25.7	-25.8	-23.9			
	Glu	0	2.6	5.3	6	7.5			
	Lac	0.38	2.37	3.84	3.93	4.25			
Clinical Liver 9	pH	7.067	7.198	7.182	7.175	7.169	7.197	7.205	
	paO2	30.32	30.58	32.17	30.23	31.89	32.49	32.49	
	pCO2	0.74	0.78	0.63	0.62	0.58	0.20	0.32	
	BE	-24.8	-22.2	-22.6	-23	-23	-22.5	-22.3	
	Glu	1.9	1.1	1.3	1.7	1.8	1.9	1.9	
	Lac	2.5	1.71	1.88	2.29	2.26	2.21	2.31	
Clinical Liver 10	pH	6.799	6.935	7.022	7.043	7.018	7.041		
	paO2	38.7	37.24	41.82	39.18	35.25	35.53		
	pCO2	1.21	1.02	0.96	0.97	0.84	0.82		
	BE	-30.9	-28.7	-26.9	-26.7	-27.1	-26.7		
	Glu	16.5	17.3	19.6	21.9	22.1	24		
	Lac	6.14	5.61	5.82	6.7	6.42	6.99		

Table 11 - Perfusate gas analysis in 10 livers transplanted after hypothermic perfusion

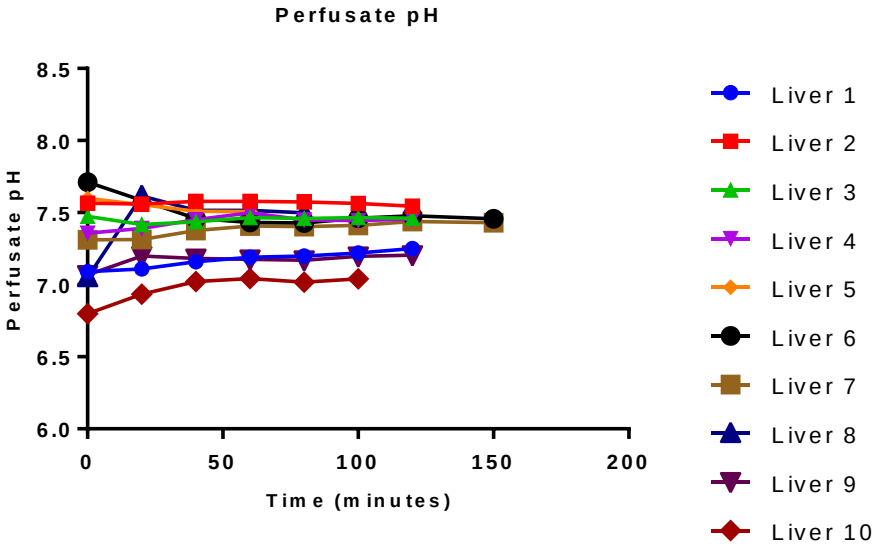


Figure 13 - Perfusate pH at each time point during perfusion

The perfusate pH was fairly static throughout all perfusions, with pH values moving in the direction of physiological pH in all livers (Figure 13).

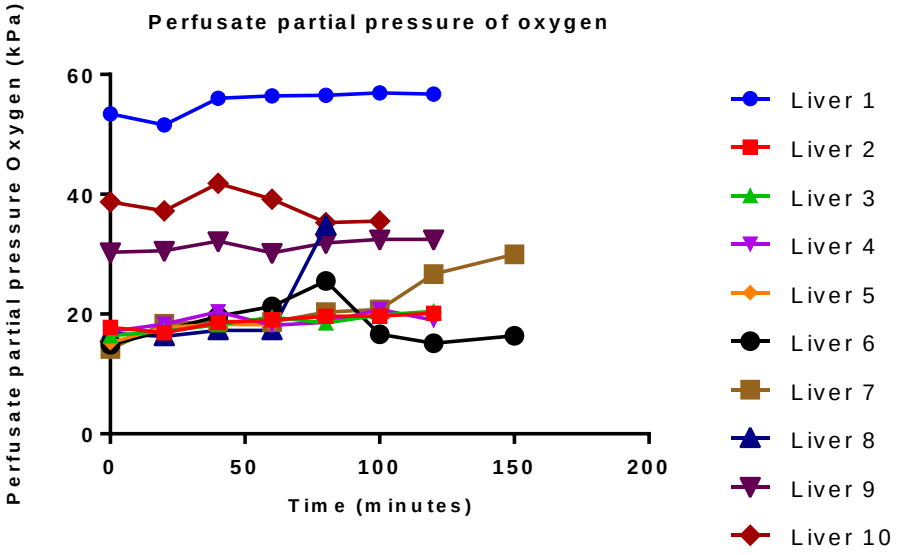


Figure 14 - Perfusate partial pressure of oxygen at each time point during perfusion

We aimed to achieve a pO2 of 20-30 kPa, however there were 2 livers that were over-oxygenated (Figure 14)

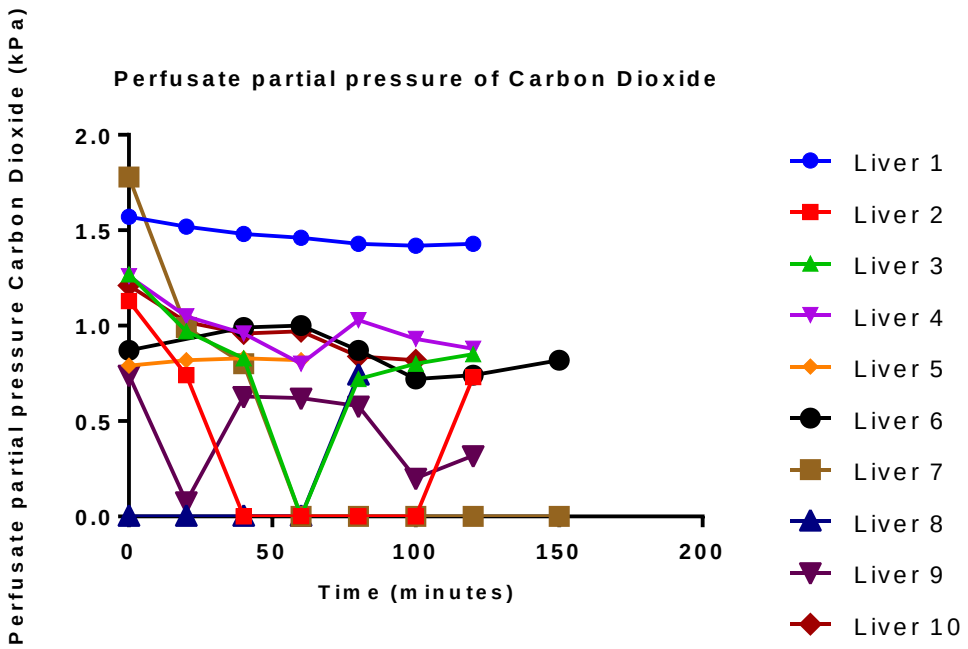


Figure 15 - Perfusate partial pressure of carbon dioxide at each time point during perfusion

The perfusate pCO_2 levels were relatively low in all livers and remained constant throughout perfusion (Figure 15)

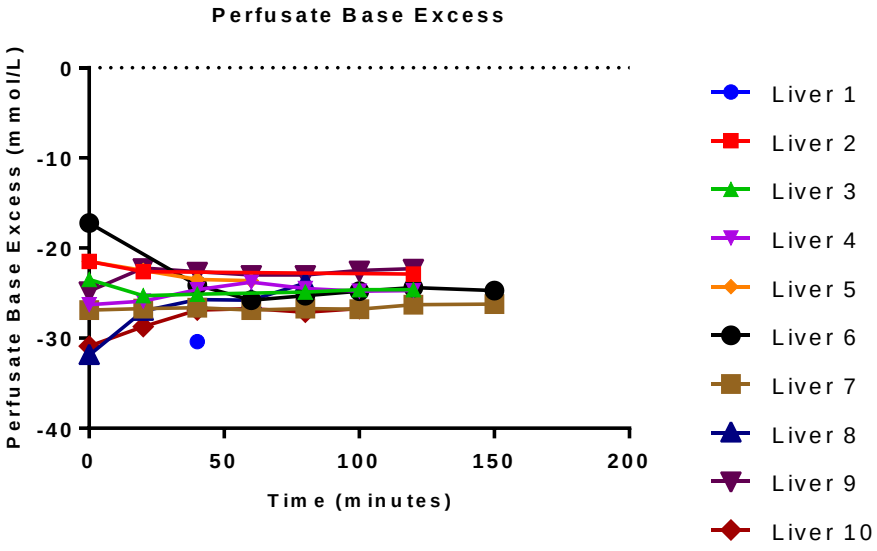


Figure 16 - Perfusate base excess at each time point during perfusion

Similar to the data on perfusate pH, the base excess normalised over time for all livers. Due to a temporary error with the blood gas analyser, there were incomplete values for liver 1 (Figure 16)

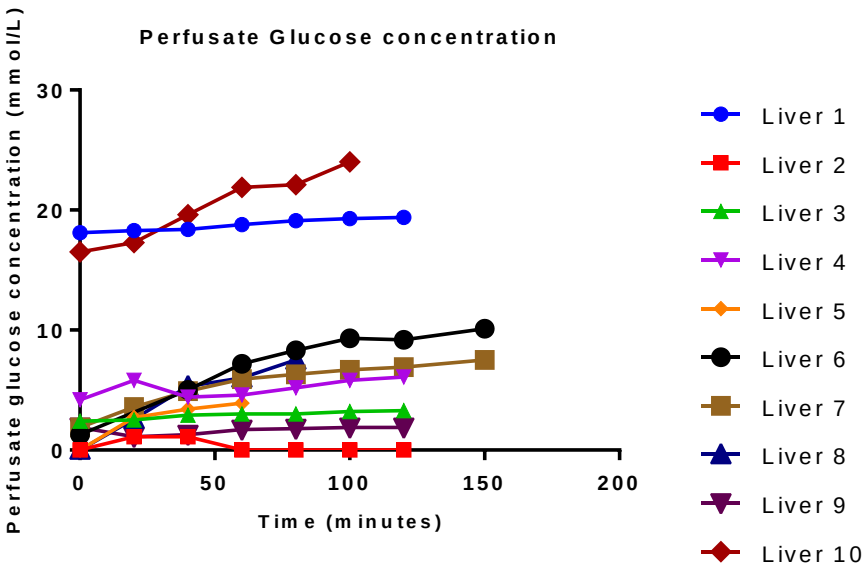


Figure 17 - Perfusate glucose concentration at each time point during perfusion

Liver 10 also has a significantly higher glucose concentration that was still climbing when perfusion finished; and liver 1 had also had a high glucose concentration, but which remained unchanged (Figure 17).

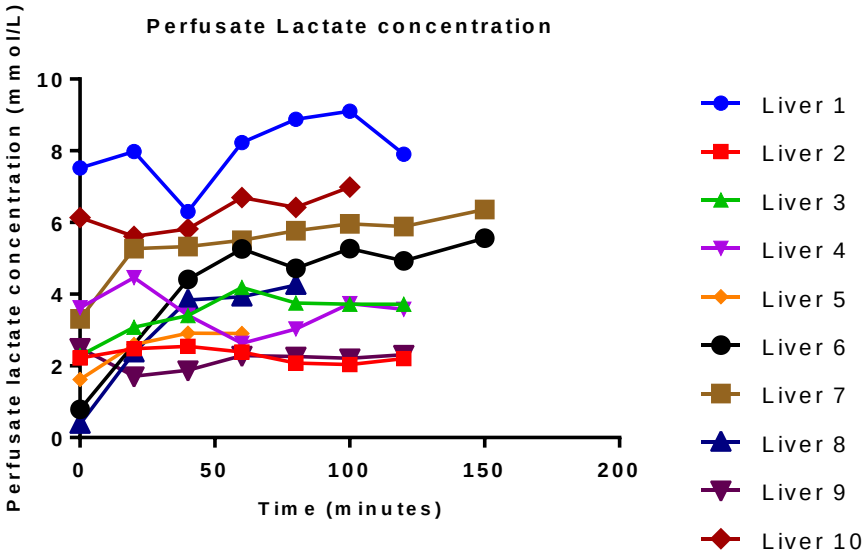


Figure 18 - Perfusate lactate concentration at each time point during perfusion

Lactate levels showed a broad range, but most remained fairly static apart from livers 6 and 8 that climbed during perfusion (Figure 18). None of these factors appear to have impacted on recipient outcomes as described below.

5.4.4 Recipient Outcomes

5.4.4.1 Cohort Control Comparison

HOPE liver recipients received standard follow up for liver transplantation including imaging where appropriate. Data on outcomes were prospectively collected without influencing standard of care.

HOPE recipient outcomes were compared to a local cohort of patients who received a standard liver transplant since 2010 that would have met our inclusion criteria for HOPE – i.e. DCD livers and DBD livers that had been declined by at least one other centre due to adverse donor factors. A total of 36 patients were included in this control cohort. Donor and recipient data were collected through analysis of prospectively maintained databases and patient records.

The 2 groups were comparable in terms of donor age, proportion DCD, and cold ischaemic times. Recipient characteristics were also well matched in terms of age (Figure 22) and presence of diabetes (Figure 23). Although the HOPE group had a significantly higher MELD score prior to transplant (21 vs 13.5, p=0.04, Figure 21) reflecting the fact they were higher-risk recipients than the control cohort. There was a higher number of HOPE recipients that had a previous liver transplant (20% vs 2.8%, p=0.11) although this did not reach significance; and a higher incidence of hepatorenal syndrome prior to transplant (20% vs 8.3%, *p* = ns, Figure 24), although also not significant.

HOPE			Historical Cohort			p-value
DCD	7		27			0.706 (Fisher's exact)
DBD	3		9			
Recipient Age						
Mean	59.7		55.9			0.44 (t-test)
Median	63.2		59			0.47 (M-W U)
Donor Age						
Mean	48.3		46.8			0.84 (t-test)
Median	52.6		51			0.89 (M-W U)
SCS Time (mins)						
Mean	576 mins		546 mins			0.54 (t-test)
Median	557 mins		546 mins			0.58 (M-W U)
Total Preservation Time (mins)						
Mean	690 mins		546 mins			**0.006 (t-test)**
Median	676 mins		546 mins			**0.007 (M-W U)**
MELD Score						
Mean	20		14.6			0.066 (t-test)
Median	21		13.5			**0.041 (M-W U)**
Previous liver transplant						
n	2/10	20%	1/36	2.8%		0.11 (Fisher's exact)
Graft Survival						
12-month	90%		84%			0.60 (log rank)
5 years			52.6%			0.18 (log rank)
Retransplant						
	1/10	10%	4/36	11%		1.0 (Fisher's exact)
for ischaemic cholangiopathy	0/10	0%	2/36	5.6%		1.0 (Fisher's exact)
Patient Survival						
12-month	100%		96%			0.54 (log rank)
5 years			75%			0.18 (log rank)
Clinically significant ischaemic cholangiopathy						
all	0/10	0%	9/35	25.7%		0.09 (Fisher's exact)
DCD only	0/7	0%	9/25	36%		0.15 (Fisher's exact)
Recipient ALT first 7 days						
	Median	Range		Median	Range	
Peak	706	150-3020	Peak	1123	224-4351	0.63 (M-W U)

Table 12 - Demographics and outcome comparison of HOPE recipients vs historical cohort

5.4.4.1.1 Cold Ischaemic Times

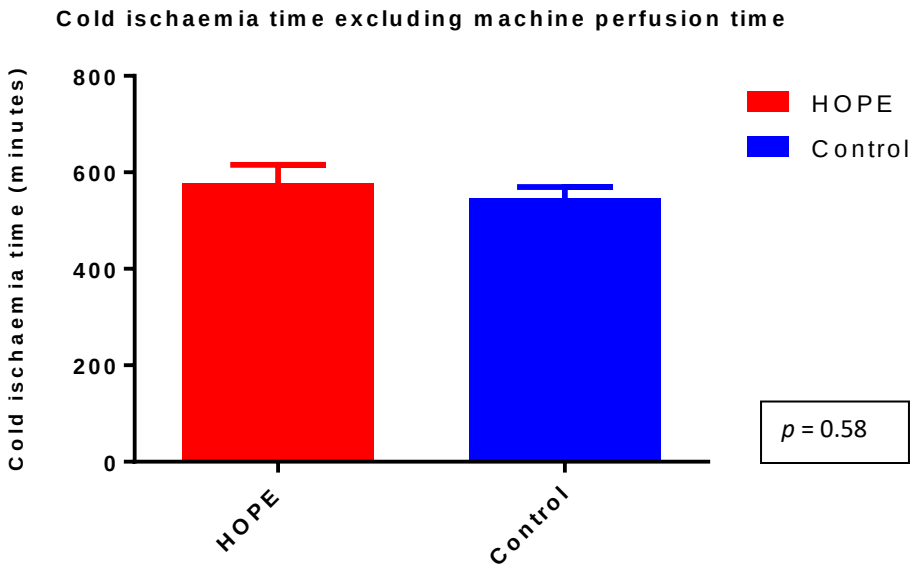


Figure 19 - Cold ischaemic times in HOPE livers vs historical controls. Showing median with SEM

Static cold storage times were unchanged by the introduction of HOPE (p = 0.58, Figure 19). However, there was a significant increase in total preservation time, which includes the perfusion period (p = 0.007, Figure 20).

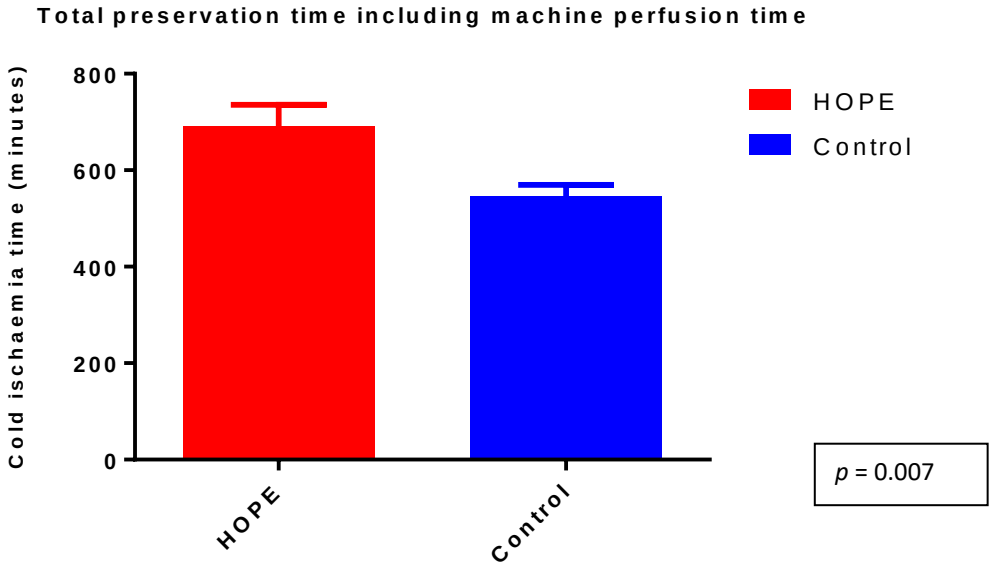


Figure 20 - Cold ischaemic times including machine perfusion time in HOPE livers vs historical controls showing median with SEM

As described above, the only significant difference between the two groups was a higher MELD score in the HOPE recipients, reflecting higher risk recipients (Table 12, Figure 21).

5.4.4.1.2 Donor and Recipient Risk Factors

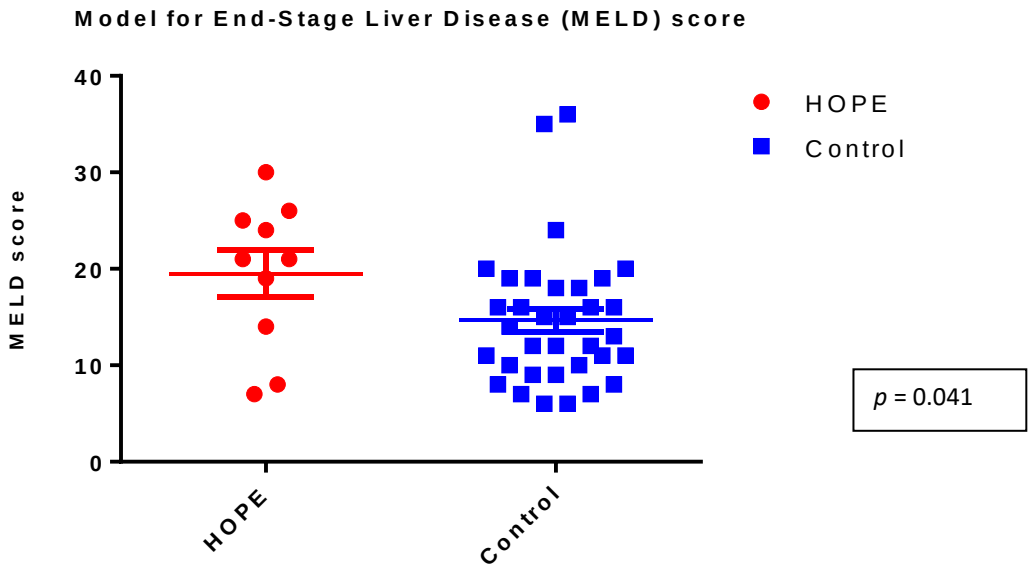


Figure 21 - MELD score in HOPE recipients vs historical cohort. Showing mean +/- S.D.

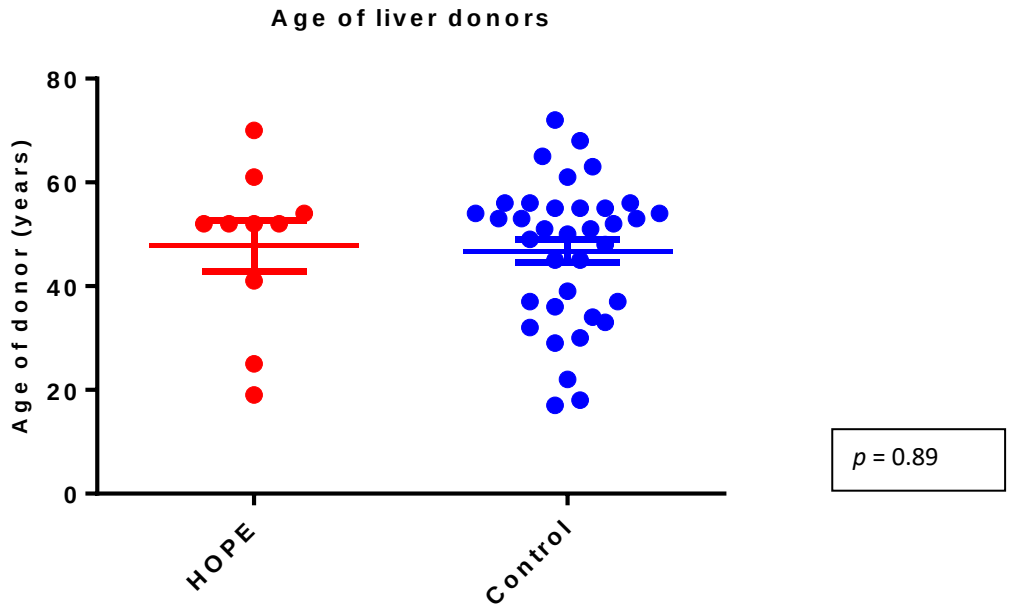


Figure 22 - Donor age in HOPE transplants vs historical cohort. Showing mean +/- S.D.

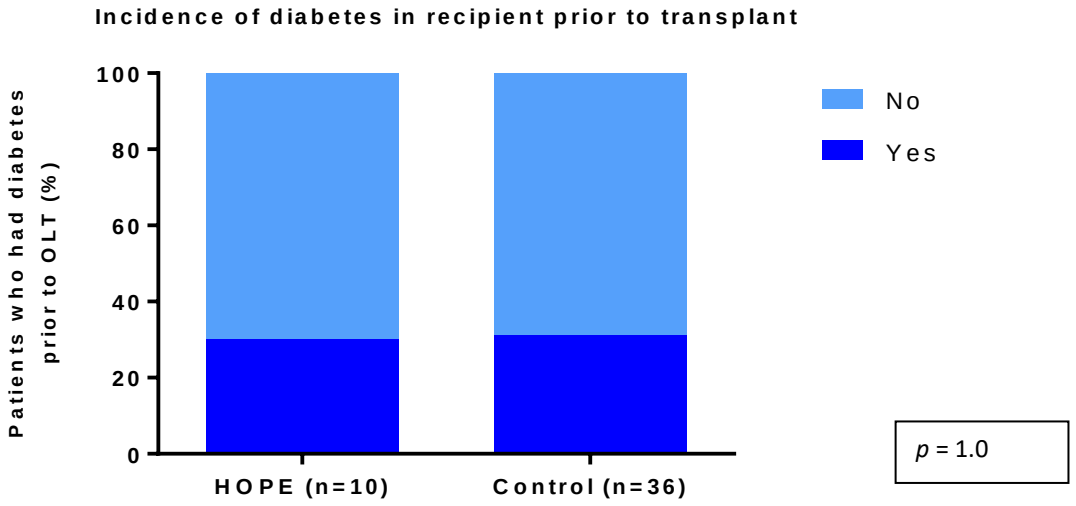


Figure 23 - Incidence of diabetes mellitus in recipient prior to transplantation in HOPE transplants vs historical cohort

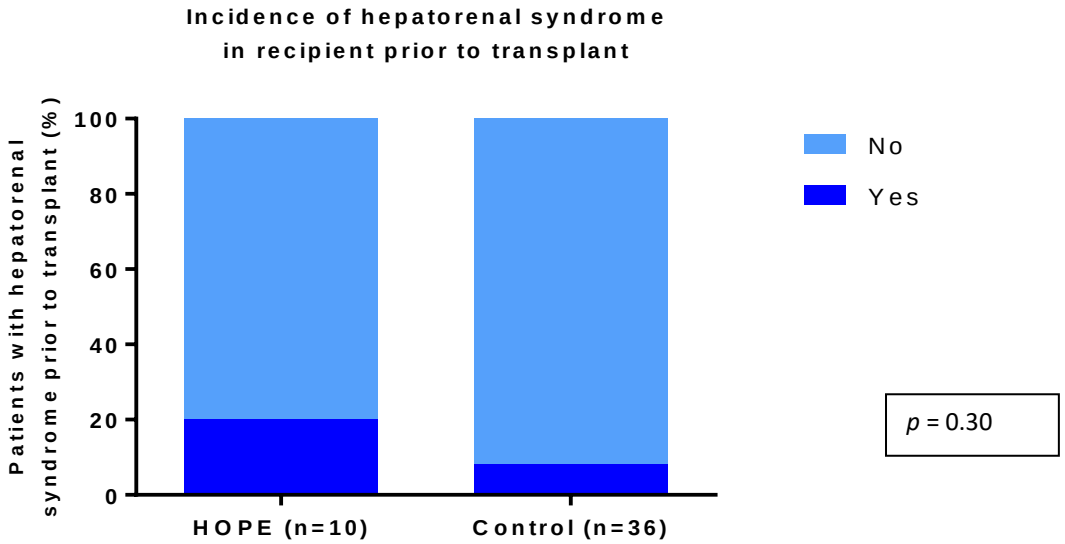


Figure 24 - Incidence of hepatorenal syndrome in recipient prior to transplant in HOPE transplants vs historical cohort

There was no significant difference in donor age (Figure 22), incidence of recipient diabetes prior to transplant (Figure 23) or incidence of recipient hepatorenal syndrome prior to transplantation (Figure 24).

5.4.4.1.3 Recipient Outcomes

Median follow-up in the HOPE group is now 52 months (43-58), there has been 1 death, who died of sepsis with a functioning graft at 34 months after his transplant.

There has been 1 re-transplant in HOPE group, which was performed nearly 11 months after the first transplant due to rejection. To date there have been no episodes of clinically-significant ischaemic cholangiopathy in any of the 10 HOPE patients. However, one patient has developed a biliary anastomotic stricture requiring ERCP and stenting. A further patient required a PTC in the early postoperative period during an episode of biliary sepsis; however since removal of the PTC over 2 years ago, the patient has not required any further biliary interventions and has maintained a normal bilirubin. A further patient has shown mild biliary wall thickening

on CT scan, but has not proved to be clinically significant and continues to have normal serum bilirubin levels over 18 months from the date of transplant. This compares very favourably to our historical cohort, in which ITBL was a significant problem. Over 25% of the patients in our historical cohort developed clinically-significant ischaemic cholangiopathy requiring intervention, compared to none of the HOPE transplanted livers. Two of the patients in the control cohort with required re-transplantation (and a further 2 were also retransplanted due to HAT). In addition to this, there was 1 further patient in the control cohort who died of organ dysfunction having developed ischaemic cholangiopathy, but who was not fit for re-transplantation. It is also interesting to note that all 9 instances of ischaemic cholangiopathy in the control cohort were in DCD grafts, and none in DBD grafts. Subgroup analysis of only DCD transplants demonstrates that ischaemic cholangiopathy tends to be more likely in DCD grafts, but not after HOPE perfusion, although our numbers are insufficient to demonstrate significance (0% vs 36%, p = 0.15). We also observed a reduction in peak ALT within first 7 post-operative days of 37% compared with the historical cohort (706 vs 1123 iu/L, p=0.63, Mann-Whitney U, Figure 25). There were no incidences of PNF in either group.

We have not demonstrated any improvement in length of ITU stay or length of overall hospital stay (p = 0.41, Mann-Whitney U, Figure 27 and Figure 28). Contrary to published reports, we also have not demonstrated a reduction in reperfusion syndrome (p = 1.0, Fisher's exact, Figure 29) or total use of blood products (p = 0.99, Mann-Whitney U, Figure 30).

There was no significant difference in the rates of post-operative biliary interventions between the two groups (p = 0.72, Mann-Whitney U, Figure 32), and nosignificant difference in the incidence of hepatic artery stenosis (p = 0.39, Fisher's exact, Figure 34). Although higher rates of HAT in the HOPE group were observed, none were retransplanted, whereas 2 patients in the control group were retransplanted for HAT.

There was higher rate of bile leak within the index admission in the HOPE group compared with the control group (p = 0.11, Fisher's exact, Figure 35). Two patients in total in the HOPE group had bile leaks, one of which required a return to theatre for conversion to hepaticojejunostomy.

There was no significant difference in the rates of acute kidney injury (p = 0.66, Fisher's exact. Episodes of acute rejection were slightly more likely in the HOPE group (p = 0.3, Fisher's exact), with 2/10 patients requiring treatment, one of which was retransplanted for rejection.

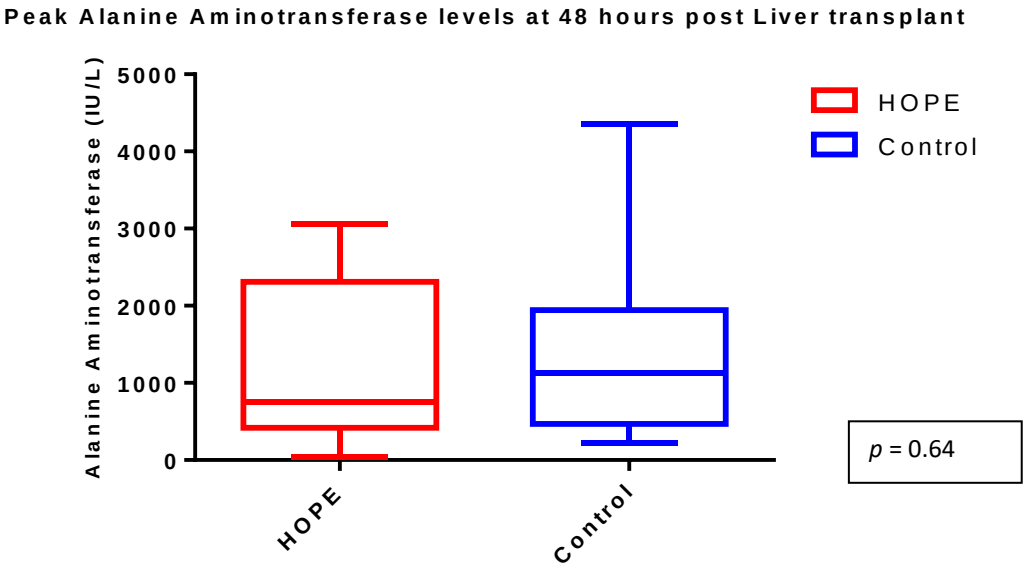


Figure 25 - Peak ALT levels within 48 hours of liver transplant in HOPE transplants vs historical cohort showing mean, S.D. (box) and minimum and maximum values (whiskers)

Post-op Day	0	1	2	3	4	5	6	7
Liver 1	530	836	681	3020	380	363	256	180
Liver 2	745	744	657	522	415	397	270	205
Liver 3	23	2684	2564	1322	859	603	411	315
Liver 4	437	667	626	490	404	278	212	133
Liver 5	389	291	212	145	116	137	133	135
Liver 6	483	599	618	475	443	406	346	302
Liver 7	291	200	150	143	128	135	145	159
Liver 8	34	32	27	29	76	144	126	150
Liver 9		2661	1918	1332	1004	780	528	447
Liver 10		1488	1045	730	517	380	304	246

Table 13- ALT levels (u/L) on each post-operative day in recipients after HOPE transplantation

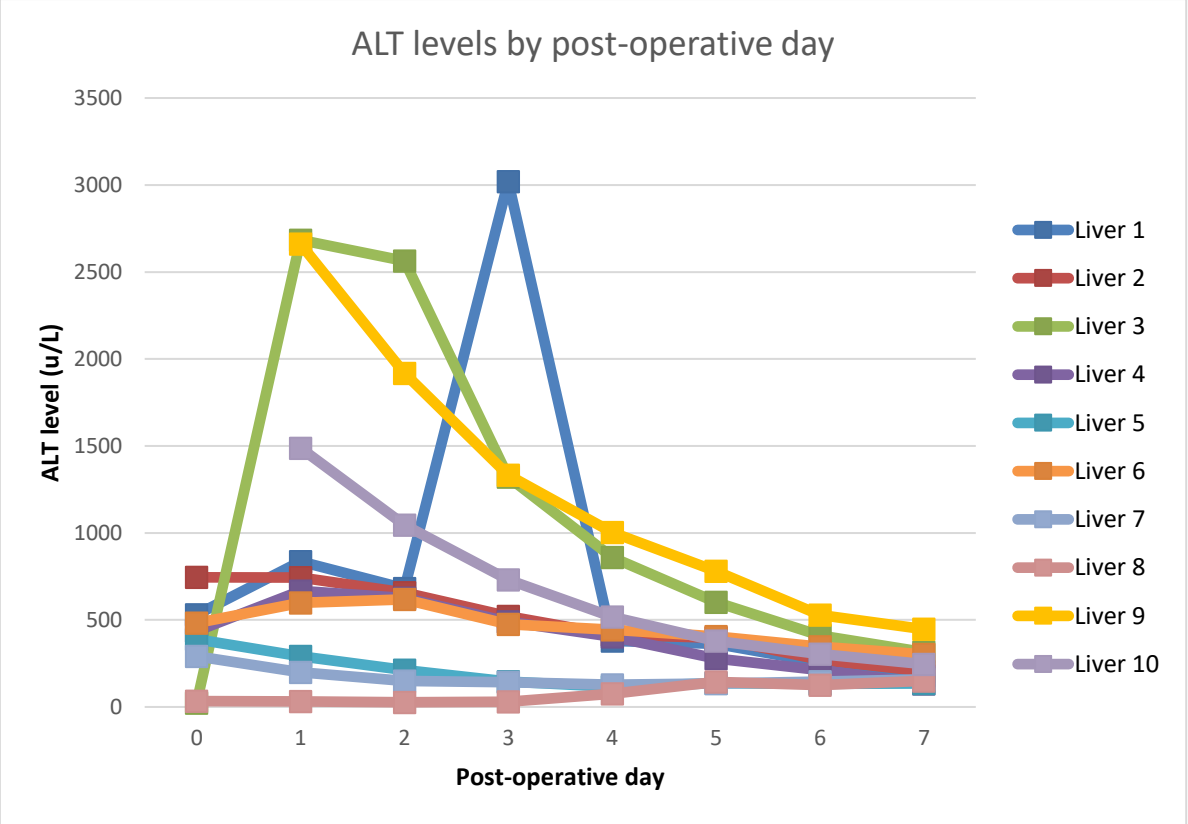


Figure 26 - ALT levels (u/L) in recipients each day after HOPE transplantation

Patient NM (blue line in Figure 26), had an isolated peak on day 3, which was due to an arterial conduit stricture. This improved following radiological intervention.

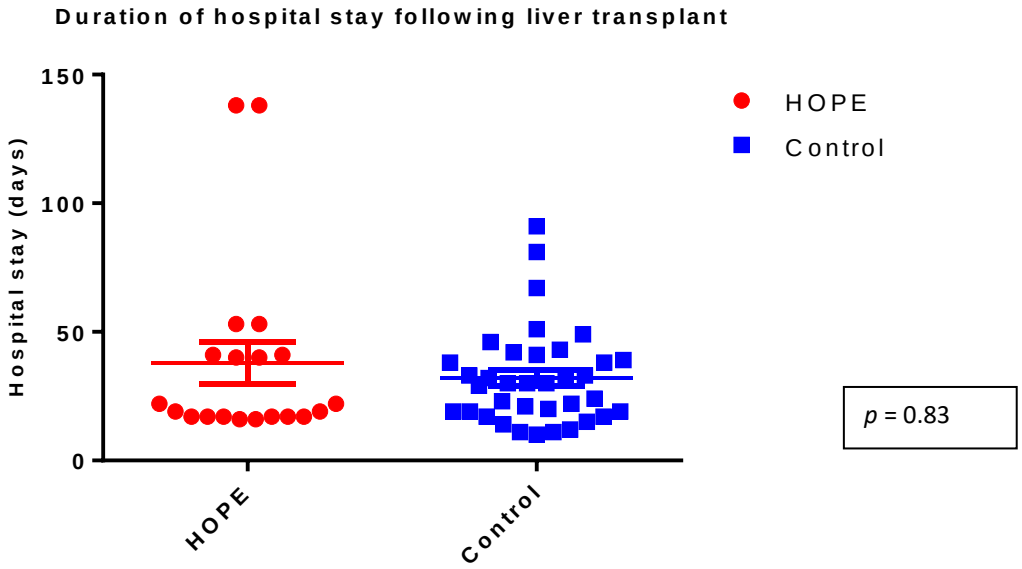


Figure 27 - Duration of hospital stay (days) after transplantation in HOPE recipients vs historical control

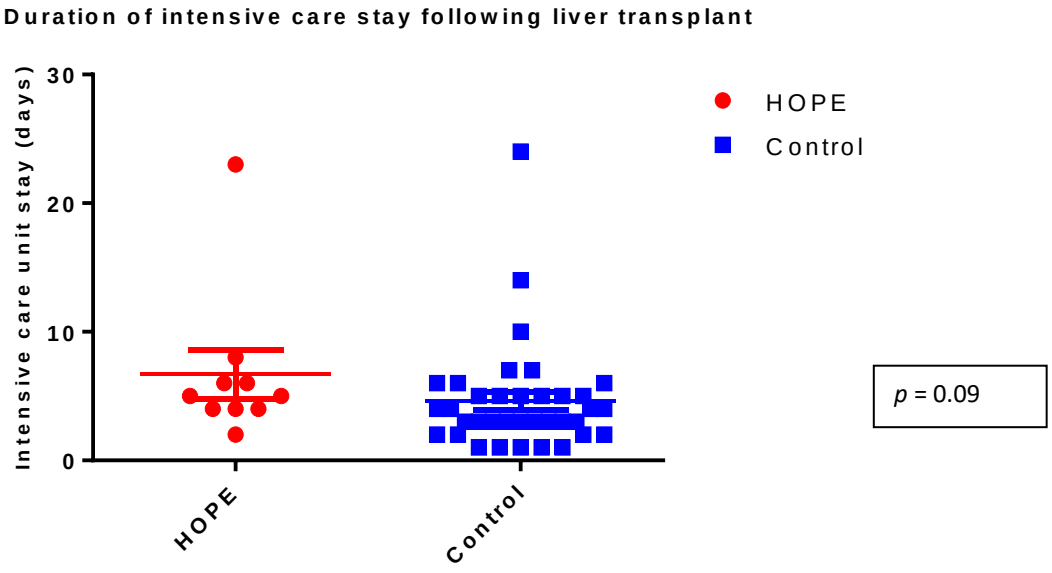


Figure 28 - Duration of ITU stay (days) following transplantation in HOPE recipients vs historical control

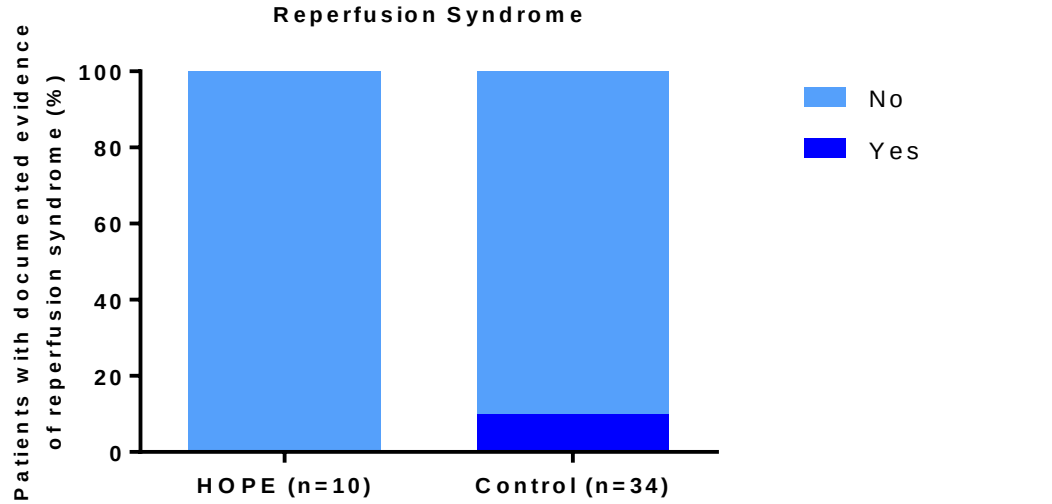


Figure 29 - Incidence of reperfusion syndrome in HOPE recipients vs historical control

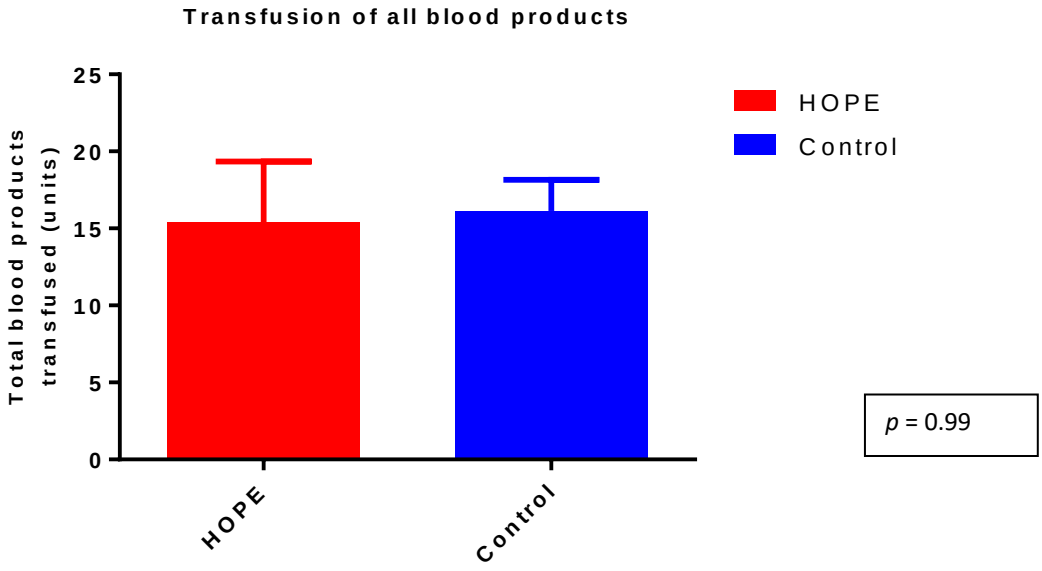


Figure 30 - Total units of blood products utilised peri-operatively in HOPE recipients vs historical control

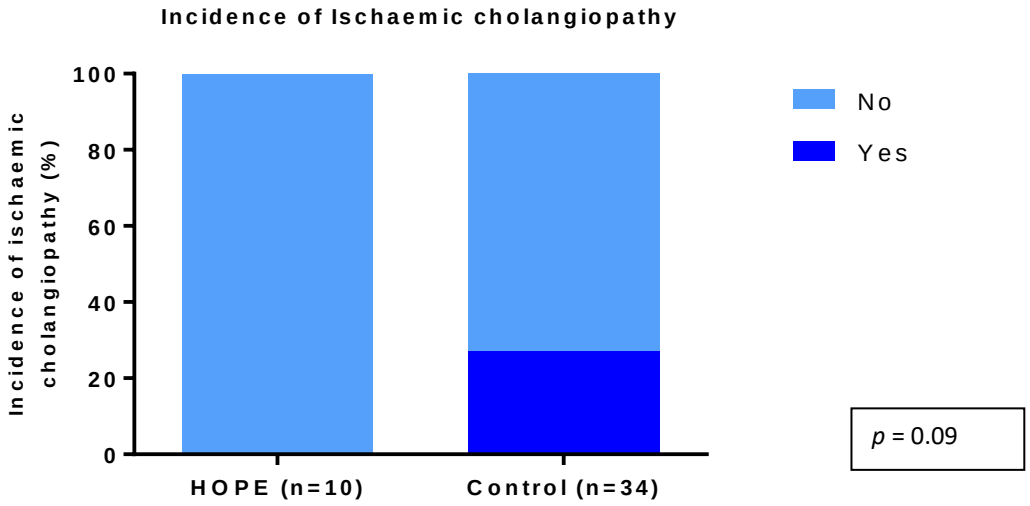


Figure 31 - Incidence of clinically-significant ischaemic cholangiopathy in HOPE recipients vs historical control

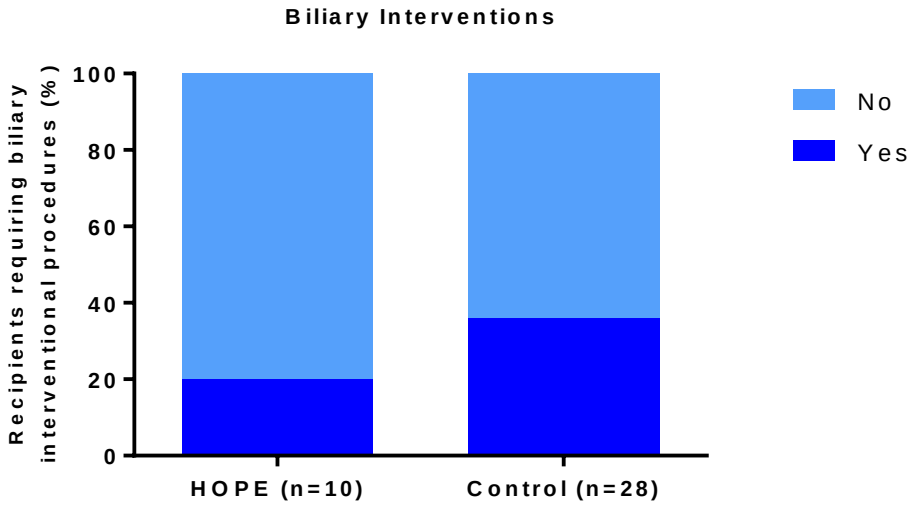
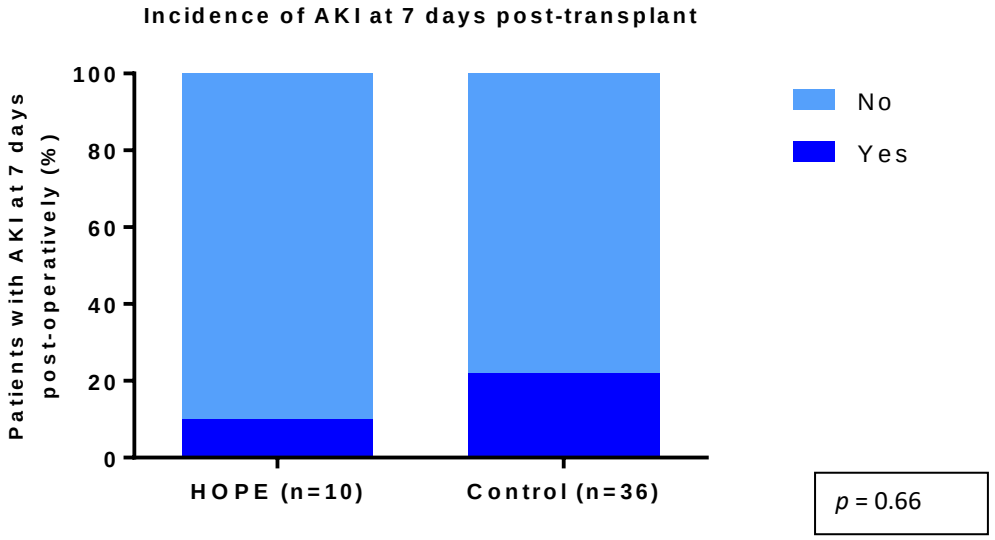


Figure 32 - Percentage of recipients requiring biliary interventional procedures in HOPE recipients vs historical control

$p = 0.72$



$p = 0.66$

Figure 33 - Percentage of recipients with acute kidney injury at 7 days post-transplantation in HOPE recipients vs historical control

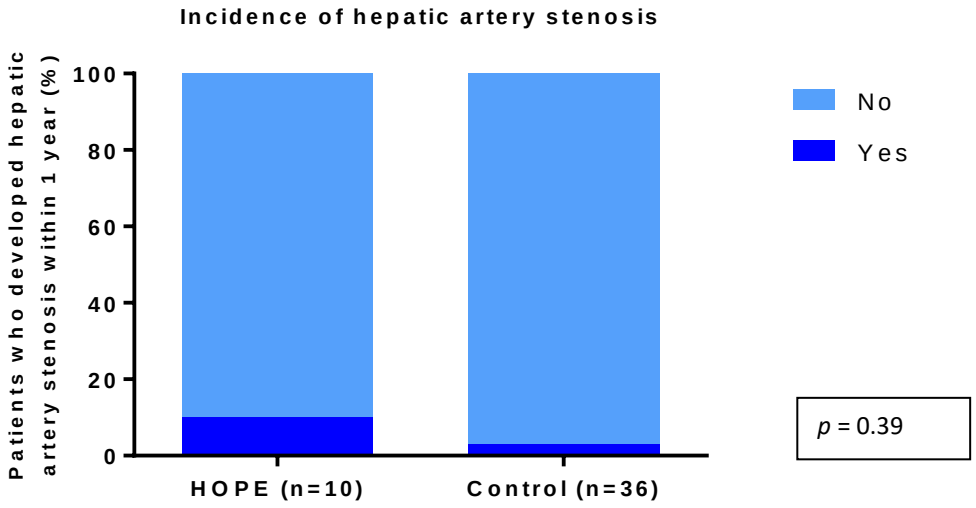


Figure 34 - Incidence (%) of hepatic artery stenosis within 1 year of transplantation in HOPE recipients vs historical control

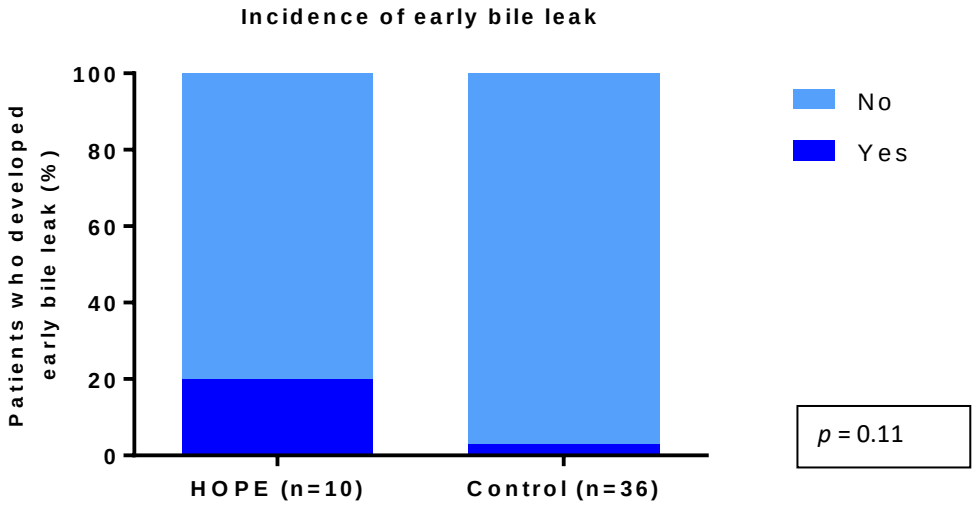


Figure 35 - Incidence (%) of early bile leak in HOPE recipients vs historical control

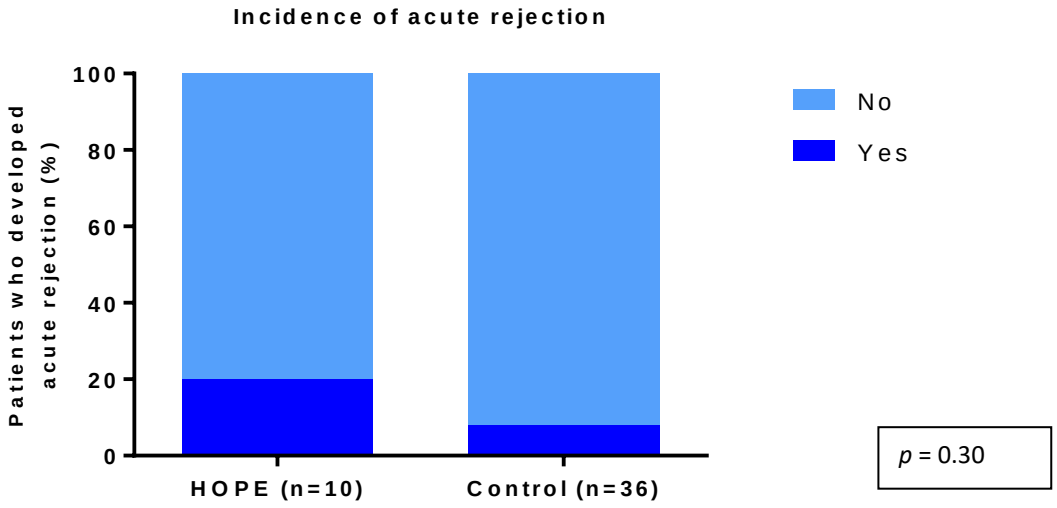


Figure 36 - Incidence (%) of episodes of acute rejection in HOPE recipients vs historical control

5.4.4.1.4 Infective Complications

Culture-positive infections were present in both groups at similar rates. In the HOPE cohort, 5 out of 10 recipients (50%) experienced a total of 9 culture-positive infections within first 30 post-operative days. The control cohort had similar rates with 21/36 (58%) of recipients experiencing 33 separate infections. The sites and organisms are summarised in table 14

	HOPE		Control	
Site	n (10)	Organism	n (36)	Organism
Wound	4	Mixed (1) S. Epidermidis (1) E. Coli (1) E. Cloacae (1)	11	Mixed (8), S. Epidermidis (1), E. Faecium (1), S. Faecalis (1)
Urine	1	E. Coli (1)	6	Mixed (2), E. Coli (2), Candida (1), S. Faecalis (1)
Blood	1	Klebsiella Oxytoca (1)	3	S. Epidermidis (1) S. Aureus (1) E. Faecium (1)
Respiratory	1	E. Coli (1)	5	Candida (1) MRSA (1) Mycoplasma hominis (1) S. Aureus (1) E. Coli (1)
Faecal			1	C. Dificile toxin (1)
Ascites/Bile	2	E. Faecium (1) E. Coli (1)	6	Mixed (3), E. Faecium (1) S. Faecalis (1) Klebsiella (1)
Skin			1	S. Aureus (1)

Table 14 – Summary of culture-positive sites and organisms in HOPE and control groups

5.4.4.1.5 Patient and Graft Survival

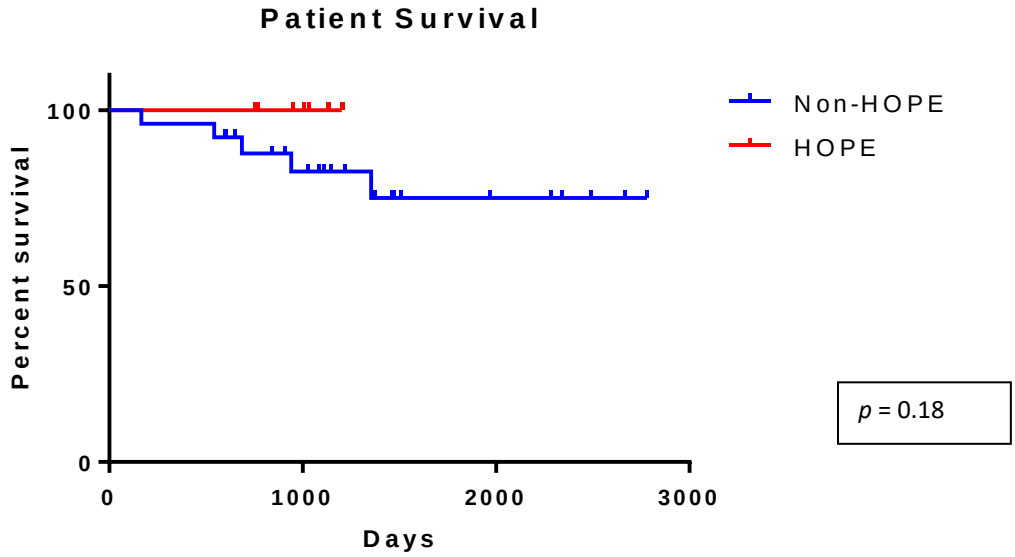


Figure 37 - Kaplan-Meier curve of patient survival after HOPE transplantation vs historical cohort

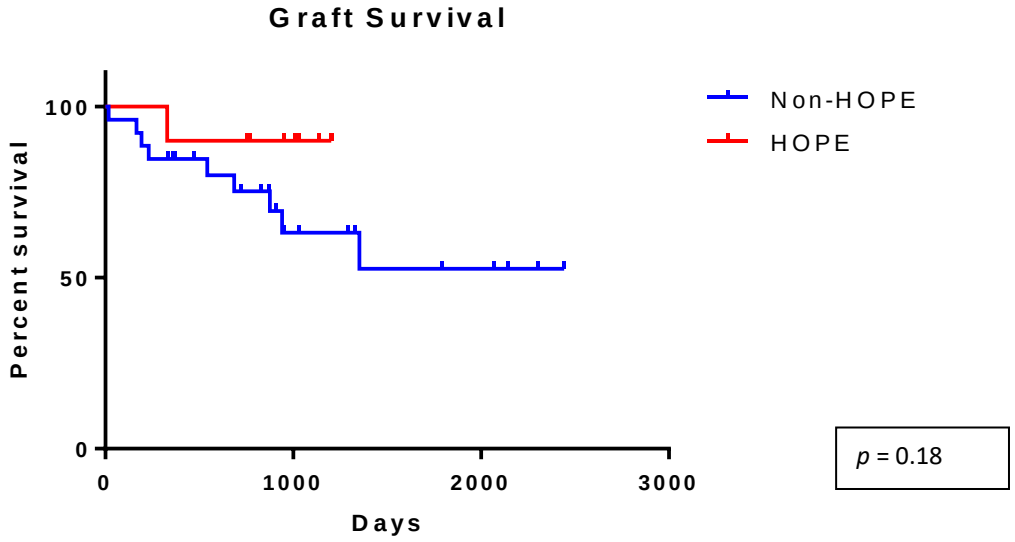


Figure 38 - Kaplan-Meier curve of graft survival after HOPE transplantation vs historical cohort

Kaplan-Meier analysis of both patient and graft survival appears to show superior outcomes in the HOPE group when compared with the historical cohort. However, our follow up remains too short for this to reach significance. We expect the difference will become more apparent in graft survival due to the relatively high incidence of retransplantation in the historic cohort, whereas the incidence of clinically-significant ITBL in the HOPE group remains 0. It remains to be seen if longer follow up will reveal simply a delay in onset of ITBL or a true reduction in incidence, although it should be noted that all the incidences of ITBL in the historical control group occurred within the first 6 months.

5.5 Normothermic Perfusion

In parallel to the introduction of clinical hypothermic perfusion a safety and feasibility cohort of normothermic machine perfusion of discarded livers was performed. Due to the increased technical risk in normothermic perfusion, it was decided that a run of 20 discarded livers would be required prior to establishing clinical ex-vivo perfusion. These 20 perfusions were carried out during the same time-frame as the 10 clinical hypothermic perfusions with a view to have the facility and expertise to perform hypothermic and normothermic clinical liver perfusion, and a long-term goal of performing a trial to compare the 2 temperature modalities. Within the remit of this research project, the series of 20 normothermic perfusions of discarded livers were performed, protocols refined and approvals obtained to perform clinical normothermic perfusion.

5.6 Discussion

A stepwise plan for clinical implementation of HOPE has been followed whereby discarded livers were first used for demonstration of safety and feasibility of the setup and protocol refinement prior to clinical translation. Protocol refinements were carefully catalogued and any team members involved educated on changes. 6 discarded livers were perfused prior to translation, and subsequently 10 clinical livers have been perfused and transplanted. High-risk donors and recipients were selected for these transplants, as these patients stood to benefit most from the technology, and were more in need of a transplant in a time-critical manner therefore accepting higher risk donors was deemed more appropriate. All 10 transplants had very significant donor and recipient risk factors. As a comparator, data was collected on a historical comparison cohort of patients transplanted in our centre since 2010 with livers that would have met our inclusion criteria for HOPE perfusion. The control cohort was comparable to the intervention group in all demographics except MELD score, which was significantly higher in the intervention group. This reflects the risks undertaken in these transplants as described above. Despite this, we have been able to demonstrate good clinical outcomes that compare favourably to the control cohort. There has been a single retransplant among the HOPE recipients, and this was due to rejection, which perfusion would not have been able to prevent. As yet there are no cases of clinically-significant ischaemic cholangiopathy, although it is worth noting that due to increased surveillance, routine scans on two patients have had biliary interventions, but not for ischaemic cholangiopathy. Whereas, in the control 9 out of 34 patients developed ischaemic cholangiopathy requiring retransplantation or intervention. However our numbers were insufficient to achieve statistical significance. All 9 patients who had cholangiopathy in the control group had received DCD livers, and cholangiopathy occurred within 6 months post-transplant. Of our 10 HOPE liver transplants, 7 were DCD; none of whom have developed cholangiopathy. Even in subgroup analysis of DCD-only grafts our sample size was again insufficient to demonstrate significance. However, given the relatively high rate of ischaemic cholangiopathy in our control cohort of DCD livers, it is likely that if our reduction in IC proves to be significant, that such livers stand to benefit most from perfusion. Peak ALT in the 7 days post-transplant is lower in the HOPE group than the historical cohort, but again insignificant. The patient with highest peak ALT in the HOPE group had a single isolated significantly elevated ALT for one day when he developed arterial compromise of the graft requiring

radiological intervention. 7 out of the 10 HOPE transplants had extremely low post-operative transaminases, but 3 recipients had ALTs >2000 which skewed our results slightly. We observed no differences in length of ITU stay or overall hospital stay, so attempts to offset cost of perfusion by reduced hospital stay are not possible in our series. No difference in rates of transfusion of blood products was observed either. Some authors have emphasised a reduction in reperfusion syndrome intra-operatively [48]; none of our 10 patients had significant reperfusion instability, but only a small proportion of the control cohort suffered this, so no significant difference was evident. There was no significant difference in rates of AKI at day 7 post transplantation in the control group. In contrast, there were higher rates of hepatic artery stenosis in the HOPE group and higher rates of bile leak, but both without significance. However, the rates of HAS and bile leak will need to be monitored closely in subsequent perfusions to ensure this trend does not continue. It is theoretically possible that cannulation of the artery and bile duct could compromise their integrity and lead to anastomotic issues. In all 10 patients the point at which the cannulae were secured to the artery or bile duct was always excised.

Overall, we have demonstrated safety and efficacy of our setup and have demonstrated safe introduction of a new technology to clinical transplantation. We have demonstrated non-inferiority to our control cohort, and hope that with longer follow-up and a larger series, some benefits may become significant. Of particular note is an apparent reduction in ischaemic cholangiopathy, a problem that has been significant particularly in our previous DCD liver transplants prior to HOPE. A power calculation suggests that if the IC rate in the HOPE group were to remain 0, then 3 more HOPE livers would be required to demonstrate significance against the same control cohort.

Longer follow up will be required to fully evaluate the benefits of HOPE, however early results are very promising.

Chapter 6. Liver Graft Assessment During Ex-Vivo Perfusion

6.1 Introduction

In addition to liver graft optimisation, ex-vivo perfusion has the potential to allow for more objective assessment of liver grafts prior to transplantation. Predictive tools such as DRI have been utilised[67], and are of some value, but further information on the function of the liver rather than purely donor factors would be more beneficial. There is an ongoing quest for suitable ways to test the transplantability of livers as a means to expand the available donor pool. The potential benefit of this is to reduce the proportion of livers that are currently discarded from potential donors. One particular area of applicability is in DCD donors, where there is apprehension over transplanting such livers due to inferior outcomes. If more objective means of assessing the livers could be employed, a more accurate selection process could be developed so that more DCD livers could be used with more confidence in future recipient function.

There are multiple parameters that could be analysed, including simple non-invasive measures such as flow rates. As well as perfusion dynamics, there are other potential means to assess liver grafts undergoing ex-vivo perfusion. This includes analysis of the perfusate, as well as more specific tests including contrast-enhanced ultrasound. If reliable biomarkers can be identified, it may become possible to perfuse high-risk livers where there is a degree of uncertainty of how they will perform after transplantation, and use the biomarkers as a tool to aid the decision-making process.

In order to further assess parameters of value in viability assessment of livers undergoing ex-vivo perfusion, we analysed various factors in both hypothermic and normothermic perfusion. In hypothermic perfusion we could compare transplanted livers (i.e. livers that were deemed transplantable at surgeon's discretion) against livers that had been declined by all centres (which due to logistics factors also had significantly longer CIT). In normothermic perfusion, all livers had been declined by all centres, but a subgroup were subjected to prolonged ischaemia. The objective of the prolonged ischaemia group is to demonstrate the perfusion and perfusate characteristics of a liver that is unequivocally untransplantable. There have been studies demonstrating that certain features such as high flow, low lactate, plentiful bile production, are indicative of a healthy functioning liver; however, little is known about whether those features would necessarily be absent in a poorly functioning liver. As such, a sub-group of NMP livers were intentionally subjected to prolonged cold ischaemia (median 71 hours, range 69-74 hours) followed by 1 hour of warm ischaemia in a 37°C water bath.

6.1.1 Perfusion Dynamics

Experience in ex-vivo organ perfusion, including other organs, has suggested that increased vascular resistance through the organ is suggestive of poorer tissue viability and hence poorer function [56, 108-110]. Our own experience with hypothermic kidney perfusion has demonstrated that flow is reduced as ischaemic injury increases [110]. This is potentially because ischaemic injury causes loss of vasodilatation, or potentially vessel occlusion due to thrombosis [110]. As most perfusion setups are pressure-controlled, the flow rate is indicative of vascular resistance. Mergental *et al*. proposed an acceptance criteria for livers undergoing NMP for viability assessment that included flow rates as one parameter [63], although if the liver had a pH >7.3 and homogenous graft perfusion with soft parenchymal consistency, the flow prerequisite was not required to pass viability [63]. Similarly, the criteria for the ongoing VITTAL study include flow rates as one of the parameters to be assessed, but not essential (only lactate <= 2.5 mmol/L is essential)[73].

We therefore assessed hepatic arterial and portal venous flow rates at regular intervals in all perfusions.

6.1.2 Lactate Metabolism and Blood Gas Analysis

Perfusate analysis has the potential to deliver in-depth information on the function of the liver during perfusion. Blood gas analysis, is rapidly and easily performed and facilities for point-of-care testing are readily available in all transplant theatres.

In normal lactate production, glycolysis in the cytoplasm produces pyruvate. In aerobic conditions, pyruvate enters the Kreb's cycle, but in anaerobic conditions such as during SCS, pyruvate is converted by lactate dehydrogenase to lactic acid, which then dissociates to lactate and H^+. In normal conditions, the liver removes approximately 70% of lactate predominantly by gluconeogenesis, and to a lesser extent by oxidation to CO_2 and water. In anaerobic conditions, glycolysis is the predominant mode of energy production in the liver, as such the liver becomes a lactate-producing organ rather than clearing it [111].

To date a lot of published studies into viability assessment have placed great emphasis on the importance of lactate clearance by the liver as a sign of healthy aerobic metabolism in peri-portal hepatocytes[28, 38, 59, 63]. Whilst it makes sense that the absence of lactate metabolism should be indicative of a poorly functioning liver, there is no evidence that lactate clearance necessarily indicates adequate function.

Similarly, as part of the blood gas analysis, normal pH is suggestive of normal aerobic metabolism and lack of lactic acid dissociation. Also oxygen consumption (i.e. difference in pO_2 between inflow and outflow to the liver) would suggest the same.

6.1.3 Bile Production

Although successful evaluation of hepatocellular function by lactate metabolism or any other successful means may prevent PNF of the liver, it will not have any role to play in predicting cholangiopathy [48]. Some studies have cited volume of bile production as a marker of viability in normothermic perfusion [69], but this experience is not universal [28]. The process of bile production is affected by many factors, which may or may not correlate with function. For example, high levels of glucose often seen in NMP setups, reduces the

production of bile by reducing cholangiocyte absorptive processes. In hyperglycaemia, as cholangiocytes absorb more glucose, they also absorb more water from bile, thus reducing bile volume production [112].

Unfortunately, in hypothermic perfusion the metabolic rate is insufficient to produce meaningful volumes of bile, therefore this form of viability assessment is not available.

6.1.4 Alanine Transaminase

Alanine Transaminase is a common part of standard liver function tests. It is an enzyme that catalyses the transfer of amino groups to form the hepatic metabolite oxaloacetate. ALT is found abundantly in the cytosol of hepatocytes, thus hepatocellular injury or death results in measurable release of ALT from damaged liver cells [113]. Although ALT is not typically available as a point-of-care test, it is universally analysed in biochemistry labs and results usually available in 20-30 minutes. Some studies have analysed aspartate aminotransferase (AST), which is also a marker of hepatocellular injury by similar mechanisms, but the advantage of a faster rise to peak and shorter half-life [113]. However, its less widespread use in clinical practice in the UK, makes its rapid analysis at any time of day less feasible.

Daily recipient serum ALT levels are routinely checked in the days following a liver transplant, and are used as an indication of graft function.

The ability to predict how a liver graft will function in the recipient after transplant remains the ultimate aim of assessing livers during ex-vivo perfusion. As ALT is an established indicator of graft function in-vivo, our hypothesis is that the analysis of ALT levels ex-vivo may be an indicator of predicted graft function post-transplant.

6.1.5 Multiplex Analysis

The liver contains multiple leucocytes such as Kupffer cells and dendritic cells, which may be activated in ischaemia-reperfusion injury resulting in inflammatory proteins such as cytokines and chemokines. Cytokines are cell signalling proteins that can affect the cells around them. There are many different types of cytokines that are activated in different circumstances and have different targets and therefore distinct roles. Multiple factors can promote cytokine expression, including ischaemia, stress, trauma and reactive oxygen species. Therefore, the relative concentration of key cytokines in the perfusate of a liver undergoing ex-vivo perfusion could be indicative of the degree of injury that the liver has sustained and consequently become surrogate markers of transplantability.

During liver reperfusion, the generation of reactive oxygen species by mitochondria threaten the viability of hepatocytes. Hepatocytes stressed by ROS, leak cellular components known as damage-associated molecular patterns (DAMPs), which become potent immunostimulators in the extracellular compartment, resulting in an overt inflammatory response [114].

Some studies have demonstrated high concentrations of such proteins, reflecting activation of the immune-cell compartment of the liver [114-116]. During reperfusion, hepatocellular ROS play a critical role in detrimental activation of a localised, sterile immune response [116]. ROS-mediated activation of nuclear factor kβ (NF-kβ),

stimulates increases in highly-inflammatory cytokines, such as IL-1α, IL-1β, IL-2, IL-6,IL-8, TNF-α, and TNF-β [117].

This contrasts with the experience during hypothermic perfusion, where hypothermic oxygenated perfusion triggers significantly less oxidative damage in livers and subsequently downregulation of downstream inflammatory pathways [30, 49]. The theory being that HOPE is able to restore mitochondria from ischaemia to replete stores of ATP without triggering the same oxidative damage experienced with normothermic IRI [30, 49].

6.1.6 Contrast-Enhanced Ultrasound

An alternative to analysis of perfusate is to perform imaging studies on the liver in real-time. One potential method is contrast-enhanced ultrasound (CEUS). This technique, currently in clinical use allows for detailed examination of vascular flow, particularly within parenchyma. The technology relies on administration of a microbubble contrast agent, ordinarily this is given intravenously, but can be given directly into the ex-vivo perfusion circuit. The microbubble contrast agent reflects sound waves, and analysis of this backscatter allows for quantification of vascular flow within a particular area of interest. One would expect that the micro-circulation of a healthy liver be intact, and we hypothesise that in an unhealthy liver there is potential for micro-emboli to have formed within the parenchymal circulation, indeed fatty livers are known to have impairment of microcirculation[118]. If this is the case, CEUS is another potential tool in the real-time assessment of livers during ex-vivo perfusion. There is some existing experience in utilising this technology in the assessment of kidneys prior to implantation[119], and CEUS is in use for assessment of graft perfusion after implantation[120-124], including the potential to detect ischaemic-type biliary lesions[125]. There is also limited evidence on the use of CEUS for the assessment of porcine livers on ex-vivo perfusion[126]. This study by Alzaraa *et al*. demonstrated the ability of CEUS to delineate poorly perfused areas that correspond to ischaemia on biopsy[126].

6.2 Perfusion Dynamics

6.2.1 Methods

All livers, hypothermic and normothermic were perfused with a fixed-pressure protocol and variable flow, such that vascular flow is a surrogate of vascular resistance within the liver. HA and PV flow rates were measured using Medtronic TX50P and TX50 flow probes respectively and recorded at the same time intervals as perfusate sampling – every 20 minutes for 2 hours and progressively decreasing time intervals beyond this for normothermic livers.

6.2.2 Results

6.2.2.1 Hypothermic Perfusion

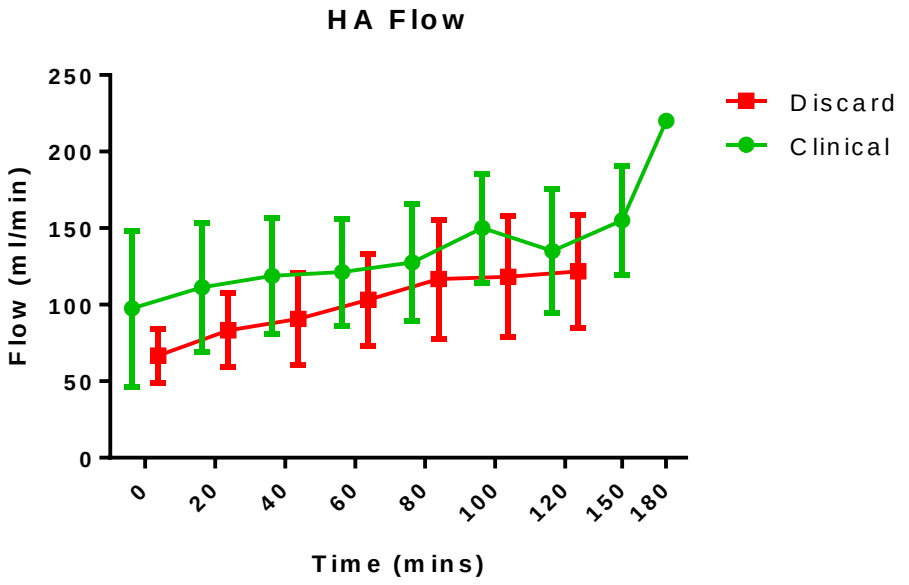


Figure 39 - Hepatic artery (HA) flow (ml/min) showing mean +/- S.D. in clinical and discarded livers during hypothermic perfusion

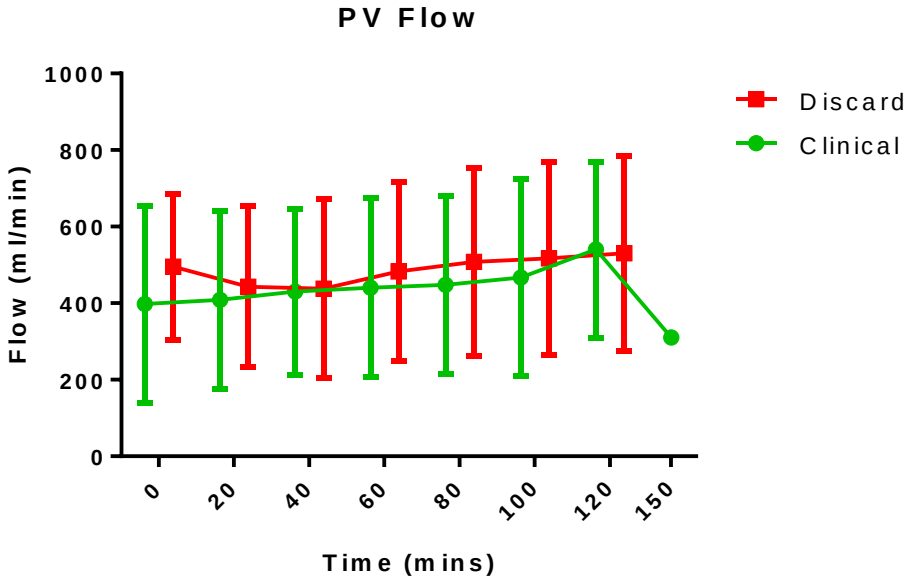


Figure 40 - Portal vein (PV) flow (ml/min) showing mean +/- S.D. in clinical and discarded livers during hypothermic perfusion

There was no significant difference in flow rates, either arterial or portal venous when comparing our clinical livers with discards (Figure 39 and Figure 40). The flow rates were relatively consistent between livers and there was a consistent trend to improved arterial flow over the course of perfusion suggesting relaxation of microvasculature or clearance of micro-emboli.

6.2.2.2 Normothermic Perfusion

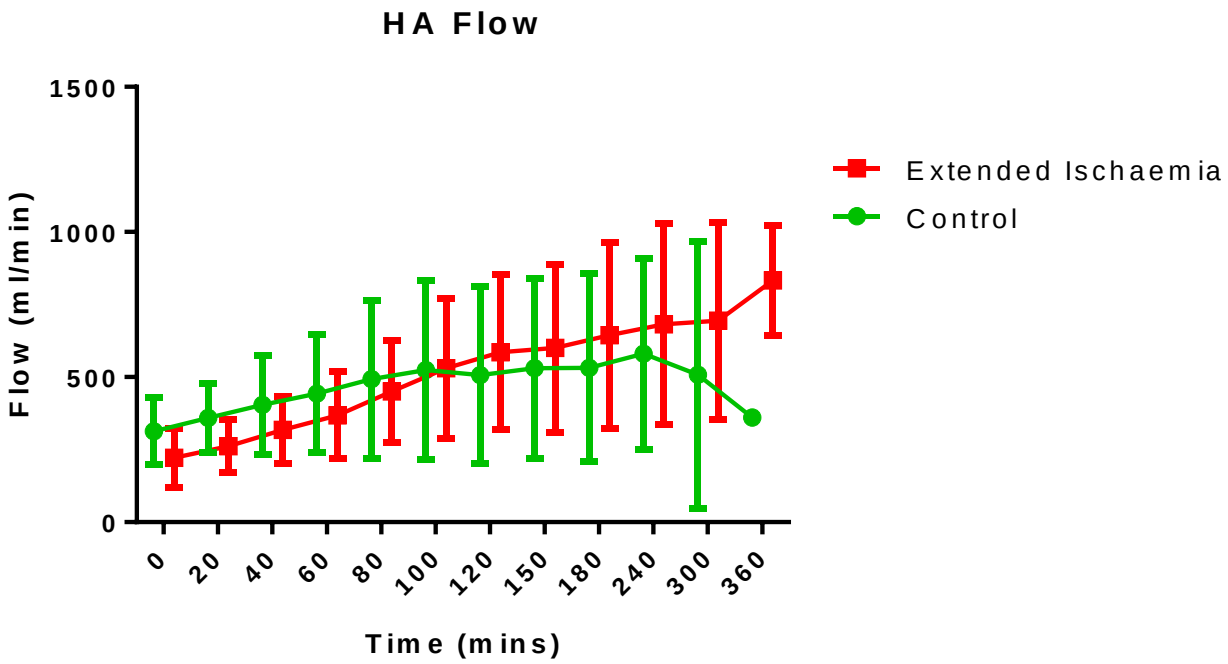


Figure 41 - Hepatic artery (HA) flow (ml/min) showing mean +/- S.D. in control and 'extended ischaemia' livers during NMP

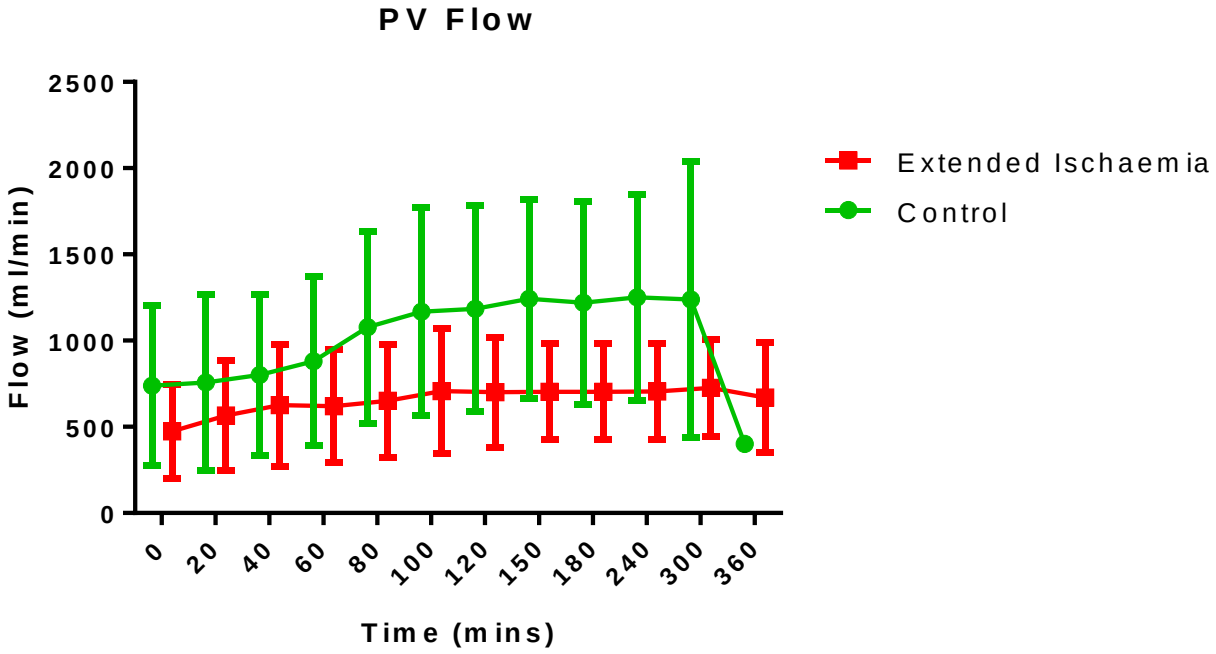


Figure 42 - Portal vein (PV) flow (ml/min) showing mean +/- S.D. in control and 'extended ischaemia' livers during NMP

The HA flow rates were almost indistinguishable between the control group and the extended ischaemia group, again with a trend towards increased flow over the course of perfusion indicative of reduced arterial resistance.

There was no significant difference demonstrated in PV flow, although there was often reduced flow in the extended ischaemia group. The lowest p-value was at 150 minutes (p = 0.07). Similarly to arterial flow, there was a general increase in flow over the perfusion time, although the change was less significant.

6.3 Lactate Metabolism and Blood Gas Analysis

6.3.1 Methods

Perfusate samples were taken every 20 minutes for the first 2 hours of perfusion followed by decreasing frequency in normothermic perfusion. Samples were taken using 1ml pre-heparinised blood gas syringes and analysed immediately using a Siemens RAPIDPoint 500 System that was regularly auto-calibrated. Sample analysis was adjusted for perfusate temperature and the same machine was used for all samples. Oxygen consumption was calculated using the coefficient of solubility of oxygen as 0.038 to calculate the volume of oxygen present, which can then be multiplied by the flow rate to calculate the volume of oxygen passing through the liver. The difference in diluted volume of oxygen between the inflow blood gas and outflow blood gas allowed a calculation of volume of oxygen consumed, which was then normalised by weight of the liver to achieve an oxygen consumption in cm^3 of oxygen per Kg of liver per minute.

6.3.2 Results

6.3.2.1 Hypothermic Perfusion

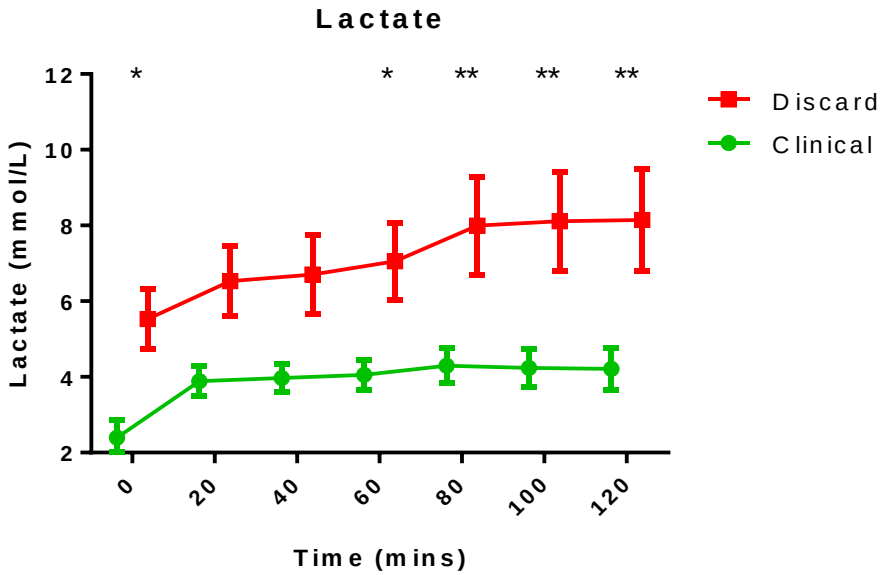


*Figure 43 - Perfusate lactate levels (mmol/L) showing mean +/- S.D. in clinical and discarded livers during hypothermic perfusion. * denotes significance at that time-point*

In our experience lactate measurements in hypothermic perfusion have demonstrated a clear distinction between the clinical and discard livers. This is statistically significant at every time-point except 20- and 40-

minutes (t-test). ROC curve analysis generates an area under the curve of 1.0, p=0.0017, with an optimum cut off value of 4.912, generating 100% sensitivity and specificity.

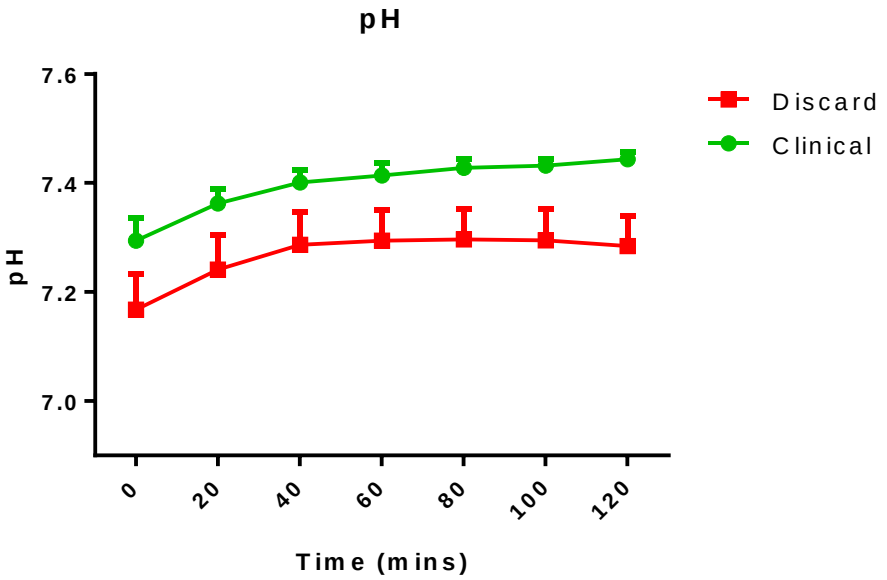


Figure 44 - Perfusate pH levels in clinical and discarded livers during hypothermic perfusion

Perfusate lactate levels were also reflected in pH measurements with perfusate from discarded livers being consistently more acidotic, at least partly due to lactic acidosis.

	Time	Start	20	40	60	80	100	120	150
Discard Liver 1	pH	7.257	7.298	7.301	7.331	7.311	7.307	7.309	
	pO2	16.43	15.46	17.97	19.29	19.21	16.11	17.23	
	pCO2	2.27	1.38	0.92	0.73	0.76	0.71	0.72	
	BE	-27.3	-27.5	-28.1	-27.7	-28	-28.2	-28.3	
	Glu	16.5	16.6	18.3	18.4	19.3	20	20.4	
	Lac	5.46	7.03	9.08	8.61	10	10.15	10.32	
Discard Liver 2	pH	7.203	7.156	7.204	7.194	7.168	7.15	7.138	
	pO2	17.3	16.58	19.86	20.52	20.69	20.4	20.54	
	pCO2	1.25	1.09	0.82	0.82	0.72	0.75	0.74	
	BE	-29.8	-31	-30.3	-30.5	-31.2	-31.3	-31.6	
	Glu	24.9	27	25.6	26.2	28.3	29.3	29.7	
	Lac	7.2	8.98	8.99	8.19	9.94	9.9	10.26	
Discard Liver 3	pH	7.111	7.273	7.326	7.333	7.303	7.314	7.318	
	pO2	26	21.05	17.47	19	20.53	21.38	21.87	
	pCO2	1.28	1.05	1.05	0.96	0.95	0.92	0.89	
	BE	-26.9	-27.1	-27.3	-27.3	-28	-28.4	-28.9	
	Glu	20.4	21.7	21.4	21.8	22.6	22.9	23	
	Lac	4.67	5.82	5.28	5.9	6.84	7.11	7.18	
Discard Liver 4	pH	7.217	7.256	7.287	7.311	7.284	7.256	7.21	
	pO2	17.95	18.11	18.88	19.15	19.45	19.16	18.84	
	pCO2	1.95	1.63	1.23	0.95	0.88	0.79	0.74	

	BE								
	Glu	18.1	18.9	19.4	19.7	20.3	20.9	21.8	
	Lac	6.11	6.86	8.04	8.99	10.23	10.36	11.02	
Discard	pH	7.458	7.492	7.46	7.567	7.505	7.484	7.491	
Liver 5	pO2	17.94	18.3	18.95	18.9	19.04	19.68	19.58	
	pCO2	1.11	0.8	1.01	0.96	0.97	1.14	1.14	
	BE	-24.2	-24	-24.3	-24.3	-23.3	-23.5	-23.3	
	Glu	10.1	11.3	11.9	13.4	13.8	14	14.5	
	Lac	3.67	4.16	4.11	4.62	3.95	3.54	3.54	
Discard	pH	7.33	7.337	7.35	7.373	7.401	7.427	7.433	
Liver 6	pO2	17.4	16.34	19.57	20.38	21.08	19.94	20.11	
	pCO2	1.69	1.52	1.43	1.18	1.25	1.19	1.23	
	BE	-26.3	-26.4	-26.2	-26.1	-25.3	-24.8	-24.5	
	Glu	6	5.4	5.7	6.3	6.9	7.3	7.8	
	Lac	3.51	3.85	4.16	4.32	3.63	3.3	3.19	
Clinical	pH	7.09	7.11	7.16	7.19	7.2	7.22	7.25	
Liver 1	pO2	53.4	51.6	56	56.4	56.5	56.9	56.7	
	pCO2	1.57	1.52	1.48	1.46	1.43	1.42	1.43	
	BE			-30.4					
	Glu	18.1	18.3	18.4	18.8	19.1	19.3	19.4	
	Lac	7.52	7.98	6.3	8.23	8.88	9.1	7.9	
Clinical	pH	7.565	7.559	7.578	7.579	7.575	7.563	7.544	
Liver 2	pO2	17.75	16.89	18.57	19	19.61	19.68	20.11	
	pCO2	1.13	0.74	0	0	0	0	0.73	
	BE	-21.5	-22.6					-22.9	
	Glu	0	1.1	1.1	0	0	0	0	
	Lac	2.22	2.48	2.55	2.39	2.08	2.04	2.2	
Clinical	pH	7.475	7.418	7.437	7.465	7.46	7.465	7.462	
Liver 3	pO2	16.39	17.14	18.21	19.45	18.53	19.81	20.45	
	pCO2	1.27	0.97	0.83	0	0.72	0.8	0.85	
	BE	-23.5	-25.3	-25.1		-24.9	-24.6	-24.6	
	Glu	2.4	2.5	2.9	3	3	3.2	3.3	
	Lac	2.29	3.08	3.4	4.19	3.75	3.72	3.72	
Clinical	pH	7.358	7.39	7.451	7.499	7.45	7.445	7.456	
Liver 4	pO2	17.08	18.32	20.38	18.15	18.62	20.8	18.99	
	pCO2	1.26	1.05	0.96	0.8	1.03	0.93	0.88	
	BE	-26.3	-25.9	-24.6	-23.8	-24.5	-24.8	-24.7	
	Glu	4.2	5.8	4.4	4.6	5.2	5.8	6.1	
	Lac	3.62	4.46	3.41	2.63	3.03	3.74	3.58	
Clinical	pH	7.599	7.556	7.51	7.508				
Liver 5	pO2	15.22	17.77	18.3	18.24				
	pCO2	0.79	0.82	0.83	0.82				
	BE	-21.5	-22.5	-23.5	-23.6				
	Glu	0	2.7	3.4	3.9				

	Lac	1.62	2.6	2.91	2.9				
Clinical Liver 6	pH	7.712		7.459	7.432	7.428	7.462	7.478	7.458
	pO2	14.9		19.6	21.3	25.55	16.64	15.15	16.37
	pCO2	0.87		0.99	1	0.87	0.72	0.74	0.82
	BE	-17.2		-24.1	-25.8	-25.3	-24.8	-24.4	-24.7
	Glu	1.3		5	7.2	8.3	9.3	9.2	10.1
	Lac	0.79		4.41	5.26	4.72	5.27	4.93	5.56
Clinical Liver 7	pH	7.312	7.313	7.377	7.408	7.403	7.412	7.438	7.431
	pO2	14.2	18.4	18.77	18.66	20.32	20.8	26.69	29.96
	pCO2	1.78	0.99	0.8	0	0	0	0	0
	BE	-26.9	-26.7	-26.6	-26.9	-26.7	-26.8	-26.3	-26.2
	Glu	1.9	3.6	4.9	5.9	6.3	6.7	6.9	7.5
	Lac	3.31	5.27	5.33	5.5	5.77	5.96	5.89	6.36
Clinical Liver 8	pH	7.05	7.616	7.515	7.516	7.498			
	pO2	16.8	16.26	17.26	17.27	34.74			
	pCO2	0	0	0	0	0.74			
	BE	-31.9	-27	-25.7	-25.8	-23.9			
	Glu	0	2.6	5.3	6	7.5			
	Lac	0.38	2.37	3.84	3.93	4.25			
Clinical Liver 9	pH	7.067	7.198	7.182	7.175	7.169	7.197	7.205	
	paO2	30.32	30.58	32.17	30.23	31.89	32.49	32.49	
	pCO2	0.74	0.78	0.63	0.62	0.58	0.20	0.32	
	BE	-24.8	-22.2	-22.6	-23	-23	-22.5	-22.3	
	Glu	1.9	1.1	1.3	1.7	1.8	1.9	1.9	
	Lac	2.5	1.71	1.88	2.29	2.26	2.21	2.31	
Clinical Liver 10	pH	6.799	6.935	7.022	7.043	7.018	7.041		
	paO2	38.7	37.24	41.82	39.18	35.25	35.53		
	pCO2	1.21	1.02	0.96	0.97	0.84	0.82		
	BE	-30.9	-28.7	-26.9	-26.7	-27.1	-26.7		
	Glu	16.5	17.3	19.6	21.9	22.1	24		
	Lac	6.14	5.61	5.82	6.7	6.42	6.99		

Table 15 - Perfusate gas analysis in clinical and discarded livers during hypothermic perfusion

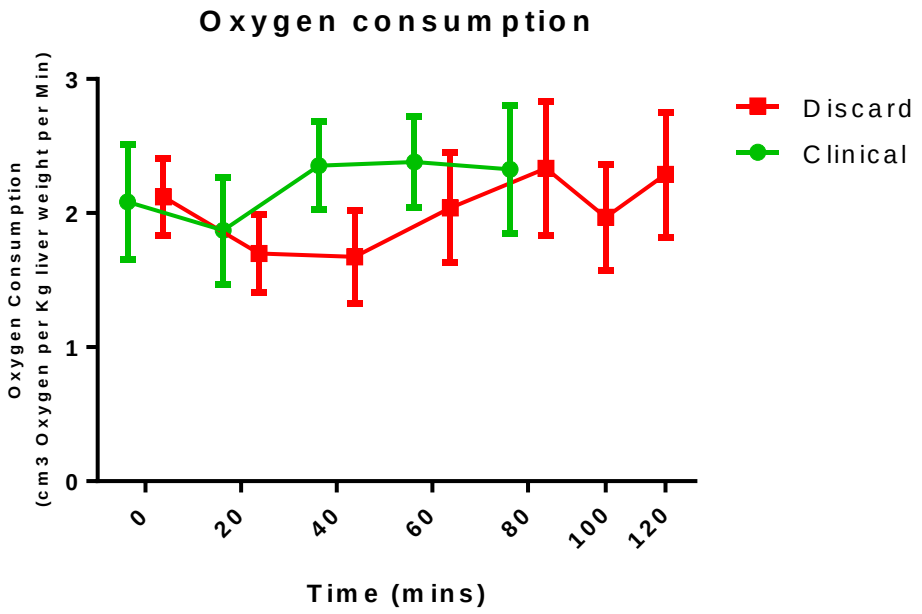


Figure 45 - Oxygen consumption (cm³ O_2/Kg liver/min) showing mean +/- S.D. in clinical and discarded livers during hypothermic perfusion

There was no significant differences in oxygen consumption between livers in each group, and all livers consumed oxygen suggesting ongoing aerobic metabolism.

6.3.2.2 Normothermic Perfusion

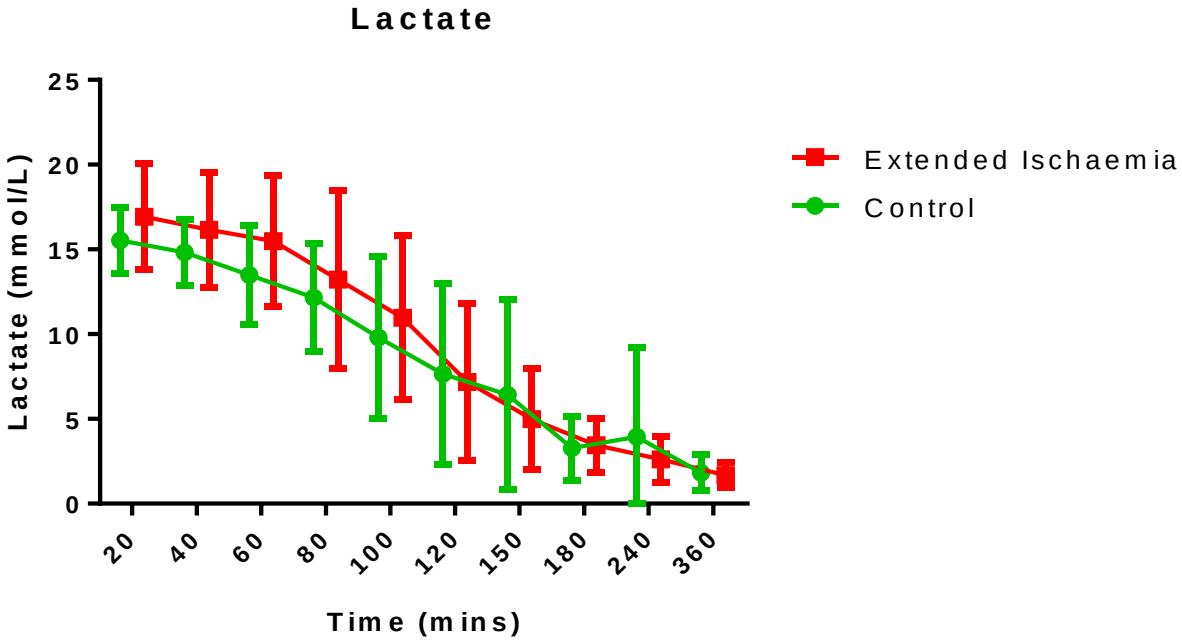


Figure 46 - Perfusate lactate levels (mmol/L) showing mean +/- S.D. in control and 'extended ischaemia' livers during normothernic perfusion

Contrasting with our hypothermic experience, in normothermic perfusion there was a reduction in perfusate lactate over the course of perfusion, and interestingly this was present in both groups. Almost all livers analysed, including livers subjected to prolonged ischaemia, cleared lactate to below 2.5 mmol/L.

When compared with pathological assessment of biopsies obtained from livers perfused, there was no significant correlation between the final lactate level and grading of levels of ischaemia-reperfusion injury, hepatocyte necrosis or steatosis, also devaluing the importance of lactate measurements (Figure 47).

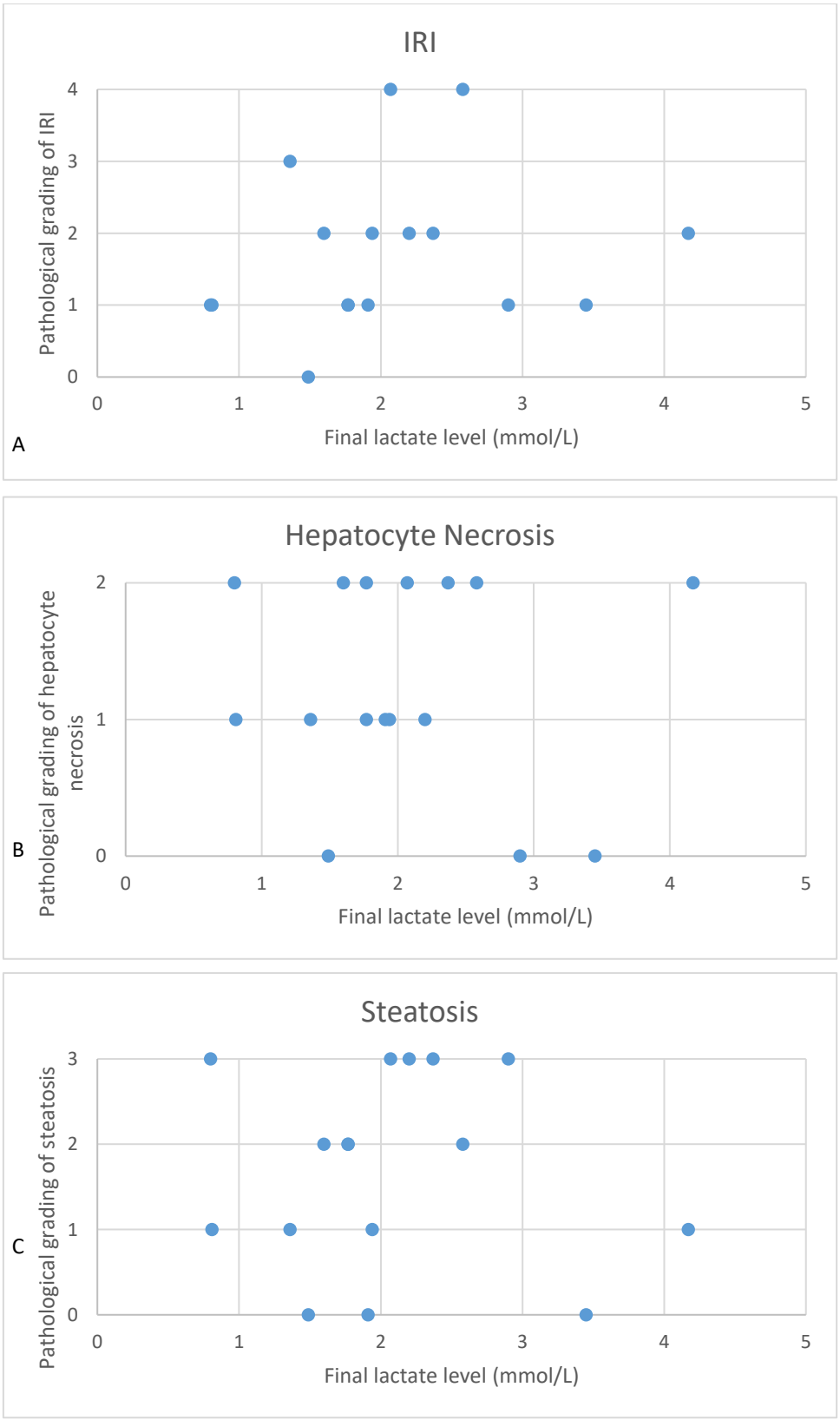


Figure 47 - Correlation of final perfusate lactate level (mmol/L) versus pathological grading of (A) ischaemia-reperfusion injury, (B) hepatocyte necrosis, (C) steatosis

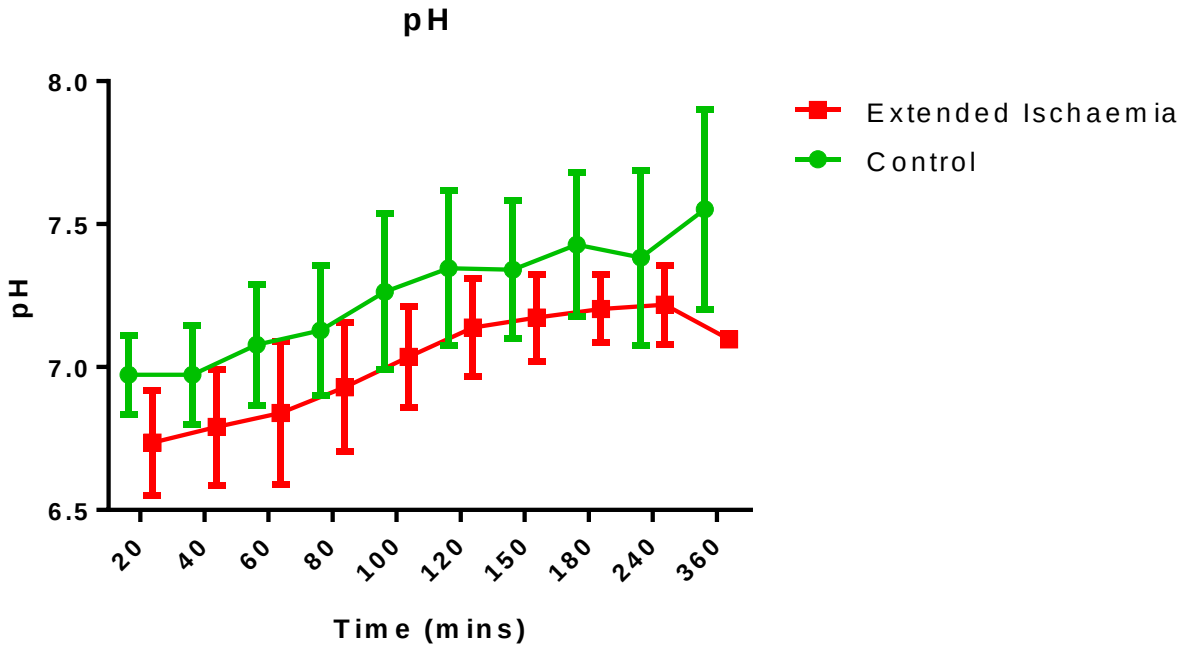


Figure 48 - Perfusate pH levels in control and 'extended ischaemia' livers during normothernic perfusion showing mean +/- S.D>

pH values mirrored the results obtained from lactate analysis, with no significant difference between groups (Figure 48).

		0	20	40	60	120	150	180	240	300	360	420	480	500
Control Liver 1	pH	6.942	6.878	7.053	7.385	7.533	7.554	7.61	7.762	7.759	7.798			
	PaO2	19.11	19.17	18.76	20.3	19.73	18.72	18.58	19.96	19.19	20.56			
	PvO2	7	6.15	5.16	2.91	3.91	4.27	3.81	3.49	3.2	3.7			
	Glu	17.9	23.7	27	25.3	23.9	24.5	23.3	19.5	15.7	14.5			
	Lac	15.7	11.94	12.61	16.05	13.64	13.15	11.94	5.81	3	2.9			
Control Liver 2	pH	6.89	6.93	6.947	7.074	7.099	7.186		7.249					
	PaO2	15.76	23.23	29.67	18.33	18.48	18.6		15.76					
	PvO2	7.03	8.11	9.94	7.68	6.71	6.75		5.78					
	Glu	33.7	35.7	37	36.3	37.6	30.7		23.1					
	Lac	15.98	17.02	16.25	12.44	12.63	9.65		6.43					
Control Liver 3	pH	7.038	7.147	7.2	7.248	7.272	7.36	7.375	7.402	7.433				
	PaO2	26.04	23.42	23.33	22.7	23.46	23.79	16.47	12.96	22.35				
	PvO2	7.21	8.02	8.52	8.49	7.07	10.1	4.74	4.42	6.25				
	Glu	16.9	18.5	19.2	18.6	20.8	20.5	21.5	21.4	20.3				
	Lac	12.34	13.95	11.8	10.42	9.02	6.52	5.28	1.86	1.6				
Control Liver 4	pH	6.681	6.729	6.782	6.85	6.912	6.98	7.036	7.007	6.977				
	PaO2	19.96	20.61	21.53	20.37	21.56	20.52	20.7	21.06	19.43				
	PvO2	9.25	8.42	8.06	7.96	7.83	7.29	8.05	7.39	8.11				
	Glu	25.9	28.8	32.7	35.1	36.5	37.5	33.2	29.4	24.5				
	Lac	12.68	13.82	16.81	15.86	15.46	10.85	6.55	4.16	2.58				
Control Liver 5	pH	6.916		6.991		7.029	7.025	7.028		6.961				
	PaO2	12.23		11.48		12.38	12.09	13.52		12.64				
	PvO2			6.47		6.9	6.91	7.63		7.44				
	Glu	37.9		39.3		37.1	39.7	40.5		40.7				
	Lac	18.15		15.14		14.94	16.81	17.32		17.84				
Control Liver 6	pH	7.155		7.444		7.706	7.75		7.745	7.758				
	PaO2	21.38		19.51		15.29	26.5		24.86	25.22				

	PvO2	5.8		1.86		1.42	2.07		4.09	4.08				
	Glu	20.3		23.4		22.6	18.8		11.1	6				
	Lac	15.61		11.42		4.96	2.39		2.11	2.07				
Control Liver 7	pH	7.04	6.949	6.947	6.882	7.081	7.369	7.419	7.409	7.343				
	PaO2	49.67	26.54	26.19	52.56	23.89	16.7	23.19	23.6	24.26				
	PvO2	6.4	6.49	6.42	7.76	5.66	4.99	4.45	4.79	5.36				
	Glu	29.1	36.4	36.2	36.4	31.7	27	24.4	20.6	14				
	Lac	15.01	14.55	13.8	12.48	5.91	2.69	1.48	1.69	1.94				
Control Liver 8	pH	7.092	7.251	7.349	7.41	7.544	7.663	7.619	7.541	7.523				
	PaO2	20.95	17.74	16.88	24.92	31.79	32.08	28.75	29.1	28.65				
	PvO2	5.76	5.01	2.77	2.31	4.39	4.56	4.2	4.76	5.33				
	Glu	26	30.5	40.1	37.4	39.6	38.4	37	32.9	30.3				
	Lac	18.28	15.42	9.24	7.41	4.33	2.43	2.76	3.35	3.45				
Control Liver 9	pH	7.009	6.924	6.994	7.045	7.195	7.227	7.298	7.314	7.307	7.305			
	PaO2	40.74	31.04	27.67	27.58	26.49	18.25	22.94	22.26	25.17	22.51			
	PvO2	5.79	6.69	5.14	4.7	3.9	3.4	4.02	3.91	4.45	4.85			
	Glu	25.1	25.8	25.9	26.2	25.2	22.8	20.8	17.5	10.3	8.2			
	Lac	16.03	13.85	10.09	8.56	3.44	1.36	1.08	0.96	0.85	0.79			
Control Liver 10	pH	6.859	6.886	6.964	7.091	7.148	7.141	7.1	7.126	7.059	7.025	7.033		
	PaO2	23.04	20.12	19.06	14.55	15.29	13.46	16.37	15.11	14.79	16.3	13.96		
	PvO2	4.96	5.99	5.31	4.15	3.71	3.41	3.36	3.72	3.79	3.88	4.7		
	Glu	25.7	32.3	30.7	29.6	26.7	22.5	20	17	15.8	12.6	10.6		
	Lac	15.89	16.93	12.43	6.99	3.53	2.12	2.82	2.33	2.4	2.49	1.49		
Extended Ischaemia 1	pH	6.847	6.978	7.053	7.162	7.24	7.33	7.35	7.341	7.377				
	PaO2	25.15	16.13	16.8	17.39	16.65	14.04	14.98	15.15	13.1				
	PvO2	6.24	6.76	4.06	3.57	2.85	3.65	3.51	3.96	7.06				
	Glu	H	39.8	H	41.4	H	41.2	H	40.6	41.5				
	Lac	17.12	17.55	16.89	11.73	10.41	4.04	3.79	3.41	4.17				

Extended Ischaemia 2	pH	6.524	6.575	6.562	6.711	6.914	6.998	7.085	7.148	7.134	7.089			
	PaO2	15.97	12.6	18.05	15.52	14.59	14.12	12.12	11.56	11.53	12.33			
	PvO2	6.99	6.03	4.62	5.3	4.63	4.28	3.81	3.14	4.18	4.57			
	Glu	37.6	37.6	x	x									
	Lac	19.19	18.2	20	18.73	15.85	14.24	9.56	5.84	3.56	2.2			
Extended Ischaemia 3	pH	6.836	6.8183	6.903	6.9153	6.953	7.086	7.083	7.122	7.143	7.106	7.13		
	PaO2	16.36	17.25	16.63	18.53	19.55	19.39	18.98	19.33	19.29	19.16	20.57		
	PvO2	6.36	7.06	7.44	6.2	7.2	6.91	6.8	7.49	7.93	7.68	7.16		
	Glu	16.3	19.3	16.3	20.6	20.8	20.2	19.7	20.1	18.4	18.2	17.6		
	Lac	19.76	19.4	15.38	14.01	13.49	7.08	5.09	2.96	1.44	1.36	1.52		
Extended Ischaemia 4	pH	7.158	7.249	7.318	7.417	7.45	7.457	7.448	7.442	7.376	7.351	7.334		
	PaO2	17.12	17.42	18.78	19.28	18.62	17.59	21.71	22.91	20.64	19.87	20.23		
	PvO2	4.42	3.5	3.53	5.32	5.4	5.27	5.37	5.64	6.2	6.43	6.33		
	Glu	27.8	27.6	28.5	29.1	27.2	25.8	20.8	17.9	10.3	7.5	5.9		
	Lac	16.79	10.92	9.34	5.1	3.11	2.22	1.43	1.27	1.35	0.95	0.81		
Extended Ischaemia 5	pH	7.387	6.811	6.756	6.832	6.842	6.957	6.989	7.019	7.009	6.985	6.949	6.911	6.912
	PaO2	23.64	20.26	19.65	19.5	19.74	20.1	19.14	19.06	18.24	18.75	20.72	21.17	21.03
	PvO2	10.71	6.61	10.2	8.87	9.21	9.07	8.46	8.33	8.05	8.38	9.34	9.58	10.02
	Glu	18.4	34.3	38.1	39	high	high	high	high	38.3	35.5	32.4	29.8	27.9
	Lac	11.82	11.94	14.66	15.83	16.57	12.02	11.11	8.35	5.03	4.21	3.66	2.98	2.37

Table 16 - Perfusate gas analysis in control and 'extended ischaemia' livers during normothermic perfusion

6.4 Bile Production

6.4.1 Methods

Bile ducts were initially cannulated with an 8Fr paediatric feeding tube (36420, Bard Medical, Covington GA, USA). However, after 2 perfusions it was felt that perhaps bile viscosity was preventing successful collection, so the cannula was changed to use a Medtronic 12Fr cannula (77012) and tied in place with a 2-0 Vicryl™ tie (Ethicon, Somerville NJ, USA).

6.4.2 Results

6.4.2.1 Hypothermic Perfusion

All livers produced bile during perfusion, but all less than 5ml over 2 hours, as demonstrated in Figure 49. However, in hypothermic perfusion bile volume production was small, this was not reliably quantifiable, therefore no analysis was performed.

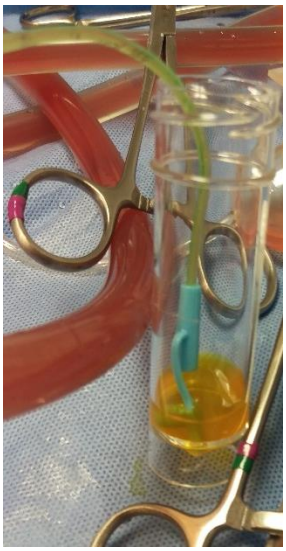


Figure 49 - Bile production on perfusion. Picture taken at 120 minute time-point

6.4.2.2 Normothermic Perfusion

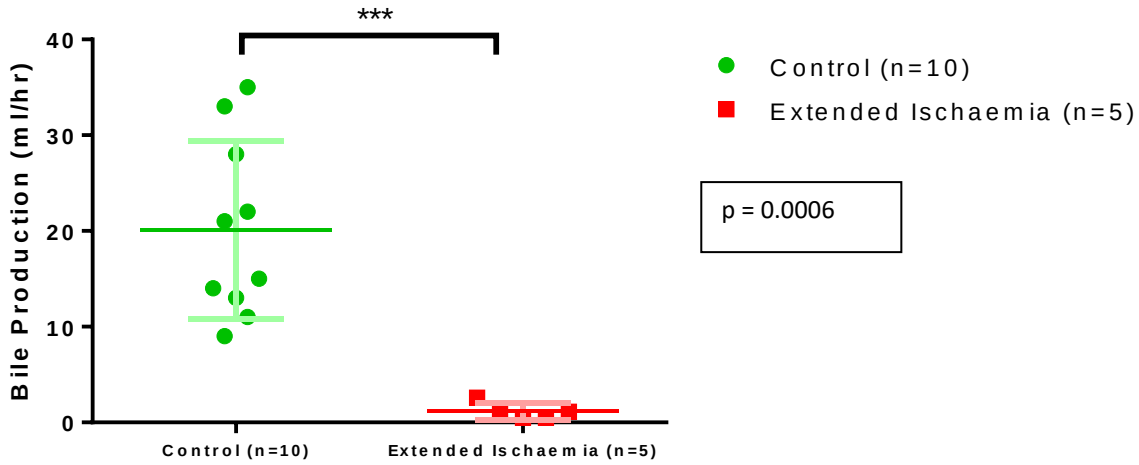


Figure 50 - Bile production (ml/hr) in control and 'extended ischaemia' livers during normothermic perfusion showing mean +/- S.D.

Although bile production was not significant enough to reliably quantify in hypothermic perfusion, however analysis of bile production in normothermic perfusion yielded very conclusive results. There is a very clear distinction between the control group and the extended ischaemia group (p=0.0006)

The liver that produced one of the smallest volumes of bile (13 ml/hr) demonstrated the greatest signs of ischaemia-reperfusion injury in histopathology, and moderate signs of hepatocyte necrosis (Figure 51). Although overall histological assessment of livers did not demonstrate any correlation between volume of bile production and levels of IRI, hepatocyte necrosis or steatosis.

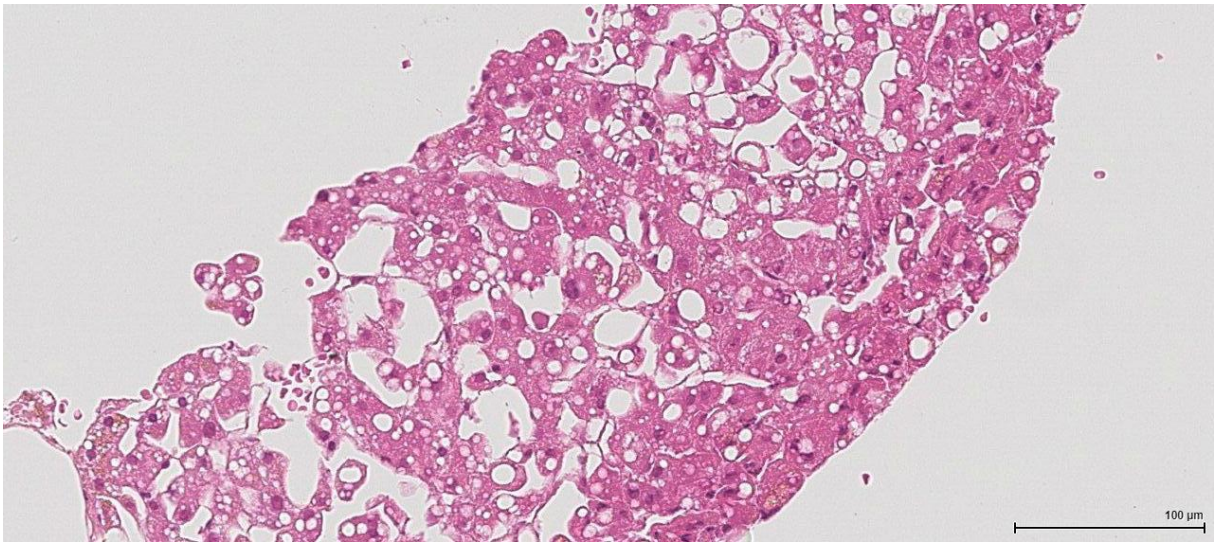


Figure 51 – H&E stained core biopsy of liver demonstrating signs of IRI and hepatocyte necrosis

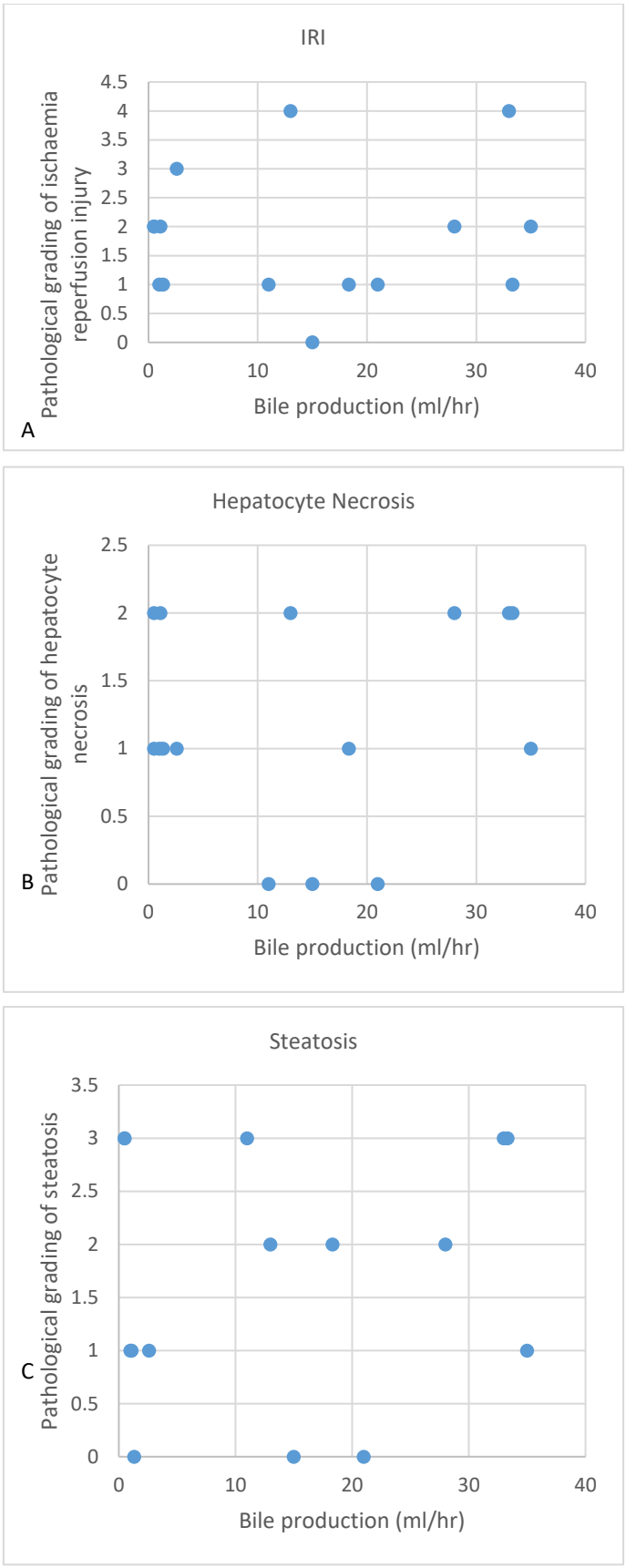


Figure 52 - Correlation of final perfusate lactate level (mmol/L) versus pathological grading of (A) ischaemia-reperfusion injury, (B) hepatocyte necrosis, (C) steatosis

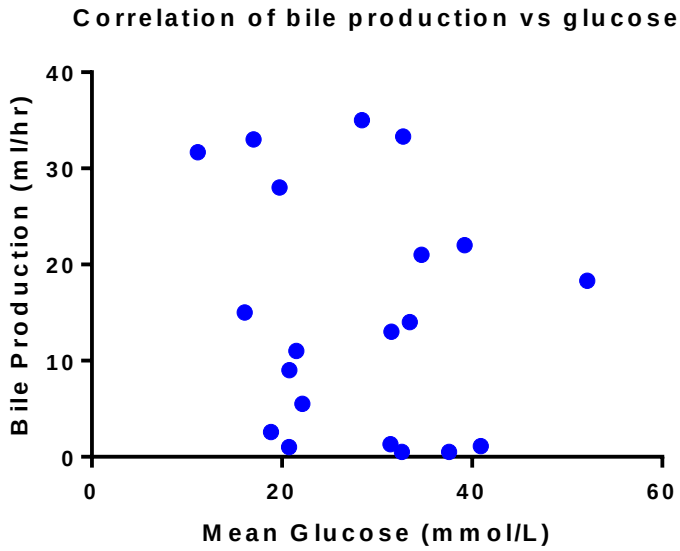


Figure 53 - Correlation of mean perfusate glucose level and bile production (ml/min) during normothermic perfusion

Although serum (or perfusate) glucose levels in theory could affect volume of bile production [112], we did not find any correlation between the two in our series (p = 0.49). It may be that with a larger cohort a difference may become apparent.

6.5 Alanine Transaminase Analysis

6.5.1 Methods

An ALT activity colorimetric assay kit (Abcam ab105134) was used for assessment of ALT levels in perfusate samples. In the ALT assay protocol, ALT transfers an amino group from alanine to α-ketoglutarate; producing pyruvate and glutamate. The pyruvate is detected in a reaction that converts a nearly colourless probe to a form that is coloured. For each plate, a set of 6 standards was prepared to create a reference curve against which the samples can be analysed.

6.5.1.1 Standard Preparation

A 1 nmol/µL Pyruvate Standard was prepared by diluting 5 µL Pyruvate Standard in 495 µL of ALT Assay Buffer. This standard was then used to prepare a standard curve dilution as per Table 17 below. These were added to each plate in duplicate.

Table 17 - ALT assay standard curve preparation volumes

Standard No.	Volume of Standard (µL)	Assay Buffer (µL)	Final Volume in Well (µL)	ALT in Well (nmol/well)
1	0	60	20	0
2	6	54	20	0.2
3	12	48	20	0.4
4	18	42	20	0.6
5	24	36	20	0.8
6	30	30	20	1.0

6.5.1.2 Reaction Mix Preparation

1000 µL of reaction mix was prepared for each plate as per Table 18 below.

Component	Volume (µL)
ALT Assay Buffer	860
OxiRed Probe	20
ALT Enzyme Mix	20
ALT Substrate	100

Table 18 - ALT assay reaction mix composition

6.5.1.3 Assay Procedure

Test dilutions were performed to establish the optimum dilution that would reliably produce a reading within the reference range. Ultimately, a sample dilution of 1/250 was optimum.

In each of 96 wells, 20 µL of either standard dilutions or sample dilutions was added, followed by 100 µL of reaction mix.

The output at wavelength of 570 nm was measured on a microplate colorimetric reader in kinetic mode every 2 minutes for a total of 60 minutes at 37°C protected from light.

6.5.1.4 ALT Calculations

The absorbance at 570 nm was used to calculate ALT enzyme activity using the formula below:

$$ALT\ Activity = \left(\frac{B}{\Delta T x V}\right) \times D$$

Equation 1 - ALT activity calculation

Where

B = Amount of pyruvate from standard curve

ΔT = Reaction time

V = Original sample volume (in mL)

D = Sample dilution factor

6.5.2 Results

6.5.2.1 Hypothermic Perfusion

There were higher levels of ALT in perfusate samples in discarded livers than clinical livers, but not statistically significant. It is likely that with additional numbers, that significance may be demonstrated. ROC curve analysis at the 20-minute timepoint achieved the highest significance (p=0.002), with an optimum cut-off of 358.1 giving a sensitivity of 100% and specificity of 88.9%.

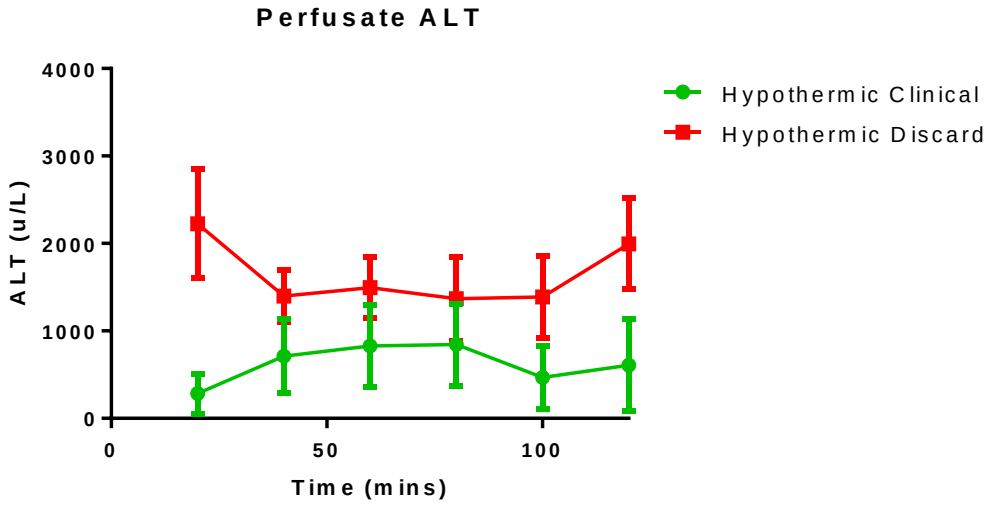


Figure 54 - Perfusate ALT levels (u/L) showing mean +/- S.D. in clinical and discarded livers during hypothermic perfusion

ALT has a further advantage as a potential biomarker as serial daily serum ALT levels are routinely checked on recipients post-transplant. This is commonly used as a marker of graft function, therefore the ability of perfusate ALT to predict recipient serum ALT post-transplant, could be very useful in graft assessment and selection. Therefore a comparison of perfusate versus recipient peak, mean and median ALTs in the first 7 days post-transplant was performed and shown in Figure 55.

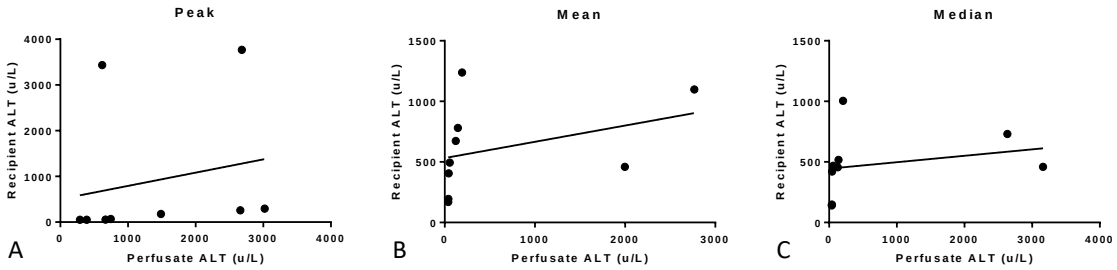
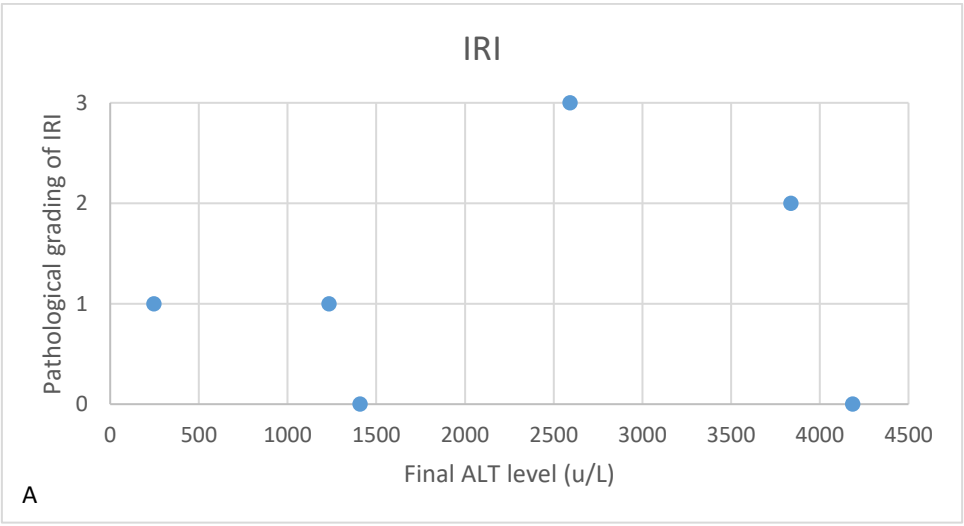
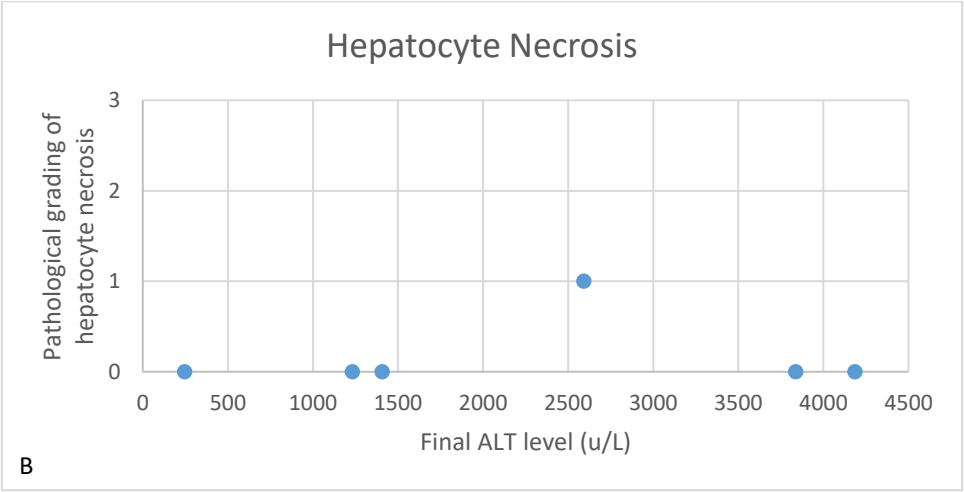


Figure 55 - Correlation of perfusate ALT levels (u/L) with recipient peak (A), mean (B), and median (C) ALT levels (u/L) in the first 7 days post-transplant

In all three, there is no significant correlation between perfusate and recipient ALT, with Pearson correlation coefficients of 0.21 (p=0.59), 0.37 (p=0.33), and 0.25 (p=0.52) respectively for peak, mean and median. Therefore, at least with the limited numbers available to us, it is not possible to state that perfusate ALT can predict recipient ALT.

No correlation was found when comparing final ALT levels with histopathological grading of IRI, hepatocyte necrosis and steatosis (Figure 56). Although it is worth noting that biopsies were only obtained from the discard series of hypothermic perfused livers, not the clinical livers that went on to be transplanted.





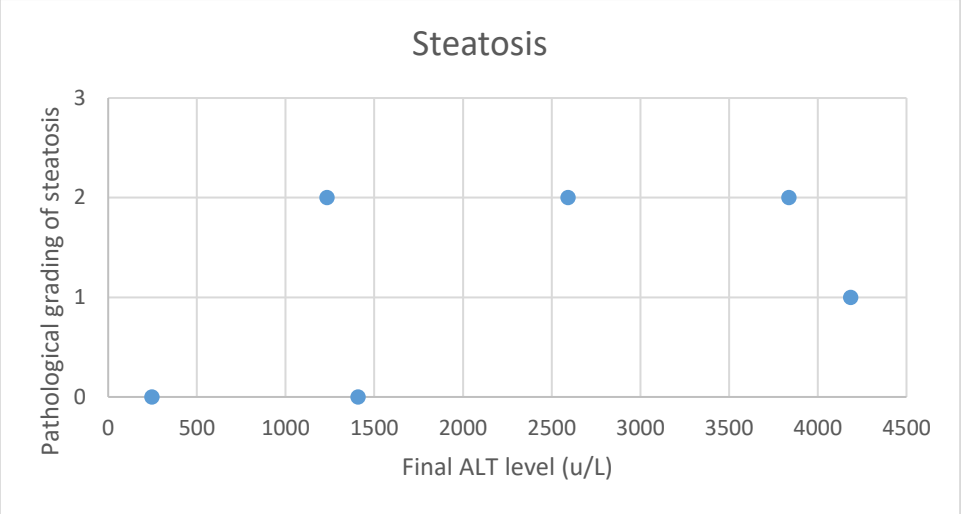


Figure 56 - Correlation of final perfusate ALT level (u/L) versus pathological grading of (A) ischaemia-reperfusion injury, (B) hepatocyte necrosis, (C) steatosis during hypothermic perfusion

6.5.2.2 Normothermic Perfusion

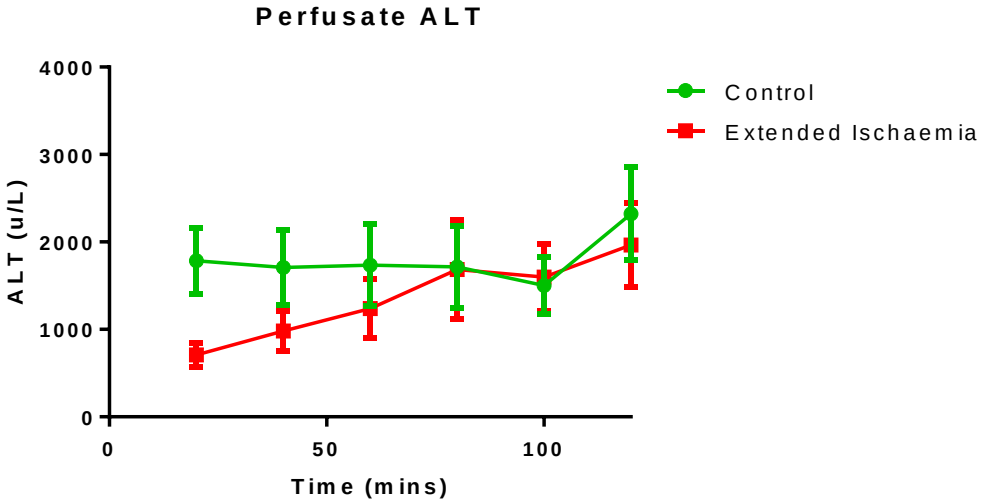


Figure 57 - Perfusate ALT levels (u/L) showing mean +/- S.D. in control and 'extended ischaemia' livers during normothermic perfusion

Interestingly, and conversely to what one might expect, ALT had a tendency to start at a lower point in livers subjected to extended ischaemia compared to control. However, this evened out over the course of perfusion to equal levels. No statistical significance was attained at any timepoint.

In our series, one liver underwent NMP with the intent to transplant, however the decision was made not to proceed to transplantation on the basis primarily of a rising perfusate ALT, which reached 3135 u/L after 4 hours of perfusion.

As with our small hypothermic discard series, there was no correlation between final ALT levels and pathological grading of IRI, hepatocyte necrosis and steatosis.

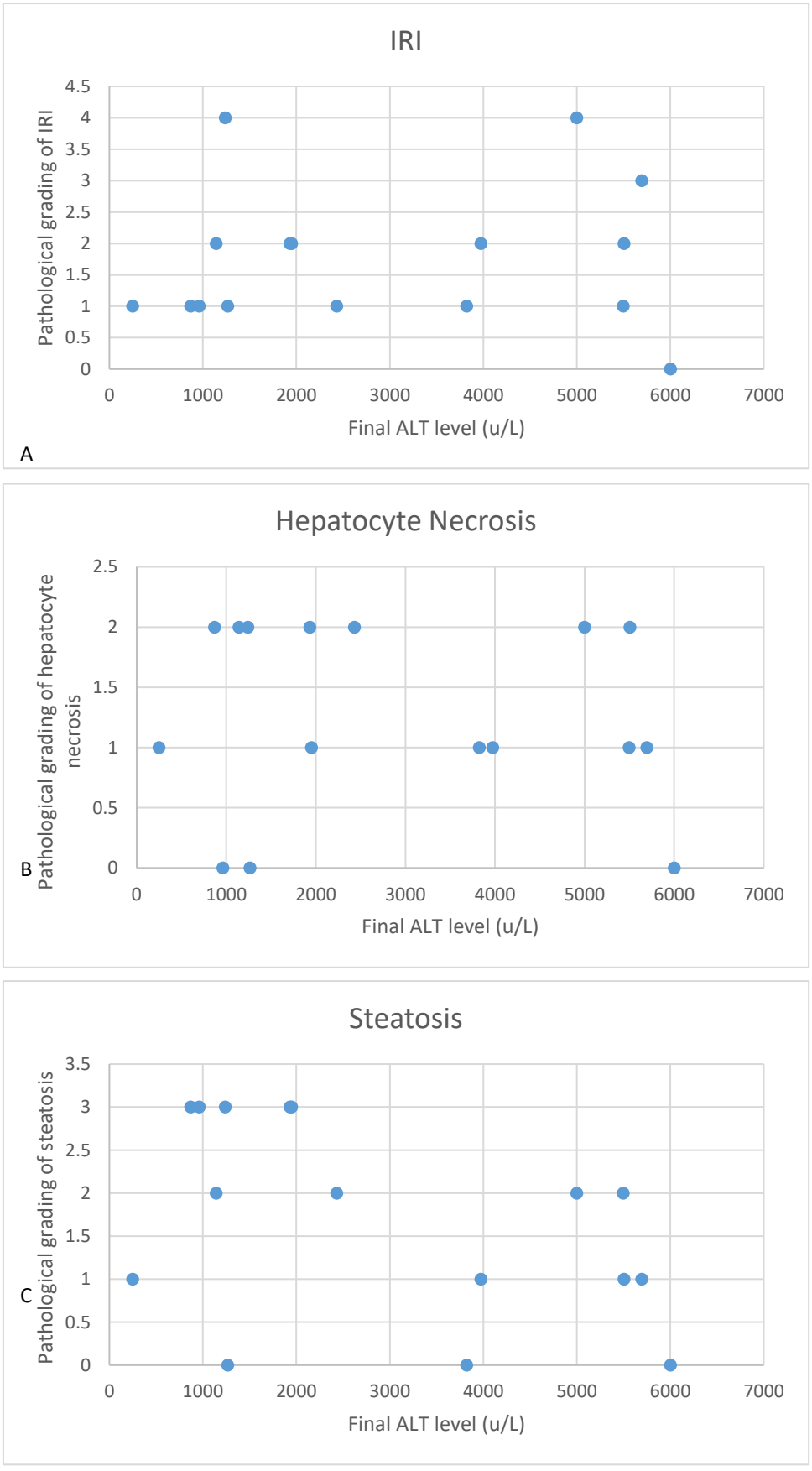


Figure 58 - Correlation of final perfusate ALT level (u/L) versus pathological grading of (A) ischaemia-reperfusion injury, (B) hepatocyte necrosis, (C) steatosis during normothermic perfusion

6.6 Multiplex Analysis

6.6.1 Methods

Perfusate samples and reagents were brought to room temperature. The following Mesoscale (Rockville MD, USA) multiplex plates were used:

V-PLEX Chemokine Panel 1

V-PLEX Pro-inflammatory Panel 1

V-PLEX Cytokine Panel 1

V-PLEX TH17 Panel 1

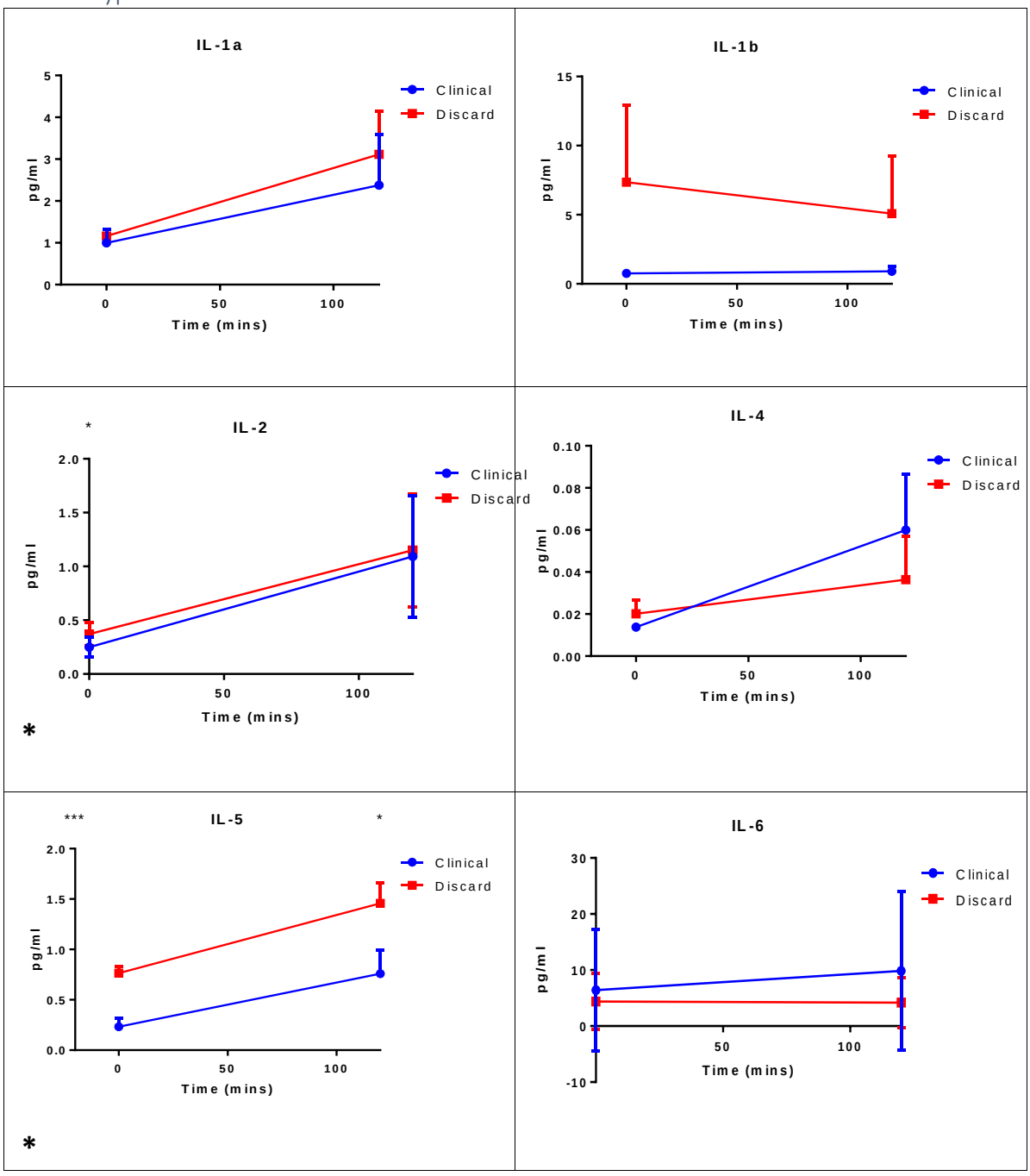
V-PLEX Angiogenesis Panel 1

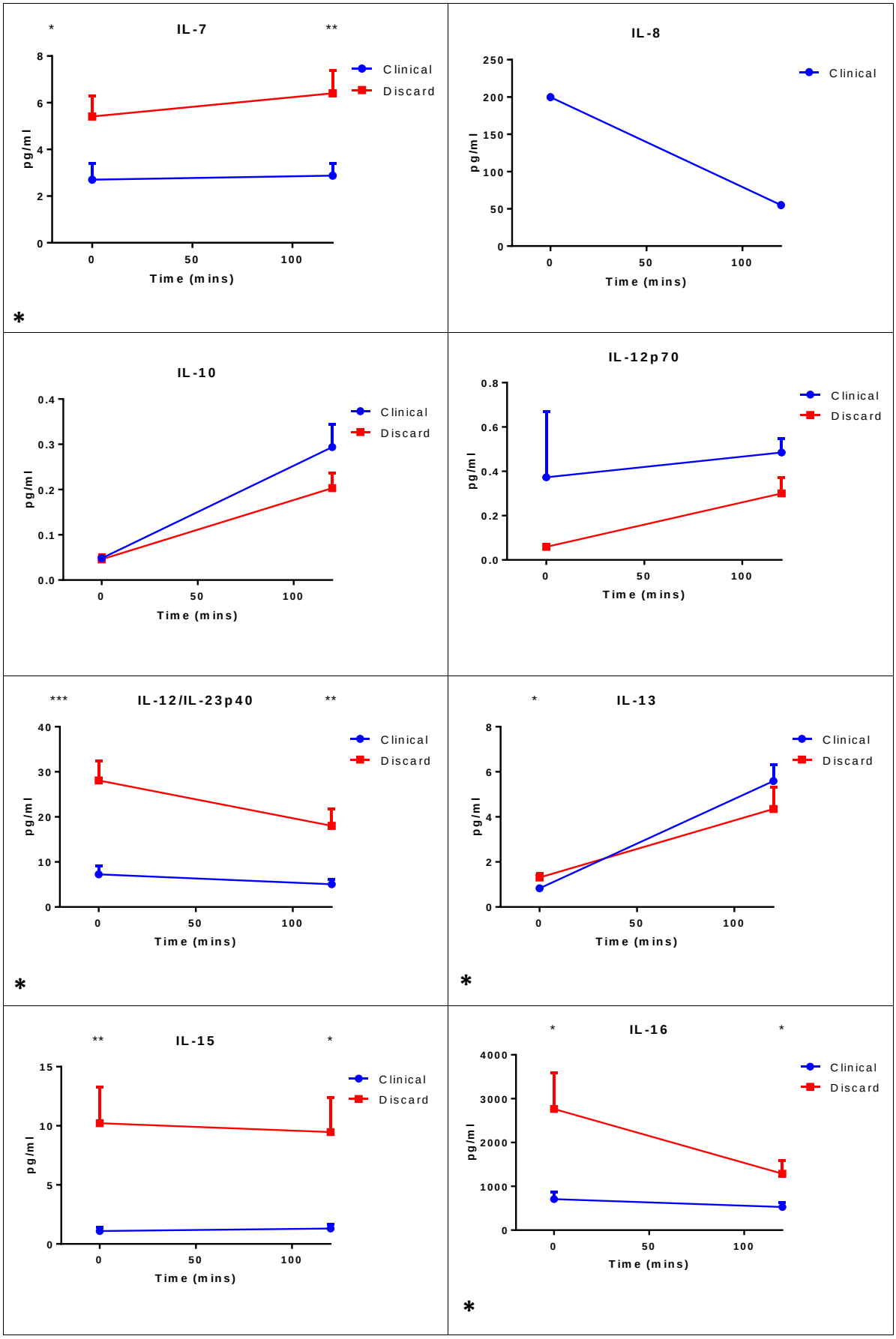
V-PLEX Vascular Injury Panel 2

Calibration solutions were prepared in the appropriate diluent for each panel, with 4-fold serial dilutions. Perfusates were diluted 2-fold for time-0 time-points and by 10-fold for end time-points in the appropriate diluent for each plate. A combined detection antibody solution for each plate was prepared, by diluting each antibody 50-fold in the appropriate diluent for each plate. Plates were washed 3 times in PBS with 0.05% Tween 20 then 50µL of diluted sample added to each well. Plates were incubated on a plate-shaker at room temperature for 2 hours. Plates were washed again 3 times then 25µL of detection antibody solution was added to each well and incubated on a plate-shaker at room temperature for a further 2 hours. Plates were washed 3 times then 150µL of read buffer added to each well and plate analysed on MESO QuickPlex SQ120 multiplex analyser.

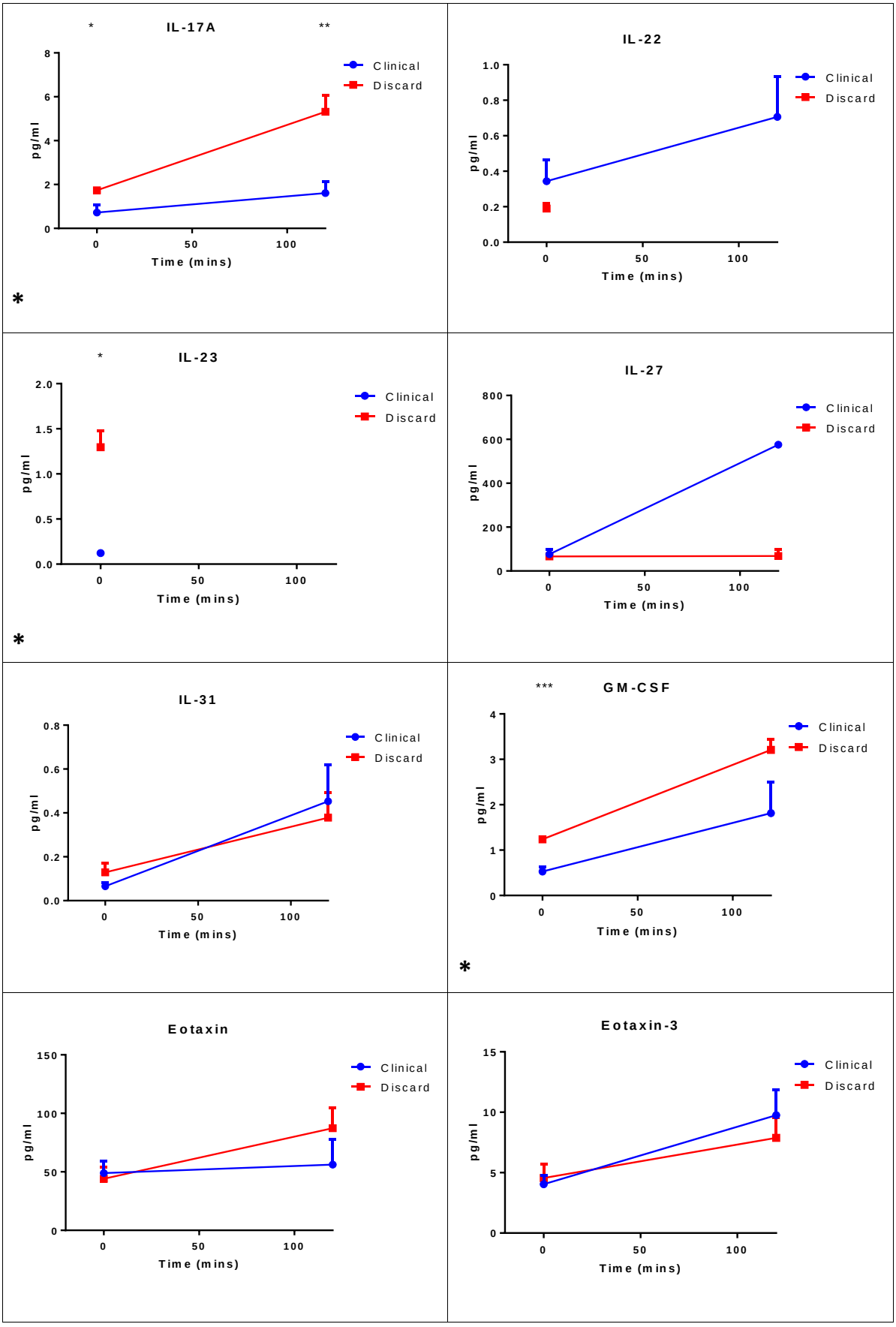
6.6.2 Results

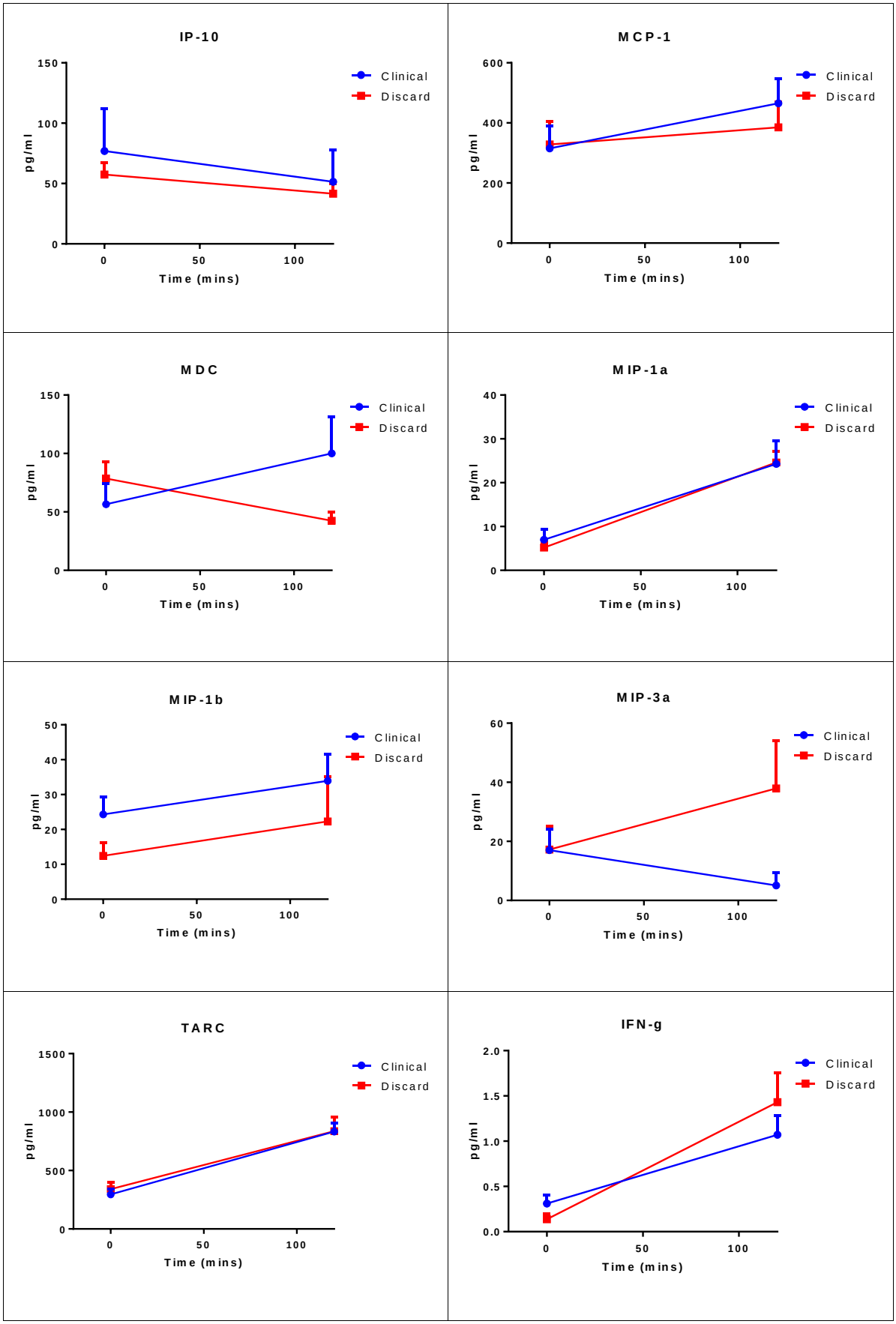
6.6.2.1 Hypothermic Perfusion

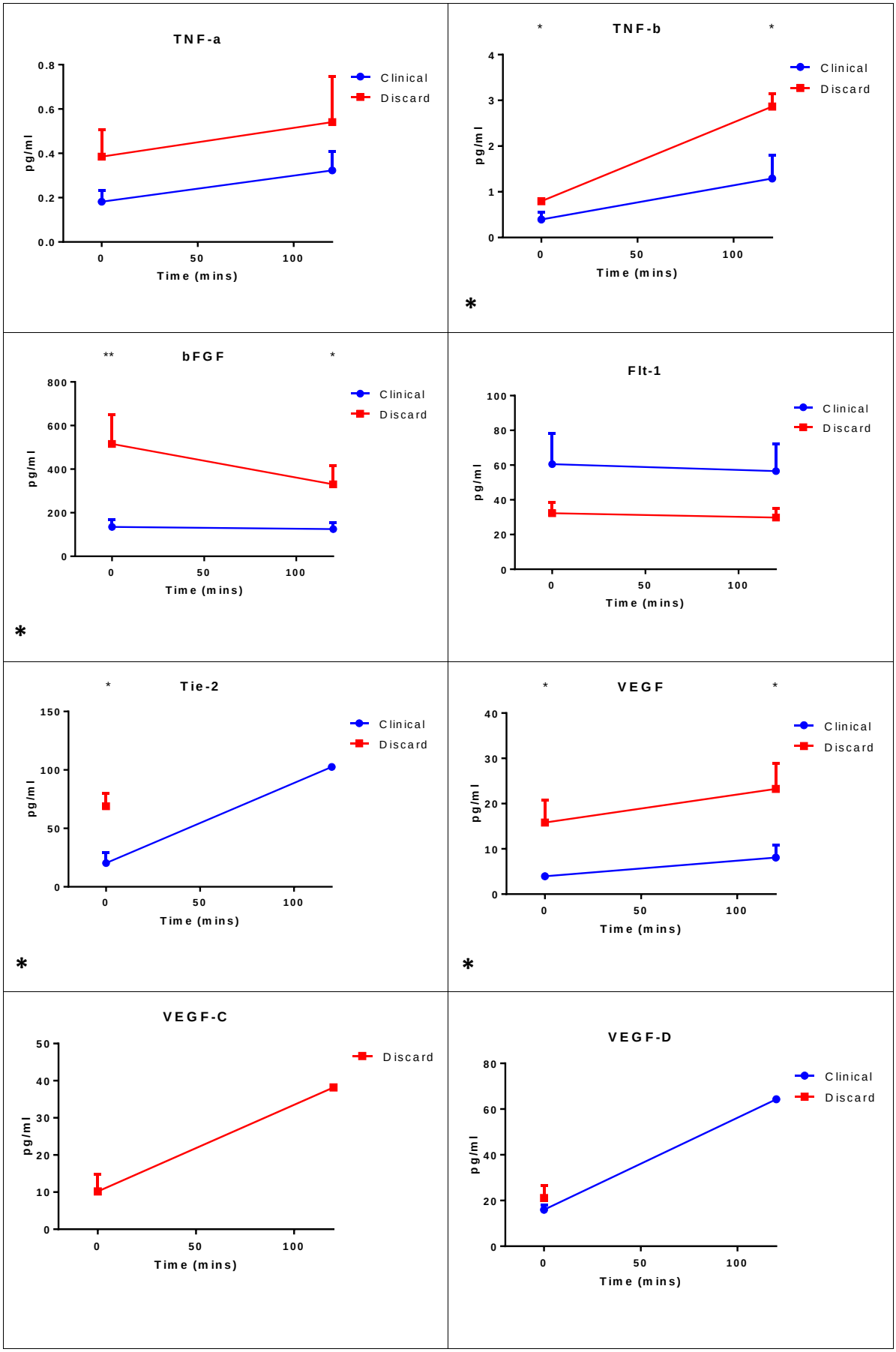


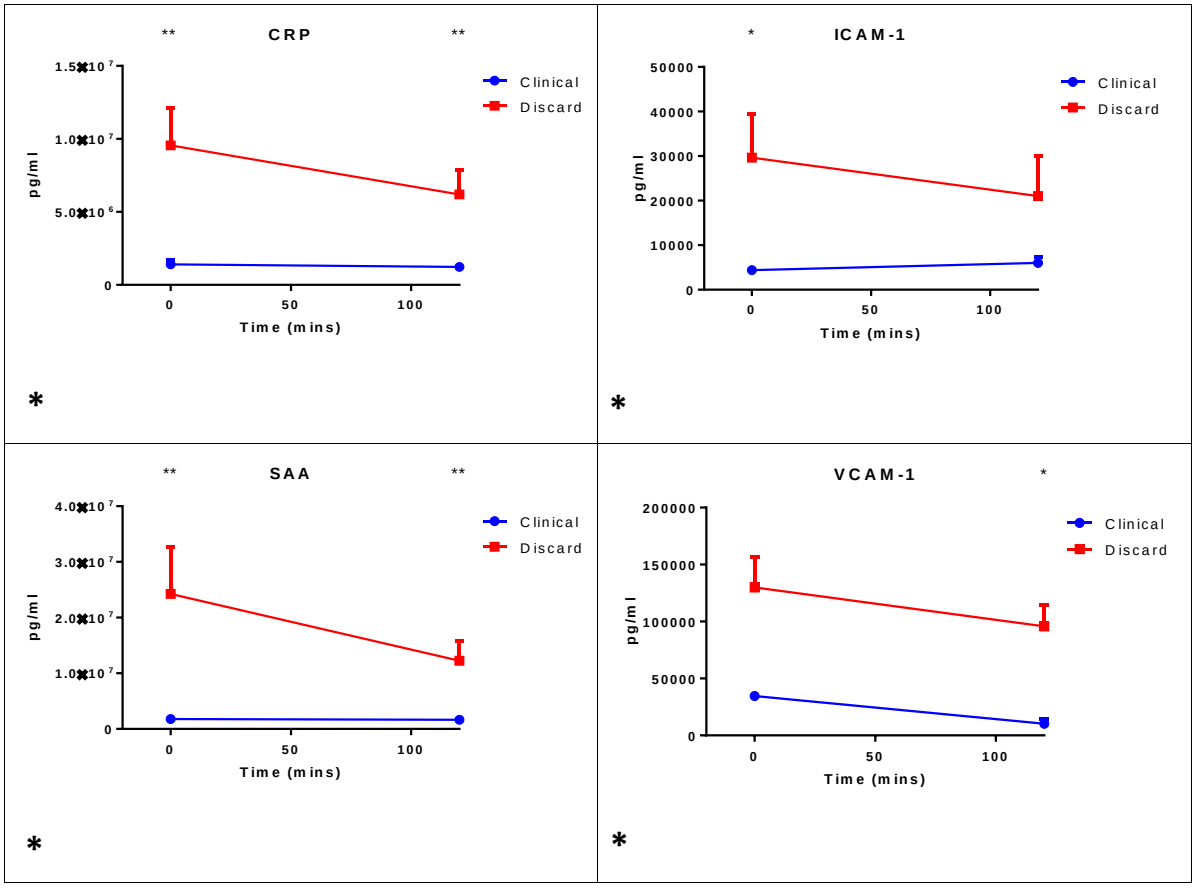

IL-7
*
**
pg/ml
Time (mins)
Clinical
Discard
*
IL-8
pg/ml
Time (mins)
Clinical
IL-10
pg/ml
Time (mins)
Clinical
Discard
IL-12p70
pg/ml
Time (mins)
Clinical
Discard
***
IL-12/IL-23p40
**
pg/ml
Time (mins)
Clinical
Discard
*
IL-13
*
pg/ml
Time (mins)
Clinical
Discard
*
**
IL-15
*
pg/ml
Time (mins)
Clinical
Discard
*
IL-16
*
*
pg/ml
Time (mins)
Clinical
Discard
*

*


*
IL-17A
**
pg/ml
Time (mins)
Clinical
Discard
*
IL-22
pg/ml
Time (mins)
Clinical
Discard
*
IL-23
pg/ml
Time (mins)
Clinical
Discard
*
IL-27
pg/ml
Time (mins)
Clinical
Discard
IL-31
pg/ml
Time (mins)
Clinical
Discard
***
GM-CSF
pg/ml
Time (mins)
Clinical
Discard
*
Eotaxin
pg/ml
Time (mins)
Clinical
Discard
Eotaxin-3
pg/ml
Time (mins)
Clinical
Discard


IP-10
pg/ml
150
100
50
0
0
50
100
Time (mins)
Clinical
Discard
MCP-1
pg/ml
600
400
200
0
0
50
100
Time (mins)
Clinical
Discard
MDC
pg/ml
150
100
50
0
0
50
100
Time (mins)
Clinical
Discard
MIP-1a
pg/ml
40
30
20
10
0
0
50
100
Time (mins)
Clinical
Discard
MIP-1b
pg/ml
50
40
30
20
10
0
0
50
100
Time (mins)
Clinical
Discard
MIP-3a
pg/ml
60
40
20
0
0
50
100
Time (mins)
Clinical
Discard
TARC
pg/ml
1500
1000
500
0
0
50
100
Time (mins)
Clinical
Discard
IFN-g
pg/ml
2.0
1.5
1.0
0.5
0.0
0
50
100
Time (mins)
Clinical
Discard


TNF-a
pg/ml
0.0
0.2
0.4
0.6
0.8
0
50
100
Time (mins)
Clinical
Discard
TNF-b
*
*
pg/ml
0
1
2
3
4
0
50
100
Time (mins)
Clinical
Discard
*
bFGF
**
*
pg/ml
0
200
400
600
800
0
50
100
Time (mins)
Clinical
Discard
*
Flt-1
pg/ml
0
20
40
60
80
100
0
50
100
Time (mins)
Clinical
Discard
Tie-2
*
pg/ml
0
50
100
150
0
50
100
Time (mins)
Clinical
Discard
*
VEGF
*
*
pg/ml
0
10
20
30
40
0
50
100
Time (mins)
Clinical
Discard
*
VEGF-C
pg/ml
0
10
20
30
40
50
0
50
100
Time (mins)
Discard
VEGF-D
pg/ml
0
20
40
60
80
0
50
100
Time (mins)
Clinical
Discard



Error! Reference source not found.

Error! Reference source not found. shows the mean and standard deviation values for the chemokines and cytokines analysed in the multiplex.

In total 18 of the markers analysed showed a statistically significant difference in levels between the 2 groups in at least one time-point. Some of the readings were unobtainable as levels were outside the reference range. Unfortunately due to the cost of the plates, it was not possible to perform trial dilutions to establish optimum dilutions.

The general trend in most markers analysed is that the levels in perfusate of discarded livers was often higher than clinical livers with some notable exceptions. Cytokines known to have anti-inflammatory actions such as IL-4, IL-10 and IL-13 seem to reverse this pattern, although of these only IL-13 was statistically significant at time-0.

The class of markers with most significant differences between the two groups were indicators of vascular injury such as serum amyloid A (SAA), vascular cell adhesion protein-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and C-reactive protein (CRP). In all of these, a statistically significant difference was evident from the first time-point and continues to the end of perfusion.

Some angiogenesis markers such as vascular endothelial growth factor (VEGF), angiopoietin-1 receptor (Tyrosine kinase with immunoglobulin-like and EGF-like domains 2, TIE-2), and basic fibroblast growth factor (bFGF) were also statistically significant, although the end time-point of TIE-2 in discard livers was outside the reference range, most likely higher than range.

Tumour necrosis factor-β (TNF-β), important in the regulation of cell survival, was also significantly elevated in discarded livers. Granulocyte macrophage colony-stimulating factor (GM-CSF), part of the immune-inflammatory cascade was also significantly elevated in discarded livers.

A number of interleukins demonstrated a statistically significant difference between the 2 groups. IL-2, IL-7 and IL-15, all involved in regulating immune response, were significantly higher in discarded livers. Similarly, IL-5 which regulates eosinophil-mediated inflammation was also elevated.

IL-12, IL-16, IL-18 and IL-23 all have pro-inflammatory targets and were significantly elevated in discarded livers compared with clinical livers. Interestingly, IL23p40 also increases angiogenesis, in keeping with our findings with angiogenic markers.

6.6.2.1.1 Analysis of test accuracy

In order for these markers to be of clinical use, they must be able to distinguish a clinical-grade liver from a discard liver with a high level of sensitivity and specificity. Receiver Operator Characteristics (ROC) curve analysis was performed to establish the optimum cut-off value and sensitivity, specificity, positive predictive value (PPV) and negative predictive values (NPV) were calculated, and the 10 tests with highest sensitivity and specificity were selected.

Test	Timepoint	Cut-off (pg/ml)	Sensitivity	Specificity	PPV	NPV	Area	p
IL5	start	0.6221	100.00	88.89	85.71	100.00	0.963	0.003
IL7	end	4.717	83.33	100.00	100.00	88.89	0.958	0.004
IL12/23p70	start	14.46	100.00	88.89	85.71	100.00	0.982	0.002
IL15	start	2.192	100.00	88.89	85.71	100.00	0.982	0.002
IL16	start	1244	83.33	88.89	83.33	88.89	0.926	0.006
IL16	end	934.3	50.00	100.00	100.00	75.00	0.833	0.034
IL17A	end	3.646	100.00	100.00	100.00	100.00	1.00	0.004
VEGF	start	6.285	83.33	77.78	71.43	87.50	0.833	0.034
CRP	start	2.394	83.33	88.89	83.33	88.89	0.907	0.009
CRP	end	4.230	66.67	100.00	100.00	81.82	0.815	0.045

Table 19 - Analysis of perfusate test accuracy

From this, 4 tests (IL5, IL12, IL15 and IL17) had 100% sensitivity, that is in a discard liver, the value recorded was above the cut-off in all livers. 4 different tests also had 100% specificity that is in a clinical liver, the value recorded was below the cut-off in all cases. PPV represents the chances that the test is above the cut-off that this correctly identified a discarded liver, this was 100% in 4 tests. NPV is probably the most clinically relevant, this means that if the test is below the cut-off level, the chances of it being a clinical liver, this was also 100% in 4 tests. The only marker to have 100% sensitivity, specificity, PPV and NPV was IL17A with a cut-off value of 3.646pg/ml.

6.6.2.1.2 Scoring System

It is possible that by combining the 10 tests above to produce a score would increase the accuracy in assessing the transplantability of a liver. As such a new scoring system was devised where 1 point was added for each test result that is above the established cut-off, giving a maximum score of 10. The total points for each liver perfused were added together and displayed below.

	Donor number	Total Score
Hypothermic Discard (6)	116476	9
	117644	8
	118050	7
	118754	10
	118768	9
	119286	6
Hypothermic Clinical (9)	118802	3
	118856	2
	120127	0
	120143	0
	121791	0
	122173	1
	123013	0
	126051	1
	126380	0

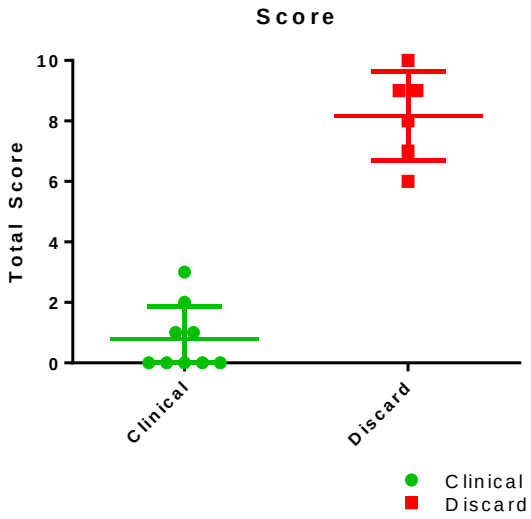


Figure 59 - 10-point perfusate scoring system in clinical and discard livers undergoing hypothermic perfusion showing mean +/- S.D.

Table 20 - 10-point perfusate scoring system

There is a statistically significant difference in the scores of the clinical and discard livers (t-test, $p<0.0001$). None of the clinical livers had a score over 3, and none of the discard livers had a score under 6.

Alternatively, to improve the clinical applicability of the scoring system, it would be beneficial to only use tests that are reliable at the start of perfusion so that a decision on transplantability could be made without having

to wait until the end of perfusion. To do this, a scoring system was applied using only the 6 tests above that were taken at the start of perfusion. The maximum score is now 6. The results are displayed below.

	Donor number	Total Score
Hypothermic Discard (6)	116476	6
	117644	5
	118050	6
	118754	6
	118768	6
	119286	4
Hypothermic Clinical (9)	118802	3
	118856	2
	120127	0
	120143	0
	121791	0
	122173	1
	123013	0
	126051	1
	126380	0

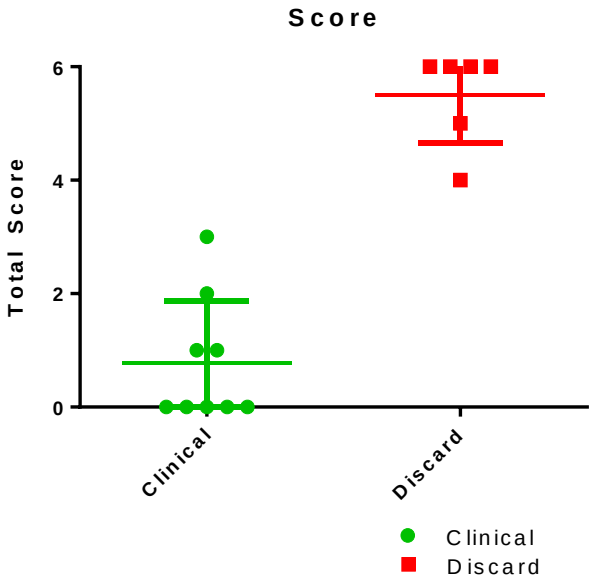
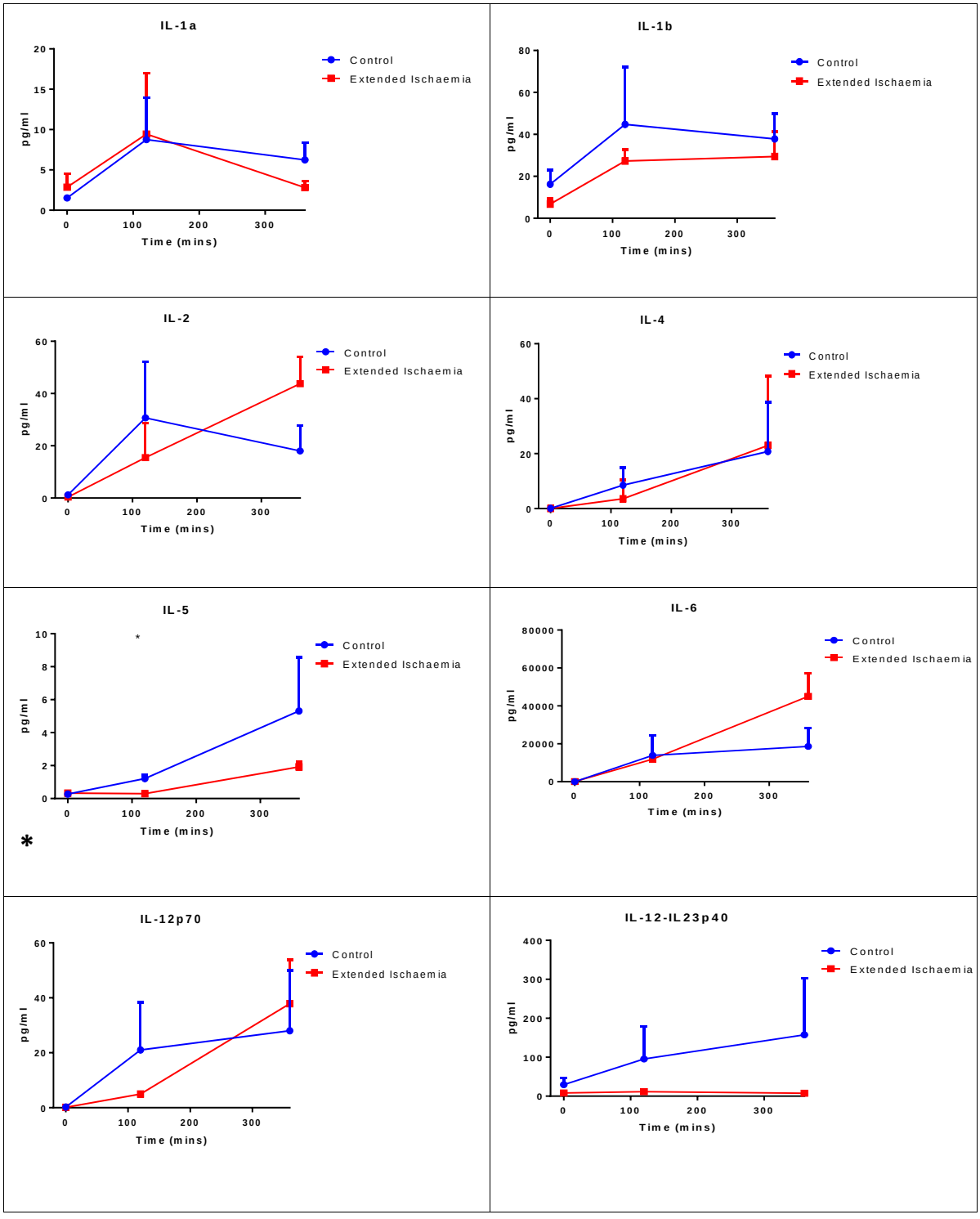


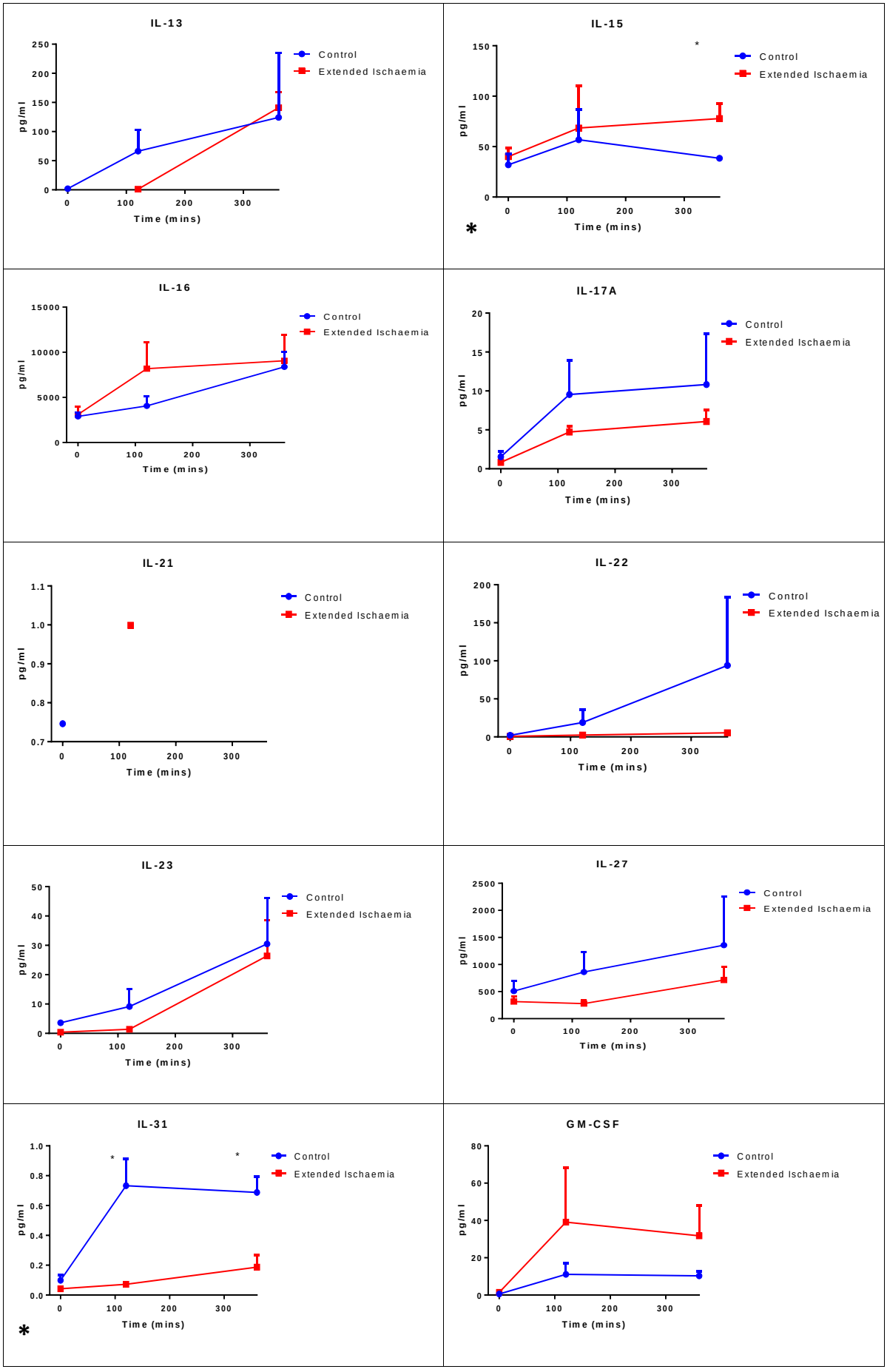
Figure 60 - 6-point start-time perfusate scoring system in clinical and discarded livers undergoing hypothermic perfusion showing mean +/- S.D.

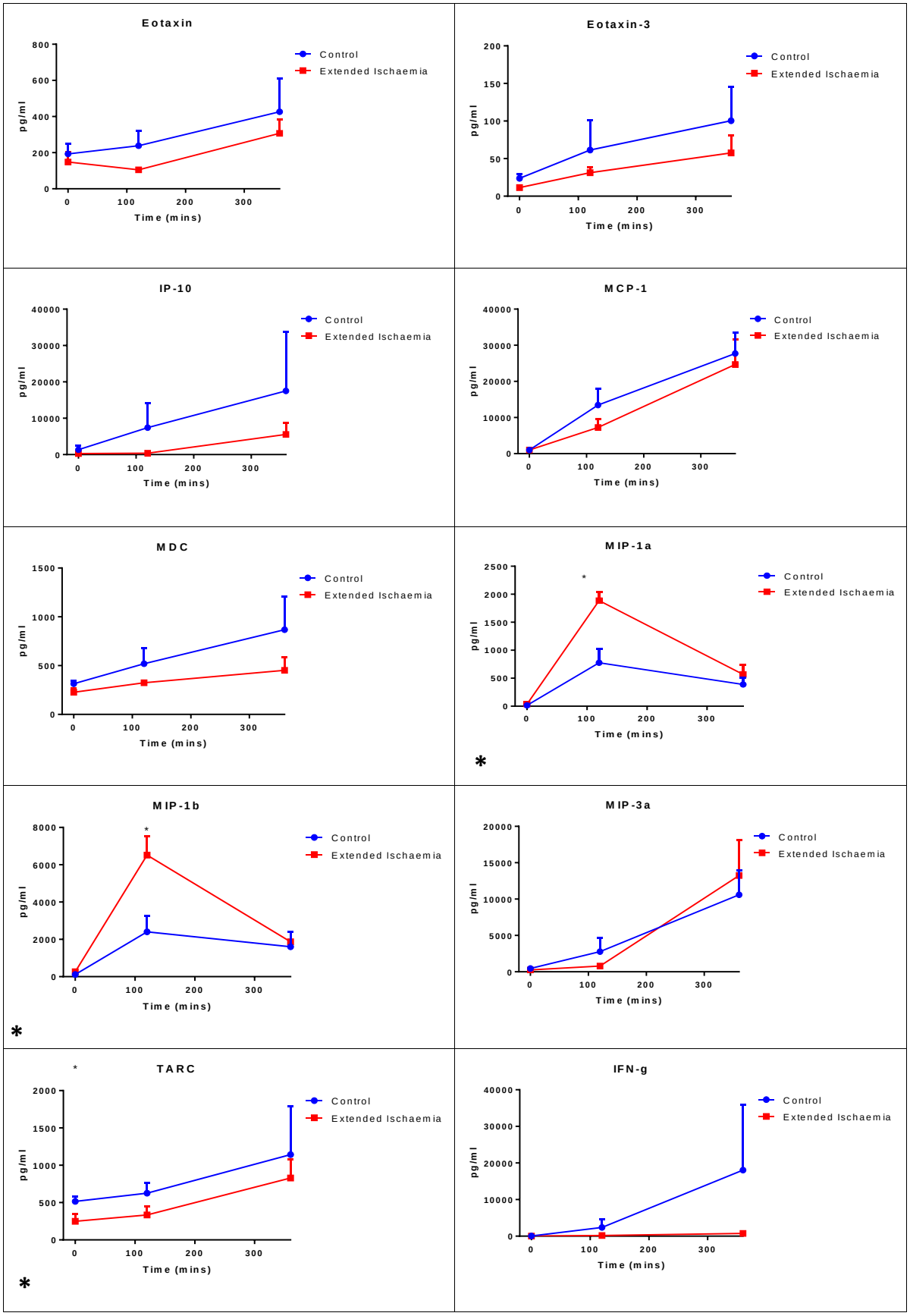
Table 21 - 6-point start-time perfusate scoring system

Using the start-point scoring system, there is less differentiation between groups, with the lowest scoring discard liver having a score of 4 versus the highest-scoring clinical liver score of 3. Nevertheless, there is still a clear statistically significant difference between the two groups (t-test, p=<0.0001). One possible clinical application is to test the 6 start-point tests and if this produces a score>2, to proceed to complete the full 10-point scoring system.

6.6.2.2 Normothermic Perfusion




IL-13
pg/ml
Time (mins)
Control
Extended Ischaemia
IL-15
*
*
IL-16
IL-17A
IL-21
IL-22
IL-23
IL-27
IL-31
*
*
*
GM-CSF


Eotaxin
pg/ml
800
600
400
200
0
0
100
200
300
Time (mins)
Control
Extended Ischaemia
Eotaxin-3
pg/ml
200
150
100
50
0
0
100
200
300
Time (mins)
Control
Extended Ischaemia
IP-10
pg/ml
40000
30000
20000
10000
0
0
100
200
300
Time (mins)
Control
Extended Ischaemia
MCP-1
pg/ml
40000
30000
20000
10000
0
0
100
200
300
Time (mins)
Control
Extended Ischaemia
MDC
pg/ml
1500
1000
500
0
0
100
200
300
Time (mins)
Control
Extended Ischaemia
MIP-1a
pg/ml
2500
2000
1500
1000
500
0
*
0
100
200
300
Time (mins)
Control
Extended Ischaemia
*
MIP-1b
pg/ml
8000
6000
4000
2000
0
*
0
100
200
300
Time (mins)
Control
Extended Ischaemia
*
MIP-3a
pg/ml
20000
15000
10000
5000
0
0
100
200
300
Time (mins)
Control
Extended Ischaemia
*
TARC
pg/ml
2000
1500
1000
500
0
0
100
200
300
Time (mins)
Control
Extended Ischaemia
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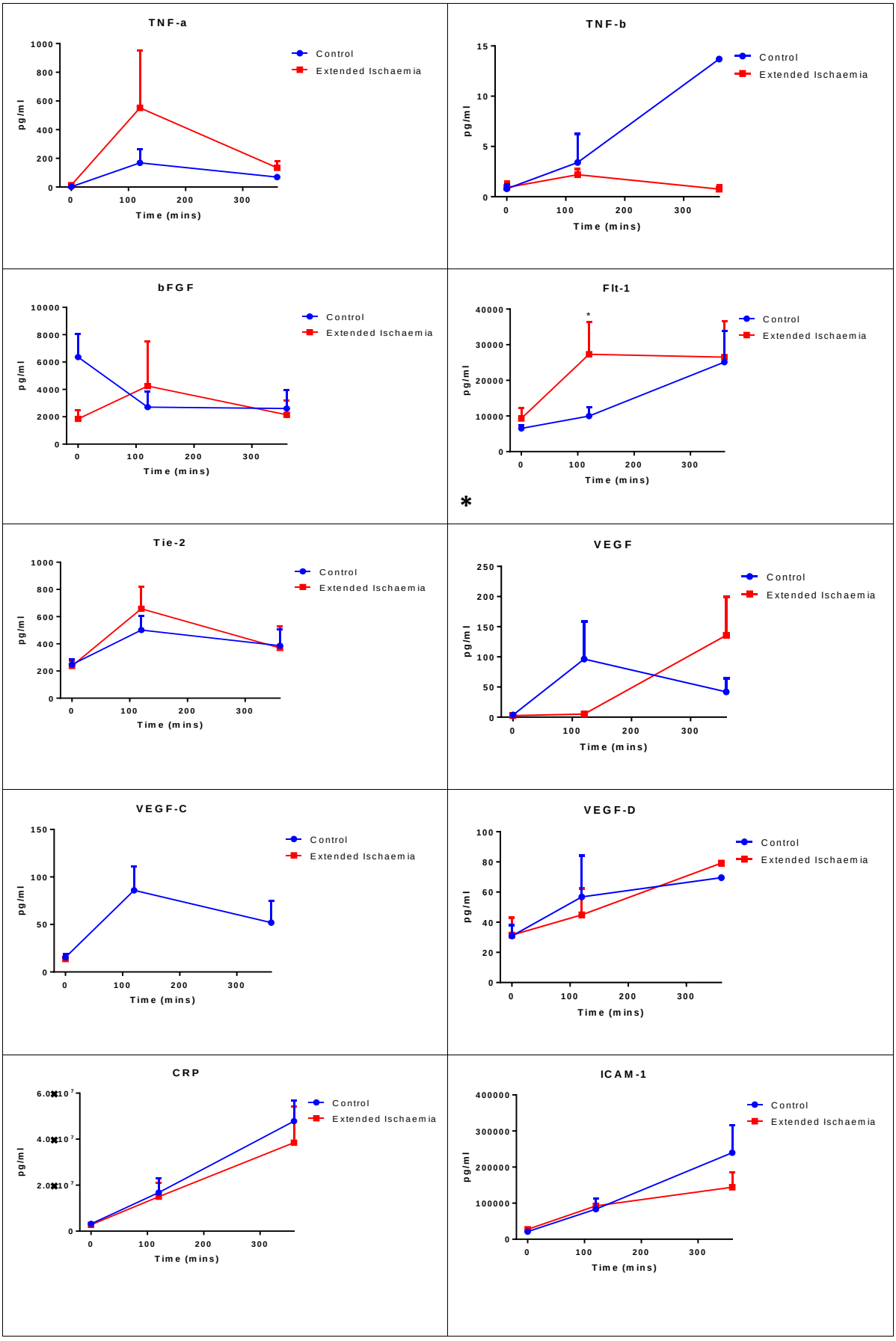
0
100
200
300
Time (mins)
Control
Extended Ischaemia


TNF-a
pg/ml
Time (mins)
Control
Extended Ischaemia
TNF-b
pg/ml
Time (mins)
Control
Extended Ischaemia
bFGF
pg/ml
Time (mins)
Control
Extended Ischaemia
Flt-1
pg/ml
Time (mins)
Control
Extended Ischaemia
*
Tie-2
pg/ml
Time (mins)
Control
Extended Ischaemia
VEGF
pg/ml
Time (mins)
Control
Extended Ischaemia
VEGF-C
pg/ml
Time (mins)
Control
Extended Ischaemia
VEGF-D
pg/ml
Time (mins)
Control
Extended Ischaemia
CRP
pg/ml
Time (mins)
Control
Extended Ischaemia
ICAM-1
pg/ml
Time (mins)
Control
Extended Ischaemia

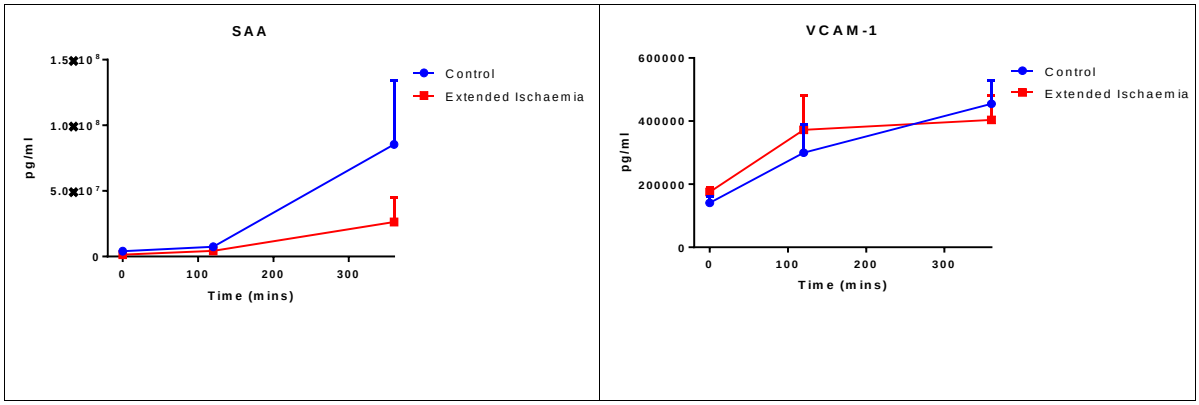


Figure 61 - Perfusate chemokine and cytokine levels in normothermic perfusion in pg/ml showing mean +/- S.D.

Figure 61 shows the mean and standard deviation values for the chemokines and cytokines analysed in the multiplex.

A number of markers again demonstrated statistically significant differences between control livers and livers that were subjected to extended ischaemia.

Macrophage inflammatory protein (MIP) 1-α, MIP1-β (also known as chemokine ligand 3 (CCL3) and CCL4 respectively), and IL-31, all pro-inflammatory cytokines were significantly elevated in the extended ischaemia group.

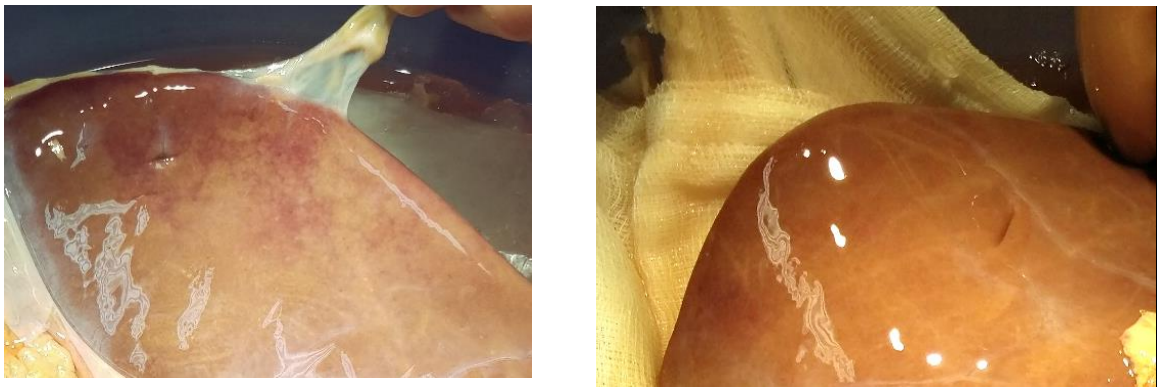
Thymus- and activation-regulated chemokine (TARC/CCL17) and IL-15, both involved in the immune response, were significantly elevated in the extended ischaemia group.

Vascular endothelial growth factor receptor 1 precursor (Flt1), an angiogenic marker, was also significantly elevated in the extended ischaemia group.

Receiver operating characteristics (ROC) curve analysis failed to identify as many markers that were reliable at identifying extended ischaemia as was identified in the hypothermic livers. Of all the markers at each time-point, only 2 generated a statistically significant ROC curve – MIP1α at the mid-time-point ($p = 0.046$) and TARC at the start of perfusion ($p = 0.036$)

6.7 Contrast-Enhanced Ultrasound

We have observational evidence of improved perfusion on the extremities of the liver. Livers that had been declined due to poor perfusion, appeared to visually improve over the perfusion period. Figure 62 demonstrates the same part of the same liver at Time 0 and Time 120. It is clear to see that better clearance of the liver tissue has occurred, possibly indicating improved peripheral circulation.



A B

Figure 62 - Prior to perfusion (A, Time 0), End of perfusion (B, Time 120)

6.7.1 Methods

Contrast-enhanced ultrasound was performed on 10 livers undergoing normothermic perfusion, 5 control livers and 5 extended-ischaemia. All livers were perfused using our standard normothermic perfusion protocol, and CEUS was performed at the start of perfusion, at the 6-hour time-point, and if possible at 2 hours also (limited by staff and equipment availability).

A Philips Epiq 7 ultrasound machine, with a C9-2 probe, loaded with up to date QLAB software was used. All contrast ultrasounds were performed by either a consultant radiologist with a special interest in contrast sonography or a senior sonographer. The probe was placed over segment 4 and centred on the porta hepatis. The arterial and portal venous circulations were analysed in isolation. This was performed by clamping the limb of the circuit not being assessed for the period of the scan. 0.7 ml of SonoVue® contrast agent was injected sequentially into the arterial limb of the circuit, then after bubble clearance was repeated for the portal venous limb. At the time of injection, curve analysis was started on the scanner and continued for 60 seconds, at which point the clamp was released to allow full arterial and portal venous perfusion. Scanning was performed for a further 30 seconds then stopped.

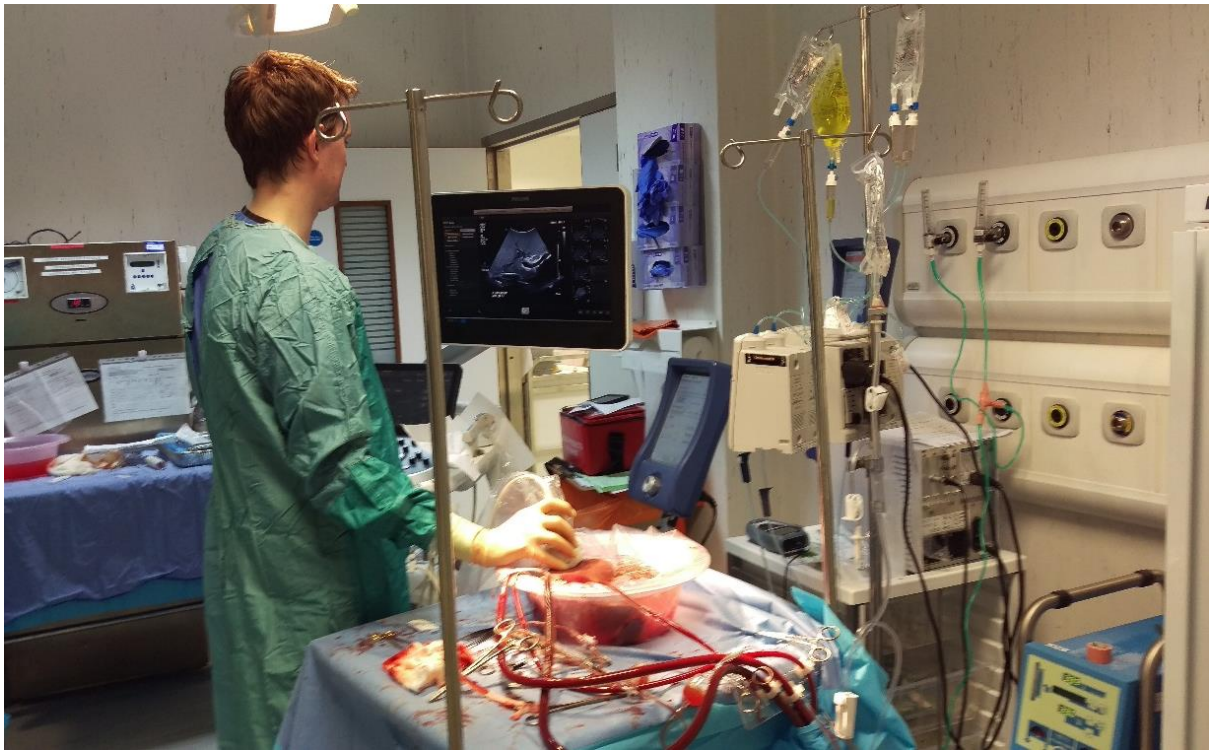


Figure 63 - Contrast-enhanced ultrasound being performed during normothermic perfusion

Area under the curve (AUC) analysis was performed using Philips QLAB software. For this, regions of interest (ROI) were identified for analysis. For each scan run, an ROI within the respective vessel lumen (hepatic artery or portal vein) that was being analysed was used, and a further ROI of an area of liver parenchyma not containing large vessels. The ROI within the vessel lumen was effectively used as a control for maximum perfusion. The AUC calculation provides a quantification of contrast backscatter within the ROI for that run of contrast, effectively a measure of perfusion within that defined area.

The value of interest is the difference in perfusion between the vessel lumen and the parenchyma. If the parenchyma is as well perfused at the centre of the vessel lumen, then there is no deficit in microcirculation. However, if the microcirculation is inadequate, the AUC in the parenchyma will be significantly lower than within the lumen. The difference between AUC in the lumen and AUC of the parenchyma was termed dAUC, where a value approaching 0 is desirable (i.e. no difference).

6.7.2 Results

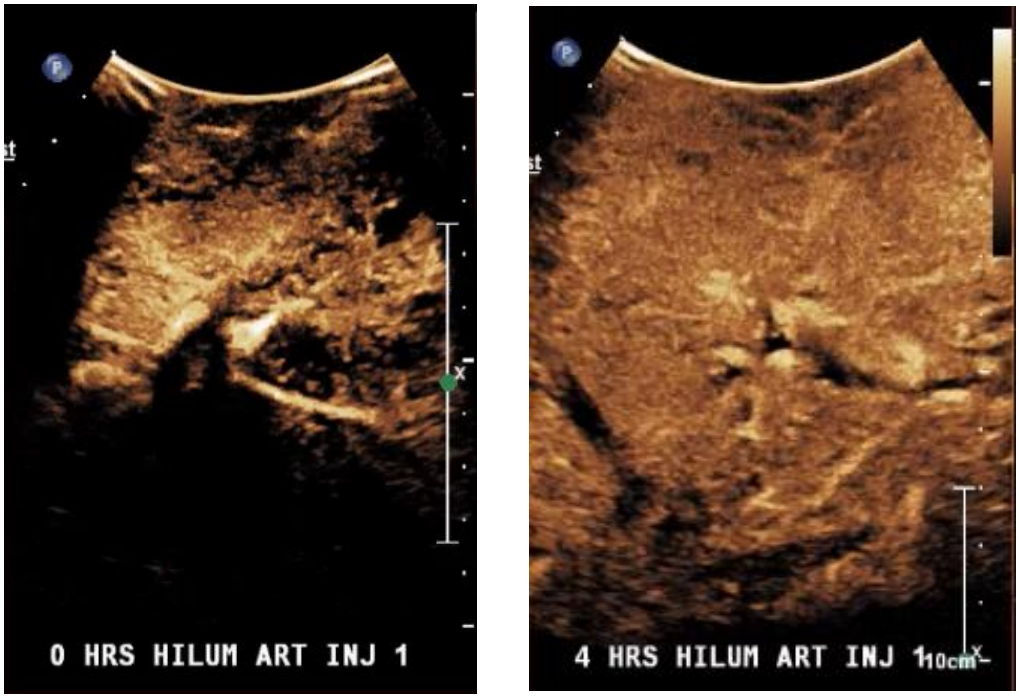


Figure 64 – Contrast-enhanced ultrasound Image capture 5 seconds after bolus of micro-bubble contrast agent in the same liver at the start of hypothermic perfusion and 4 hours later

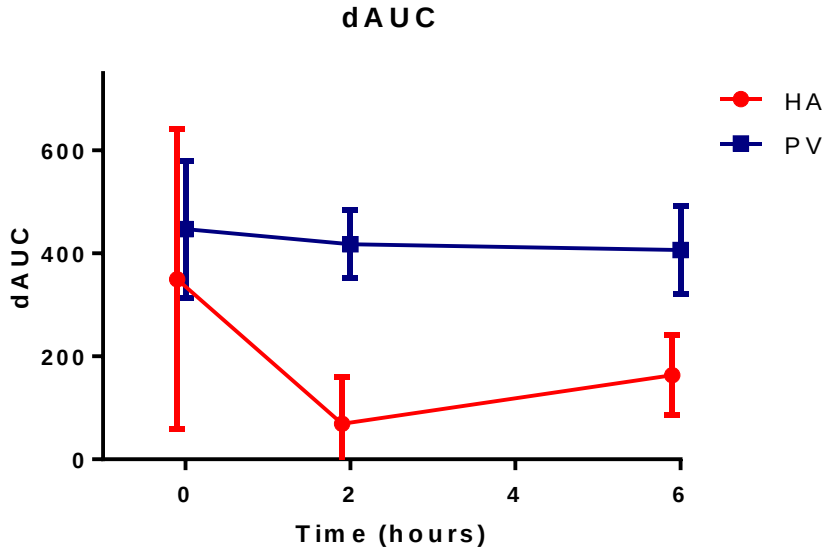


Figure 65 - Difference in area under the curve (dAUC) after hepatic artery (HA) or portal venous (PV) bolus on microbubble contrast agent showing mean +/- S.D.

Overall, analysing all livers together, there is no difference in dAUC over time for portal venous perfusion, however the dAUC reduces over time for arterial perfusion. That is to say that the difference in perfusion between parenchyma and arterial lumen reduces, therefore parenchymal perfusion improves over time.

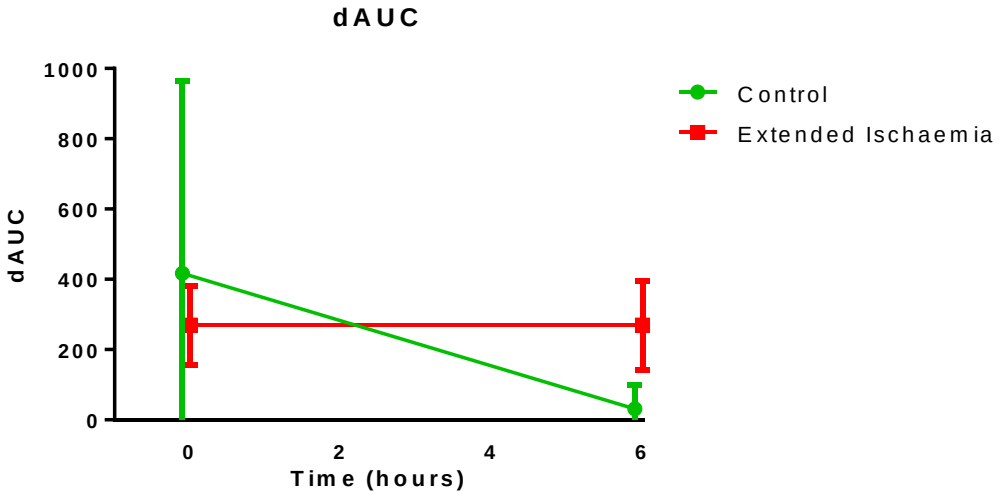


Figure 66 - Difference in area under the curve after bolus of microbubble contrast agent into arterial circulation comparing control and 'extended ischaemia' livers undergoing normothermic perfusion. Showing mean +/- S.D.

When the arterial dAUC is then analysed in isolation, comparing the two groups proves very interesting. The control group demonstrated an ability to improve its parenchymal arterial perfusion over the course of perfusion, whereas the extended ischaemia group did not.

6.8 Cold Ischaemic Times

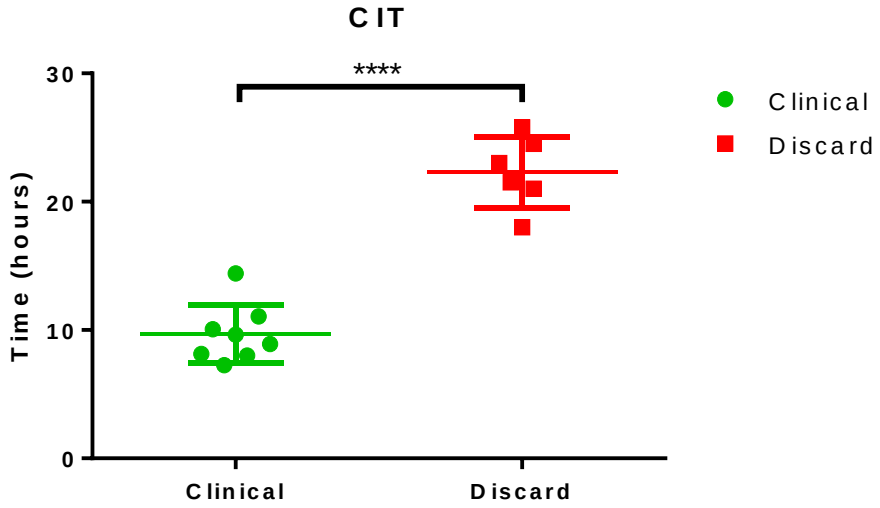


Figure 67 - Cold-ischaemic times in control and discarded livers undergoing hypothemic perfusion

It is important to note that the differences between the clinical and discard livers may not be exclusively due to differences in liver characteristics and performance as there was a significant difference in cold-ischaemic times (CIT). This discrepancy was unfortunately unavoidable due to the logistics of the offering process. In clinical livers there are obvious time-pressures to transplant as soon as is feasible and it would not be ethical to intentionally delay this. However, discard livers undergo a full offering process to all centres before being

offered for research, including sometimes awaiting biopsy results. Even after acceptance for research, the liver must then be transported to Newcastle prior to our research taking place.

The introduction of clinical hypothermic perfusion has not affected our mean static cold storage time, but did increase the overall preservation time.

6.9 Discussion

Being able to use perfusate samples to aid in the decision on whether to transplant a liver or not has the potential to increase the numbers of livers available for transplant as well as potentially improving the accuracy of that decision with more objective and repeatable measures. We have here analysed a number of potential markers ranging from lactate that is already easily available as a rapid point-of-care test, to ALT, which is already performed in all biochemistry labs, through to cytokines that are not yet available as rapid point-of-care tests, but there is potential to develop this.

6.9.1 Hypothermic Perfusion

Our analysis of hypothermic livers compared two groups. Livers were essentially allocated to groups by clinical decision-making as discard livers had been declined by all centres purely on data available on donor factors and the retrieval process including visual inspection of the liver by the surgeon. We aimed to establish whether biomarkers would also be able to make this distinction, with the ultimate aim that if reliable biomarkers could be identified, this decision could be more objective.

A number of potential biomarkers have been identified that appear to differentiate between a liver that has been clinically deemed transplantable (hypothermic clinical livers) and livers that have been declined by all centres. These include vascular injury markers and angiogenesis markers. Pro-inflammatory cytokines were significantly elevated in discard livers and conversely, anti-inflammatory cytokines tended to show the opposite effect. In addition, cytokines involved in regulating the immune response were also significantly elevated in the discard group. These results are all in keeping with what would be expected. Potentially, one or more of these in combination could aid in the decision-making process on the transplantability of a liver. Two potential compound scores using biomarkers have been proposed, both clearly distinguishing between clinical and discard livers. The 6-point scoring system has the advantage of only using perfusate taken at the start of perfusion, so would allow for an early decision on transplantability. However, although there is no overlap between the groups (i.e. 100% sensitivity and specificity), the 10-point test proved more accurate. A potential route to clinical application could therefore be to run the 6-point test at the start of perfusion, and if a more equivocal result (e.g. >2) was obtained, perfusion could be continued and a further completion 10-point test carried out at the end of perfusion before a decision on transplantability is made.

Perfusate ALT levels were much less able to distinguish between the two groups, with no significance on analysis. An advantage of this over the multiplex is that lab-based testing is already available in all liver transplant units and a result is obtainable in approximately 25 minutes (although not using our methods). Correlating the perfusate ALT levels to recipient serum ALT levels post-transplant did not yield a particularly reliable correlation. Although so many variables contribute to the recipient's ALT levels, including 2nd warm ischaemic times, drugs the patient may have been given etc, that fairly large numbers may be necessary to demonstrate closer correlation. In normothermic perfusion, Watson *et al*.[28] have demonstrated a correlation between perfusate and recipient ALT, however no other data has been published on this in hypothermic perfusion.

Perfusate lactate measurements are potentially even easier to obtain than ALT, as this can be measured by blood gas analysers, which are readily accessible in any transplant theatre. The Siemens analyser we used for our analysis is able to give a lactate reading in <2 minutes. The lactate levels on our discard livers started at a higher level than clinical livers and continued to climb over the course of perfusion, whereas clinical livers remained relatively stable and at a significantly lower level. From our tests, a lactate level above 4.912 would only ever be seen in a discard liver, and not seen in a clinical liver, and therefore has potential clinical applicability.

6.9.2 Normothermic Perfusion

At the point of perfusate analysis, we had yet to perform any clinical normothermic perfusions, thus all samples were from livers deemed unsuitable for transplantation. However, in addition to the 10 control livers, a further 5 livers were intentionally subjected to extreme ischaemia (median 71 hours, range 69-74 hours). This was performed with the aim of establishing what an unquestionably untransplantable liver would behave like on perfusion.

When comparing the control group to the extended ischaemia group, there were fewer significant results in the multiplex than comparing the 2 hypothermic groups. This is likely to be because, although the control group was not subjected to extended ischaemia, they were still livers that had been deemed untransplantable (i.e. 'discard'). Therefore, in effect the two groups are a comparison of livers that were deemed untransplantable with ones that were already untransplantable, but subjected to further significant ischaemic injury. Because none of the livers were expected to produce low levels of appropriate cytokines, it was harder to differentiate the two groups. Nevertheless, some pro-inflammatory cytokines were significantly elevated in the extended ischaemia group, as was Flt1 an angiogenic marker. In addition, immune-mediating markers were also elevated in the extended ischaemia group. This mirrors the results obtained in the hypothermic group.

Previous studies have postulated the importance of perfusate ALT in the assessment of livers, and indeed its correlation to recipient ALT[28]. However, in our experience perfusate ALT was not particularly reliable in distinguishing between these two groups, but again this may be due to a lack of a transplantable liver group for comparison. It is also worth noting that Watson *et al*. achieved a positive correlation between perfusate and recipient ALT but this was aided by a single case that was an outlier with very high ALT levels in perfusate and recipient serum. Nevertheless, our first NMP perfusion with intent to transplant was abandoned primarily due to a rising perfusate ALT level, which reached 3135 u/L at 4 hours of perfusion.

The use of perfusate lactate in liver selection for transplantation has already been postulated, and a cut-off value of 2.5 mmol/L has been suggested [63, 73]. We therefore analysed our perfusate levels, and found that it was not able to distinguish between our control discard livers and the 60 minutes group. However, more importantly, our results show that all livers, even after extended ischaemia were able to clear lactate. In fact, all of our livers would have met the cut off value set by Mergental *et al*. Lactate clearance appears to be a falsely reassuring finding, and should be disregarded in normothermic perfusion. It is likely that the ability of

hepatocytes to withstand hypoxia is surprisingly good, and therefore will still undergo aerobic metabolism even after hypoxia. However, other liver cells such as cholangiocytes are more sensitive to hypoxic conditions. Hence, although in an ex-vivo circuit, these livers can clear lactate, they will not have adequate post-transplant function. Interestingly, this is the opposite conclusion to our lactate findings in hypothermic perfusion.

Bile production was very significantly different between our 2 study groups. Livers after extended ischaemia effectively produced minimal bile, the liver that produced most bile in this group produced 2.6 ml/hr compared with the lowest in the control group being 9 ml/hr. This supports the theory that cholangiocytes are more sensitive to ischaemic insult than hepatocytes. It has been suggested that glucose concentration can affect volume of bile production, but we found no correlation in our series.

Interestingly, contrast ultrasound appeared to be the best measure by which to distinguish between the control livers and those that had been subjected to extended ischaemia. When the dAUC for arterial perfusion was analysed in these livers, the parenchymal perfusion in control livers improved over time, whereas there was no improvement at all in extended ischaemia livers. It is possible therefore to conclude that there is a role for CEUS in the assessment of livers during normothermic machine perfusion. Although further data has yet to be acquired on the dAUC of livers that go on to be transplanted. Full analysis of this data has the potential to include CEUS in liver assessment.

6.10 Conclusion

A number of very interesting patterns were identified, demonstrating that reduced levels of pro-inflammatory markers, vascular injury markers and angiogenesis markers are all desirable and have the potential to differentiate transplantability in livers undergoing ex-vivo perfusion. A scoring system is proposed for hypothermic livers, which in our experience was able to reliably differentiate between clinically transplantable livers and discarded livers. There is certainly potential for clinical application of this scoring system. The current limiting factor is the availability of these tests at the point-of-care, however there is work underway to make some of these available as a rapid assay. ALT in our experience appears to be of limited value, but may have a role as part of a more complex compound score. Lactate proved to be useful in the assessment of hypothermic livers, but not during normothermic perfusion, where all livers were able to successfully clear lactate. CEUS was only performed in normothermic perfusion, but provided very interesting data on the potential for ex-vivo perfusion to improve parenchymal perfusion. We found that ex-vivo perfusion does improve parenchymal circulation, but this benefit is lost in livers that have suffered excess ischaemia. Further work is required to correlate levels to clinical outcomes and in higher numbers to confirm our findings before clinical implementation as an added tool in the decision-making process of whether to transplant a liver. From our data, it is unlikely that a single biomarker can be identified that can in isolation predict the future viability of a liver graft. Increasingly complex scoring systems taking into account perfusion dynamics, bile production, a number of biomarkers, and improvement on CEUS may be necessary to radically improve liver selection.

Chapter 7. Steatosis

7.1 Introduction

Donor liver steatosis is a well-documented risk factor for post-transplant outcome, including an increased risk of PNF. This was first described by Todo *et al* almost 30 years ago in report of 2 cases of PNF following transplantation of what was described as 'greasy'-feeling livers [127]. Two further studies shortly after this describing similar experiences resulted in widespread avoidance of macrosteatotic livers [128, 129]. The study by D'Alessandro *et al*, reported 100% rate of PNF in livers with severe fatty infiltration [128]. However, increasing pressure for utilisation of marginal grafts has renewed interest in steatotic livers and the acceptable limits of transplantation. There is still a lack of consensus on the degree of acceptable hepatic steatosis, and practice varies widely between transplant units. There is evidence that >30% hepatic steatosis is associated with worse clinical outcomes [75], and that <30% hepatic steatosis is not associated with poor graft outcome [75]. However, the dilemma in graft selection is greatest in grafts displaying 30-60% hepatic steatosis (often labelled as 'moderate steatosis'). Whilst it is generally accepted that >60% steatosis results in a degree of recipient risk of morbidity and mortality that is not acceptable, it is arguable that 30-60% steatotic livers may be utilised in some circumstances (e.g. the absence of other risk factors, short ischaemic times and degree of urgency). A transatlantic survey of liver transplant surgeons in the UK and USA demonstrated the extent of dichotomy of opinions regarding utilisation of steatotic grafts, particularly the significance of microvesicular steatosis [130]. In this survey, the most common method for assessment of steatosis was that performed subjectively by the retrieving surgeon based on appearance and texture. In UK respondents, liver appearance was the only method used by 50% of respondents. Although severe macrosteatosis is easily assessed, macroscopic appearance of moderate steatosis has been proven to be unreliable when assessed by surgeons [131].

The two methods of assessing hepatic steatosis prior to transplantation of a liver currently available are either visual assessment by the retrieving and/or transplanting surgeon or biopsy. Both these options have major drawbacks – visual assessment is inherently subjective and opinions will differ, whereas histopathological assessment is time-consuming and can delay surgery. Given the importance of accurate assessment to differentiate mild-, moderate- and severe steatosis, the ability to perform this assessment with a rapid test that could be performed at the point-of-care would be a great asset in the real-time assessment of liver grafts prior to transplantation.

Oil-red O (ORO) is a staining method utilised in pathology to demonstrate the presence of lipids in pathological sections of tissue. It was therefore utilised in the assessment of our biopsies for grading of steatosis. ORO had a specific absorbance frequency, therefore we aimed to investigate the potential for automated quantification of the amount of absorbance at 518 nm.

7.2 Oil-Red Staining and Positive Pixel Analysis

Our first step was to stain fixed slides with ORO and use automated image analysis software to quantify the proportion of pixels within the slide that fall within the spectrum of ORO absorbance. Figure 69 below demonstrates the software in use. A cohort of mouse livers from mice that had been fed either a control diet or a high-fat diet were used for calibration and reference.

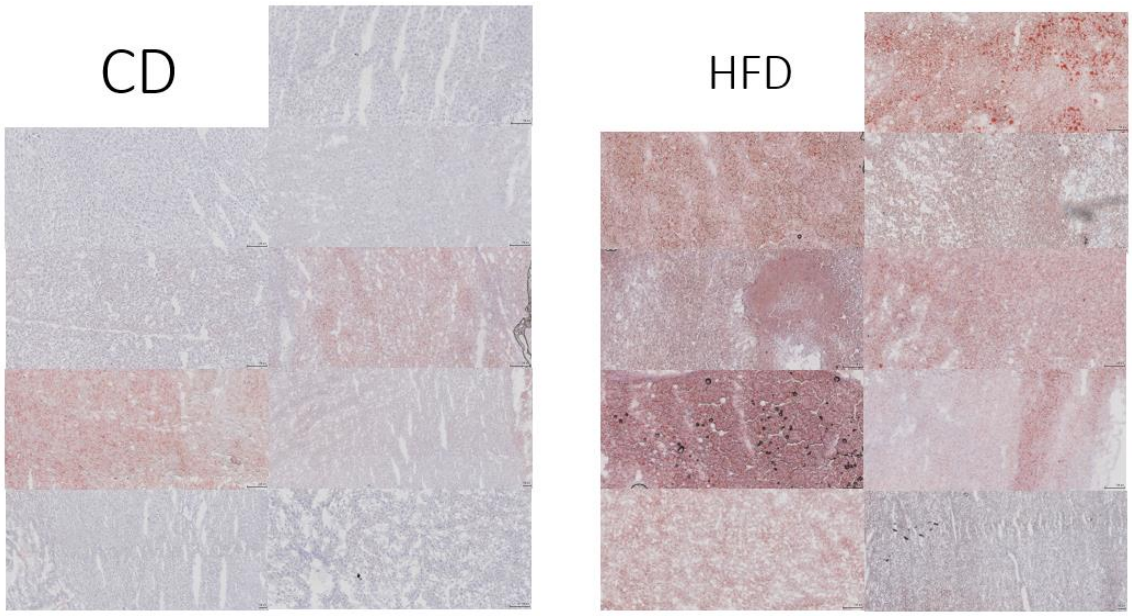


Figure 68 - Compound image of slides obtained from mice fed control diet (CD) and high-fat diet (HFD), all stained with oil-red

It is evident from Figure 68 that livers from mice that were fed a high-fat diet had noticeably increased oil-red staining.

Figure 69 demonstrates software analysis of the images to identify pixels consistent with oil-red staining

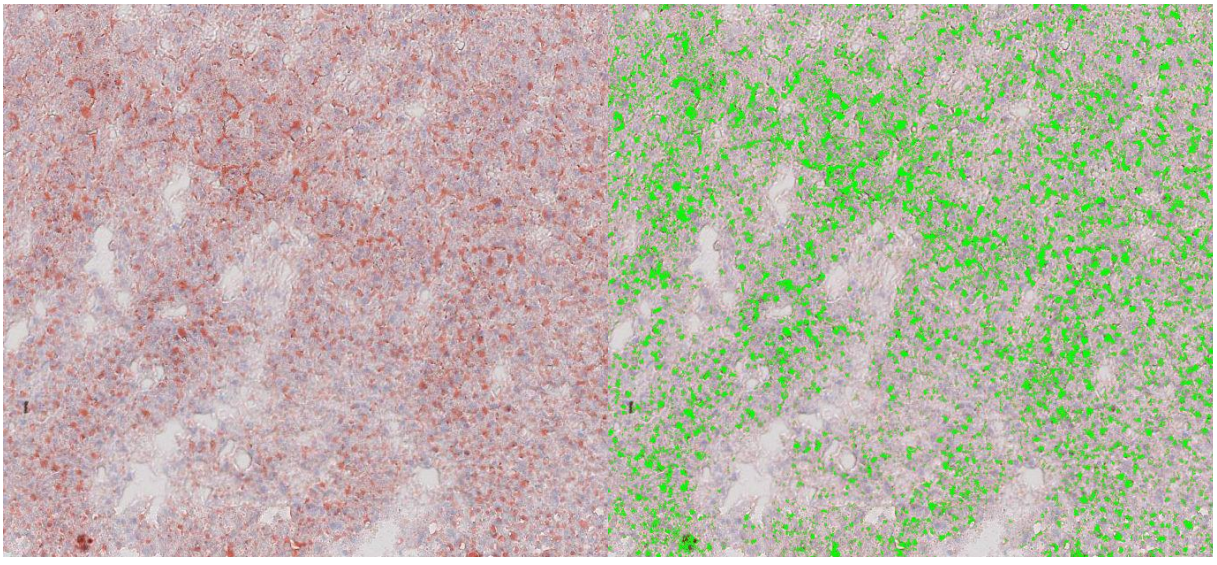


Figure 69 - Software identification of oil-red staining (marked green post-identification)

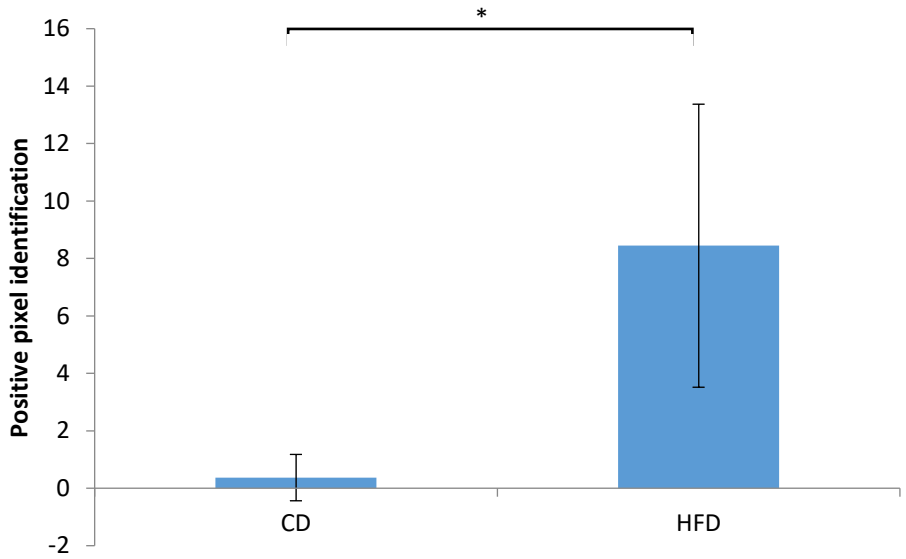


*Figure 70 - Quantification of steatosis by software positive pixel identification of oil-red staining (arbitrary units), control diet (CD) vs high-fat diet (HFD). *denotes significance*

There is a significant difference (p=0.0002) in oil red O staining between the two groups when analysed by positive pixel analysis (Figure 70).

7.3 Oil-Red Colorimetry

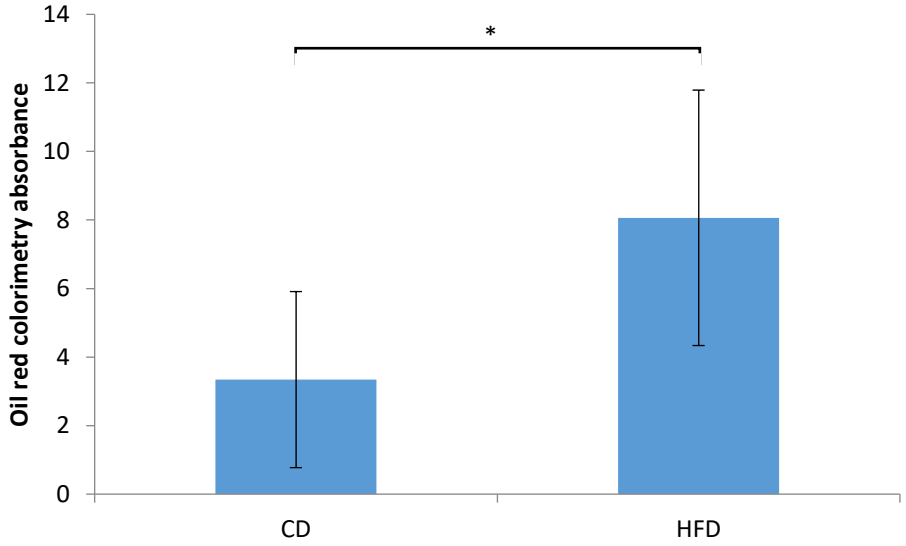


*Figure 71 - Colorimetry absorbance at 515nm following oil-red staining, control diet (CD) vs high-fat diet (HFD). *denotes significance*

The results are shown in Figure 71, and again demonstrate significance (p=0.0065).

The positive pixel value was correlated with the colorimetry value as demonstrated in Figure 72. There is a correlation between an elevated colorimetry absorbance with a high arbitrary value on positive pixel analysis. Pearson's correlation co-efficient is 0.6889, demonstrating a moderate positive correlation.

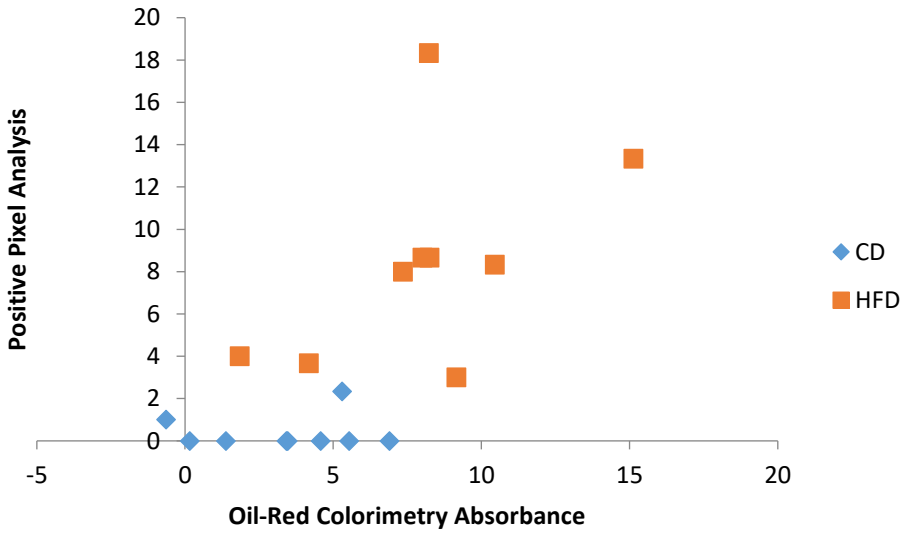


Figure 72 - Correlation of positive pixel value and colorimetry absorbance, control diet (CD) vs high-fat diet (HFD)

7.4 Triglyceride Determination

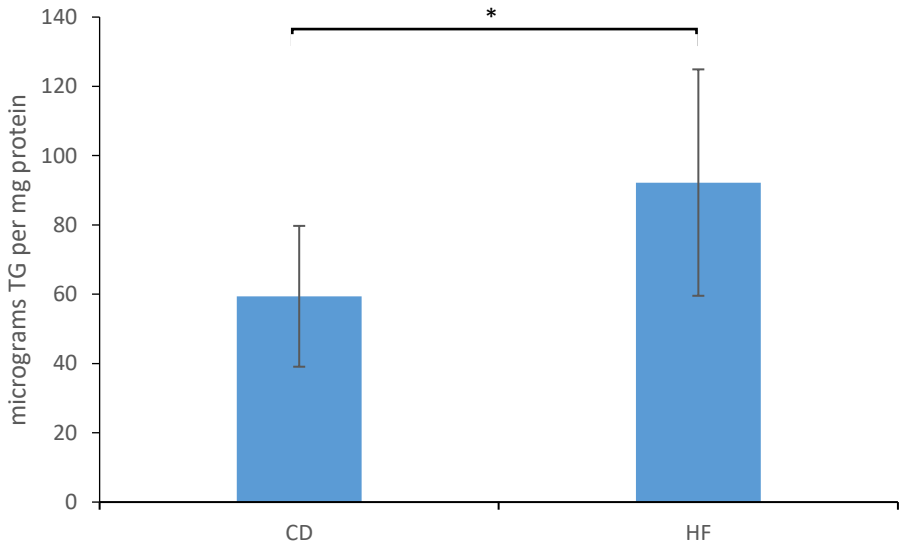


*Figure 73 - Triglyceride content in mice livers, control diet (CD) vs high-fat diet (HFD). Showing mean +/- S.D. *denotes significance*

When analysed (Figure 73), this did reach statistical significance (p=0.021), but ORO colorimetry demonstrated a more significant p-value, and was a faster test to perform.

7.5 AR42J-B13/H Cells

7.5.1 RT-PCR

Over the 14-day period of differentiation from B13 to B13H cells, CYP2E and albumin expression increases and becomes equivalent to the liver control. Apolipoproteins A2 and B both demonstrate a similar pattern, although A1 and E are less convincing. Nevertheless, the RT-PCR results demonstrated hepatocyte-like behaviour.

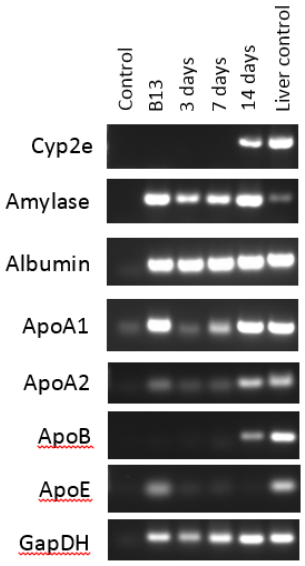


Figure 74 - RT-PCR for the indicated transcripts. Number of days indicates duration of treatment with dexamethasone

7.5.2 Effect of Steatosis on Viability in Hypoxic Conditions

There was a significant fall in viability when exposed to hypoxia in both the control group and the fatty acid group, but the viability fell further in steatotic cells (Figure 75). This demonstrates that steatotic cells were less able to cope with hypoxic incubation.

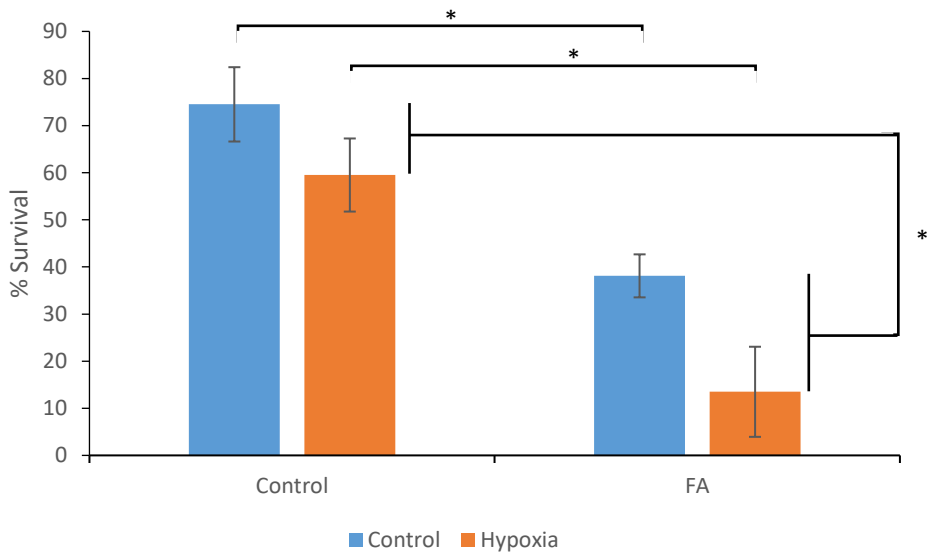
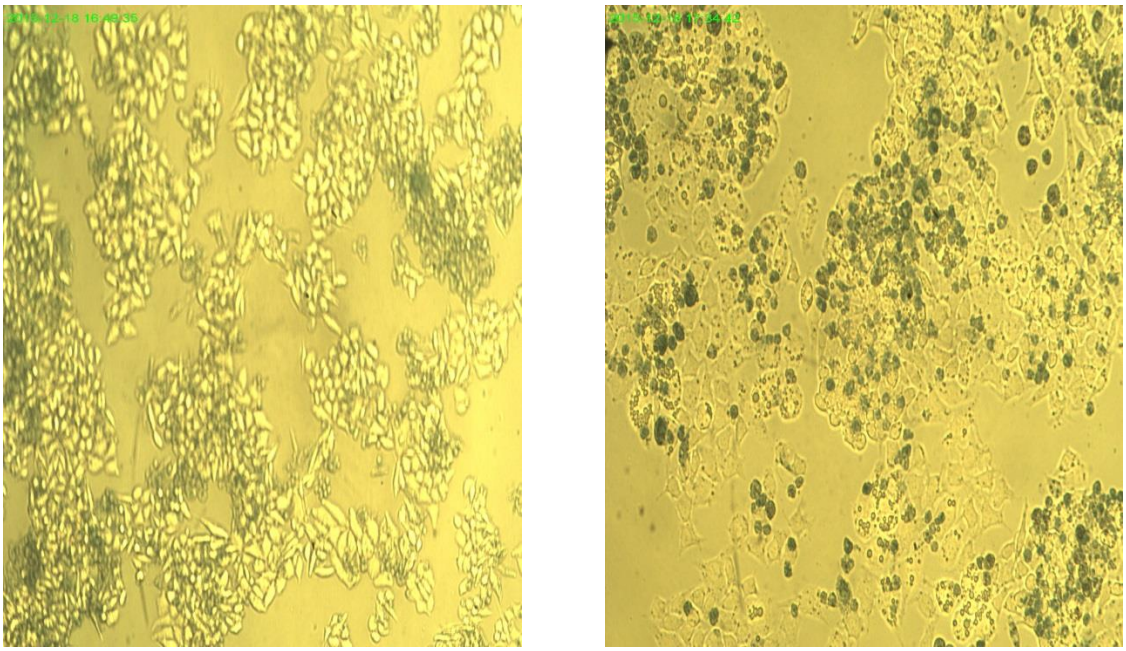


*Figure 75 - Viability of B13/H cells when exposed to hypoxia. *denotes significance*



A B

Figure 76 - Slide photographs of control cells following hypoxic injury (A), and steatotic cells following hypoxia (B)

7.6 Colorimetric Assessment of Human Liver Biopsies

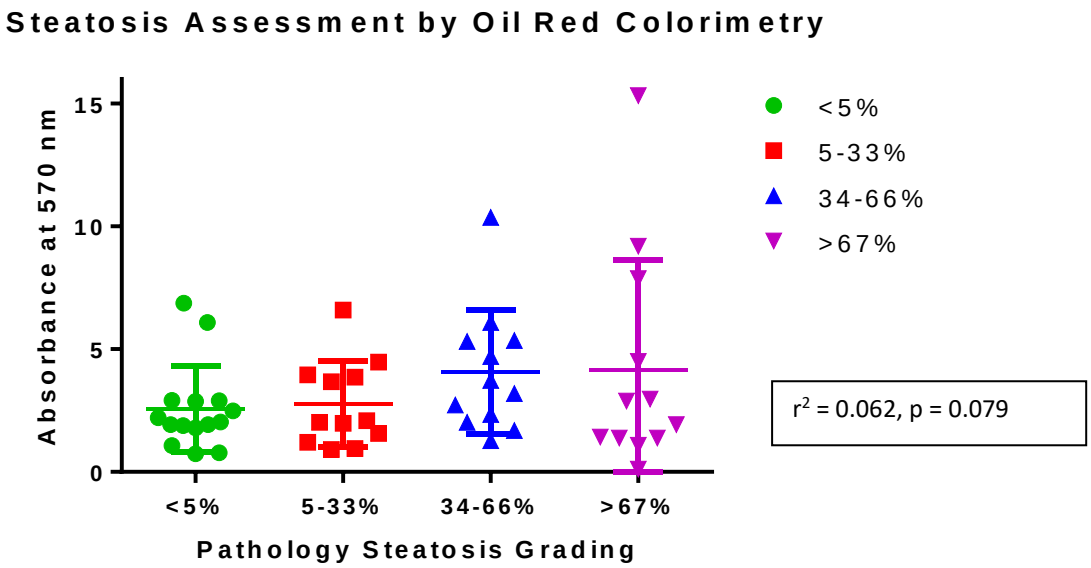


Figure 77 - Steatosis assessment by oil-red colorimetry against pathological grading of steatosis

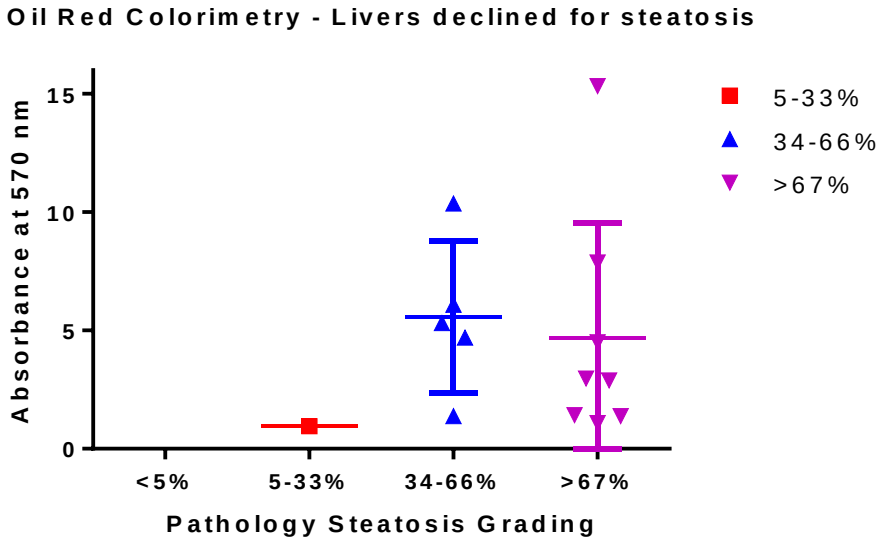


Figure 78 - Steatosis assessment by oil-red colorimetry against pathological grading of steatosis only in livers declined due to steatosis

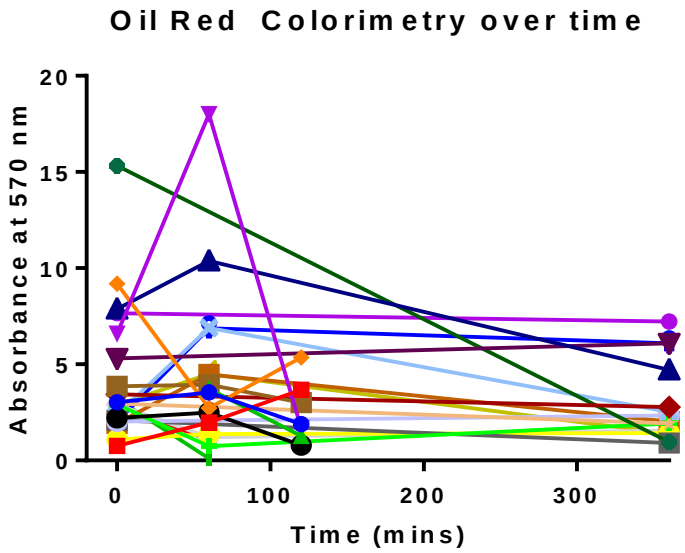


Figure 79 - Changes in oil-red colorimetry assessment over time of perfusion

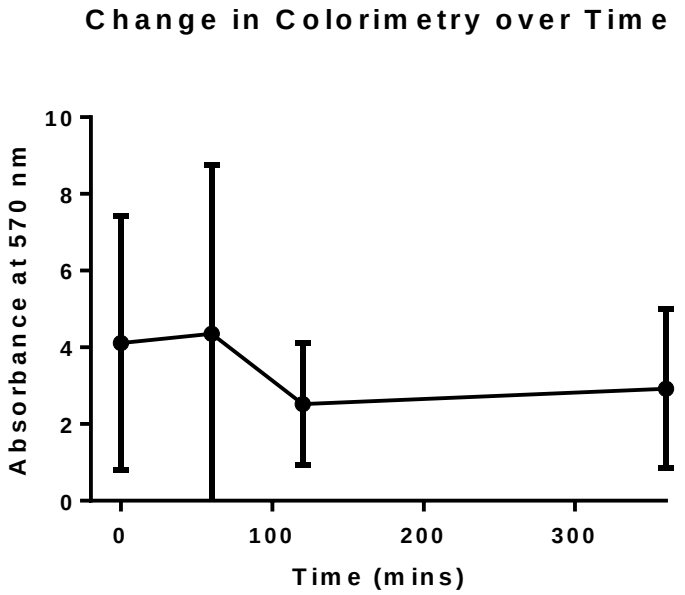


Figure 80 - Median +/- SE changes in oil-red colorimetry assessment over time of perfusion

One liver declined primarily due to steatosis was found both on colorimetry and then confirmed by oil-red histopathology to have only mild steatosis. (Figure 81).

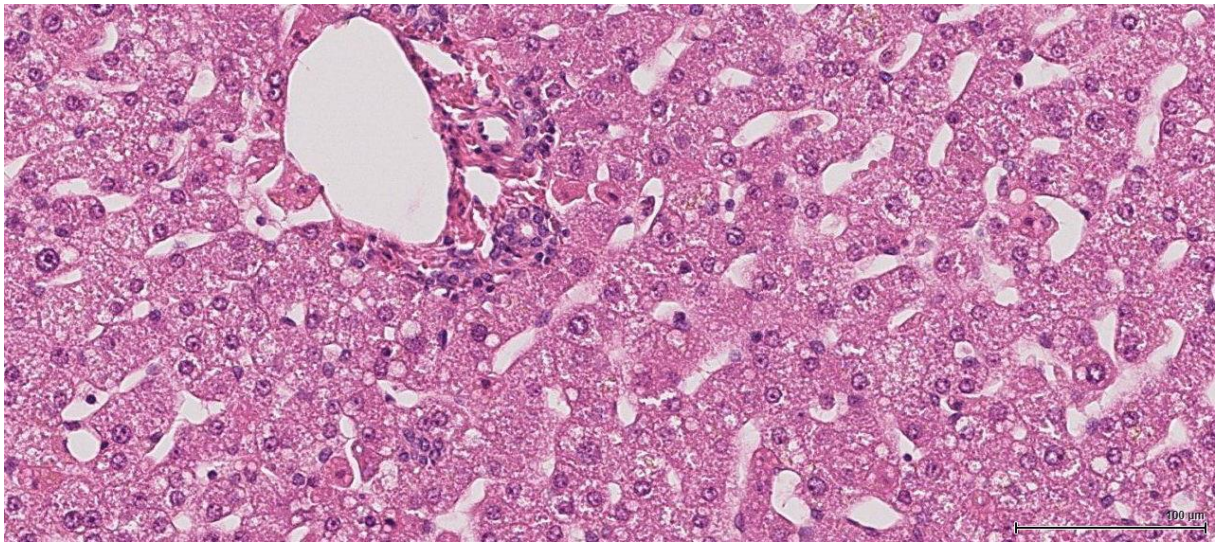


Figure 81 - H&E stained slide of liver declined primarily due to steatosis, but only demonstrating mild steatosis

7.7 *In-Vivo* Non-Invasive Steatosis Estimation (LIQu)

Steatosis quantification at the time of liver retrieval is currently largely performed by visualisation of the organ by the retrieving and implanting surgeons. The basis for establishing level of steatosis relies on the colour of the liver as well as size and appearance of the edges of the liver. We aimed to establish an automated, software-based image analysis tool to help quantify this more objectively from an image taken of the liver with a calibration slide overlying the liver. We collaborated with a private company to develop the software required for this and manufacture the calibration slides. We encountered significant difficulties in engaging with the company as the project matured and found it difficult to have them on board with the timelines required in this project. Nevertheless, the platform remains in development and an image bank of livers is in acquisition for later analysis. Regretfully, this data could not be acquired within the timescale of this thesis.

7.8 Conclusion

The ability to accurately assess hepatic steatosis in a point of care test has great value as a screening test. To date, there is no available option, so a number of modalities were explored in this study. Histopathological staining was performed from murine liver samples that had been fed either a control diet or a high-fat diet, . With ORO staining there was a clear difference in ORO uptake, visible even to the naked eye. Software-based image analysis was able to accurately identify ORO stained areas and therefore adequately score the level of steatosis. Although this was a reliable method of steatosis quantification, it is not possible to perform this prior to implantation and therefore does not provide any value as an aid to assessing transplantability of a liver. It did provide a gold-standard by which to compare potential point-of-care tests such as ORO colorimetry or triglyceride determination.

ORO colorimetry is a lab-based technique that could be adapted as a point-of-care test. It is relatively rapid to perform and easily repeatable. The results using the same mouse livers did also demonstrate a significant difference between the 2 groups. Correlation against automated image analysis of histopathological stained slides yielded a moderate correlation. In contrast, triglyceride determination would be more challenging to translate to a point-of-care test and yielded less significant results.

Our hepatocyte-like cell line demonstrated the effects of macrosteatosis on survivability in hypoxic conditions. Both the control group and the steatotic group showed a fall in viability when exposed to hypoxia, but the death rate was significantly higher in the macrosteatotic group of cells.

Finally, ORO colorimetry was performed on human liver biopsies and correlated to histopathological grading by a blinded liver histopathologist (our current clinical gold standard). Although there was some correlation between colorimetric absorbance and pathological grading of steatosis, this was not significant. However, a high colorimetric absorbance had good positive predictive value for severe steatosis, even though the negative predictive value was poor. In our series of perfused discarded livers, one liver had been declined from clinical use by the transplanting surgeon due to visual appearance of steatosis, but both colorimetric assessment and then pathological grading confirmed there was only mild steatosis, further reinforcing the need for accurate point-of-care testing.

Chapter 8. Pregnane-X Receptor and Utilisation of Perfusion as a Platform for Drug Delivery

8.1 Introduction

The increases in the liver transplant waiting list, against a largely static donor pool, and subsequent increased use of DCD donors have been associated with increased incidence of ischaemic-type biliary lesions (ITBL)[35, 132, 133]. Ischaemia and the inflammatory cascade subsequent to reperfusion are thought to play a major role in the pathogenesis of these lesions, which usually present as biliary strictures several months after transplantation[133]. DCD livers are subject to increased ischaemia reperfusion injury secondary to long primary ischaemic warm times.

Pregnane-X receptor (PXR) is a member of the nuclear receptor superfamily of ligand-activated transcription factors[99] and is a potential target for the treatment of inflammatory liver disease[93, 98, 134-136]. PXR activation promotes the metabolism, transport and excretion of toxic compounds through induction of the CYP3A subfamily of cytochrome P450[137, 138]. PXR activation plays a key role in regulating metabolism [139], fibrosis[103], inflammatory response [140], cell proliferation [141], glucose, cholesterol and fat metabolism [142], bilirubin homeostasis[143] and endocrine homeostasis [144]. Its use as a drug target has been postulated in the treatment of inflammatory bowel diseases[145-148] among other diseases.

Furthermore, PXR activation has been shown to significantly reduce NF-κB-induced peri-portal inflammation and fibrosis as well as directly inhibiting inflammatory responses in hepatocytes[149].

There has been some research linking fatty liver disease to PXR as well as other ligand-activated nuclear receptors such as proliferator-activated receptor-α (PPARα) and constitutive androstane receptor (CAR)[150]. Ligand-activated nuclear receptors, including PXR are critical for nutrient/energy homeostasis through the gut-liver-adipose axis. Nuclear receptors are ligand-activated transcription factors regulating the expression of several genes through direct modulation of the transcriptional activities and epigenetic changes[150]. These nuclear receptors are subclassified into 7 groups (NR0 to NR6)[151]. The NR1 family (of which PXR is a member) is closely associated with energy/nutrient control in the liver, and therefore may play a vital role in hepatic steatosis regulation. Notably, Spruiell found that in mice, PXR elevated energy metabolism and energy levels, but severely impaired glucose tolerance[152]. Recent studies have also identified a self-detoxification role to PXR[153]. PXR as a xenobiotic sensor could trigger self-detoxification in the liver[103, 154], which is supported by the role of PXR in liver injury [155, 156].

There is also increasing evidence supporting a link between PXR expression and cholestatic liver injury, suggesting new paradigms in the potential treatments for hepatic cholestasis [157, 158]. After bile-duct ligation, PXR-knockout mice exhibit more hepatic damage than wild-type mice[136], and PXR activation reduced liver injury in wild-type mice, but not knock-out mice[137]. In addition, rifampicin (a potent PXR activator) has been shown to improve liver function tests in patients with primary biliary cholangitis [159].

8.2 Rodent Ischaemia-Reperfusion Model

Our group has previously studied the effects of PXR on ischaemia-reperfusion injury in a rat model and found that sustained isoprostane E2 elevation, inflammation and fibrosis are all reduced by PXR activation. This was performed by selectively clamping the lobar vessels supplying the left and middle hepatic lobes for 90 minutes. Animals were kept alive for up to 28 days post reperfusion, at which point under terminal anaesthesia, blood, bile and liver lobes were retrieved [106]. In this study, rats that were administered a rodent-specific PXR activator (Pregnenolone-16α-carbonitrile, PCN) demonstrated significant reductions in cholestasis, hepatic injury, ischaemic lobe isoprostane E-2 levels, peri-portal inflammation, and fibrosis [106].

In order to verify the target of benefit to IRI in rodents we aimed to compare the effects of PXR status and activation on IRI in mice. We hypothesized that PXR activation will be beneficial to IRI, and that this benefit will be lost in PXR-knock-out mice.

8.2.1 Materials and Methods

Pregnelone-16α-carbonitrile (PCN) is a rodent-specific PXR activator, which was purchased from Sigma Aldrich (St. Louis, MI, USA). All other drugs and anaesthetic agents were obtained through the Comparative Biology Centre at Newcastle University. Genotyping primers were obtained from Sigma Aldrich (St. Louis, MI, USA).

SJL/J mice were re-derived by the MRC Harwell from stock archived by the group [103] with or without a deletion in the PXR gene (see Appendix 1, http://www.mousebook.org/stocks/sjl129b6-nr1i2h-0) and selectively bred. Genotyping was then performed for confirmation.

8.2.1.1 Genotyping

An ear notch was obtained from mice and DNA purification performed from this as per protocol below.

8.2.1.1.1 DNA Purification

- Add 200 µL genomic prep buffer (50mM Tris-HCl, pH 8.0, 100mM NaCl, 10mM EDTA, 0.5% NP-40) to pellet
- Add 20 µL 20 mg/ml proteinase K solution
- Incubate overnight at 55°C
- Allow to cool and add 50 µg RNAaseA
- Incubate at room temperature for 20 minutes
- Add 200 µL phenol/choloroform/isoamyl alcohol (25/24/1 v/v/v)
- Vortex and centrifuge for 5 minutes

- Keep upper aqueous layer
- Add 1 µL Glycogen
- Add 1/10th volume (i.e. 20 µL) 3M Sodium acetate pH 5.2
- Add 2x volume (400 µL) 100% ethanol (stored at -20°C)
- Place on dry ice for 20 minutes
- Centrifuge at 13000rpm for 10 minutes at 4°C, discard supernatant
- Wash pellet with 70% ethanol (stored at -20°C)
- Place on dry ice for 20 minutes
- Centrifuge at 13000rpm for 10 minutes at 4°C
- Air dry
- Resuspend in water

8.2.1.1.2 PXR +/+ Genotyping

Set up a mastermix:

	FOR 10 SAMPLES
x10 Taq buffer (w/out $CaCl_2$)	40
25mM $MgCl_2$	50
dNTPs	8
Pharmacy grade H_2O	217
US primer PXR-ko+1 GCTGTACCACACCCCTCAACCC (10nmoles/ul)	40
DS primer PXR-ko-1 AGACTCCAGTGGATCCCCCACACCTAT (10nmoles/ul)	40
Taq (added just before use - keep in freeze block)	8
Volume Mastermix	40 µL
Volume of Sample	2 µL

Table 22 - PXR +/+ genotyping mastermix composition

PCR Block conditions:

95°C	2mins						
95°C	1mins	⟶	60°C	1mins	⟶	73°C	1mins (35 cycles)
73°C	8mins						

PCR product size = 204bp

RUN ON A 1.5 - 1.8% Agarose Gel

The mouse Wild Type PXR Gene

cccagcgaactccgaaaacccatcttctgggtcctgaagtgaaatggaaa
tttaagcagaggacctcccaccagtggcccaccactatctgggactcagt
ctcatcccataaagtggcctgacatgtaagaactttgtcaaatctttctc
aggagacaagccagtcctg**ctggtcatcactgtt**gctgtaccacacccct
caacccagctacggtttccttctcccagcatgggatccttgggaagttc
tcactaggatccattgtttcagtagtctgacagttagactgagaaacact
agtgtctgtttagaacaccttcttgtccacccagcccattacactaccac
tgtatatgcacataggtgtgggggatccactggagtctgggtggaccacc
agtagaacaatctctccatcaacagccaccaactgtcaatagctcctcag
ttaaaaaaaataggagcttgtcactcatttatttttggaactctagaat
aacttgtcctatgctgctttaaacacaaaatgacataaaaacaattatac

8.2.1.1.3 PXR -/- Genotyping

Set up a mastermix:

	FOR 10 SAMPLES
x10 Taq buffer (w/out $CaCl_2$)	40
25mM $MgCl_2$	40
dNTPs	8
Pharmacy grade H_2O	227
US primer PXR-F1 CTGGTCATCACTGTTGCTGTACCA (10nmoles/ul)	40
DS primer PXR-R3 CTAAAGCGCATGCTCCAGACTGC (10nmoles/ul)	40

Taq (added just before use - keep in freeze block)	8
Volume Mastermix	40 µL
Volume of Sample	2 µL

Table 23 - PXR -/- genotyping mastermix composition

PCR Block conditions:

95°C 2mins

95°C 1mins ⟶ 59°C 1mins ⟶ 73°C 1mins (35 cycles)

73°C 8mins

PCR product size = 265bp

RUN ON A 1.5 - 1.8% Agarose Gel

The mouse knock out gene

cccagcgaactccgaaaacccatcttctgggtcctgaagtgaaatggaaa
tttaagcagaggacctcccaccagtggcccaccactatctgggactcagt
ctcatcccataaagtggcctgacatgtaagaactttgtcaaatctttctc
aggagacaagccagtcctg**ctggtcatcactgttgctgtacc**ac.....

..... GCAGTCTGGAGCATGCGCTTTAG ..neo gene

8.2.1.3 Ischaemia-Reperfusion Injury Protocol

All mice were weighed 2 days pre-procedure and administered either PCN or vehicle control via intraperitoneal route once daily for 2 days prior to ischaemic insult.

On the operative day, they were weighed again. Any that had suffered significant weight loss or showed signs of distress prior to surgery were culled by schedule 1 at this point.

Mice were administered ketamine/medetomidine 10 µL/g as induction anaesthetic. Once asleep, were shaved for laparotomy and administered morphine 10 µL/g and long-acting amoxicillin (Clamoxyl™ L.A.) via subcutaneous route. Eye ointment was applied.

Maintenance anaesthetic was achieved by isofluorane 0.5-1% given through a face mask. On completion of laparotomy, atipamezole 5.5 µL/g was given for reversal.

Chlorhexidine 0.5% was used for skin preparation.

Whilst under anaesthesia, mice were kept on a heat mat and approximately 1cm midline laparotomy was performed. The porta hepatis was identified and isolated. A Pringle manoeuvre was performed using a 5mm x 0.8mm micro-vascular clamp applying 10g pressure (World Precision Instruments, Hertfordshire, UK). This was kept in situ for 30 minutes. At the end of the ischaemic period, the clamp was removed and the laparotomy

closed in 2 layers. Interrupted 6-0 Vicryl® on TF taper-point round-bodied needle was used to close the muscle layer and skin was closed with a continuous subcutaneous 5-0 undyed Vicryl Rapide® on a P-1 11mm reverse-cutting.

Mice were recovered in a warmed cabinet for 24 hours and given buprenorphine 2-5 µL/g 4-6 hourly as required for 24 hours.

Mice were kept in individual cages and monitored regularly for up to 7 days at which point they were culled by schedule 1 and their liver retrieved.

If at any time prior to the end-point the mice showed signs of distress exceeding moderate severity they were culled by schedule 1.

8.2.1.4 Confirmation of PXR activation

Liver biopsies obtained from mice at the experiment end point were used for confirmation of PXR target gene induction. PCR was performed on liver tissue samples for induction of CYP3A11, the rodent PXR-regulated gene.

PCR was carried out as per standard protocol.

8.2.2 Results

8.2.2.1 Genotyping

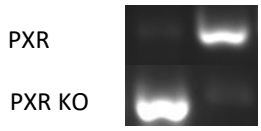


Figure 82 – RT-PCR for indicated transcripts in PXR genotyping

Genotyping was performed by ear-notch samples, and successfully demonstrated a clear and expected difference in the two groups.

8.2.2.2 Ischaemia-Reperfusion Injury Survival to 7 days

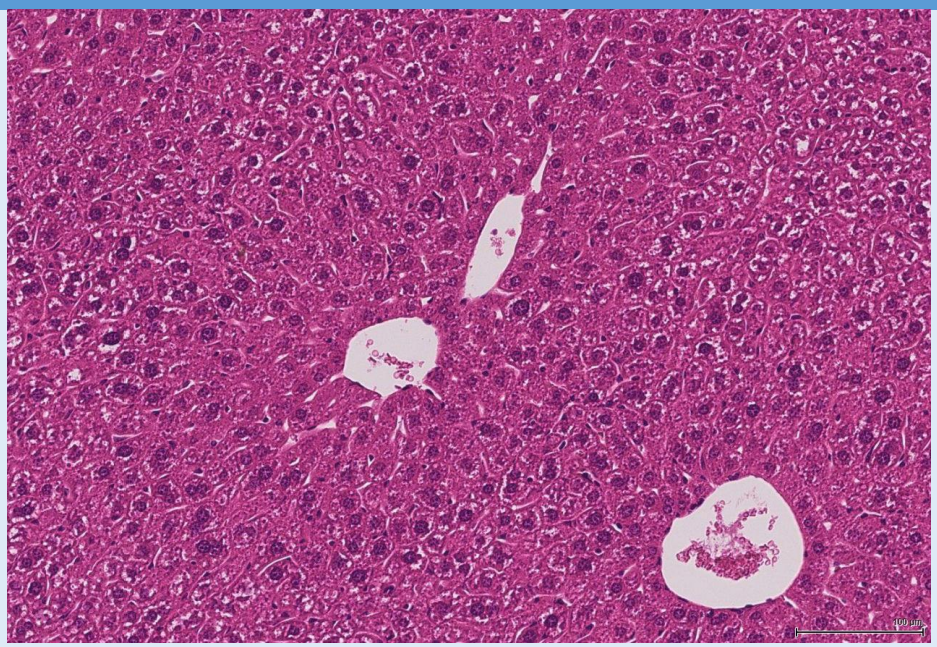
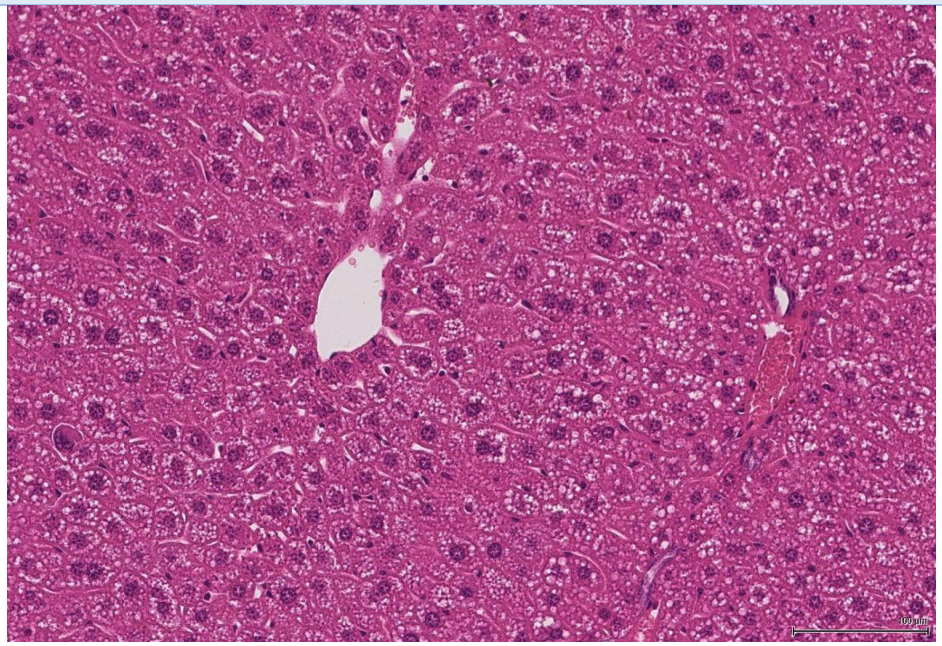
Group	Survived to 7 days	Schedule 1 within 7 days	7-day mortality (%)	*p*
Wild Type Vehicle	3	20	87	0.02
Wild Type PCN	7	8	53.3	
Knock-out Vehicle	8	5	38.5	0.56
Knock-out PCN	7	7	50	

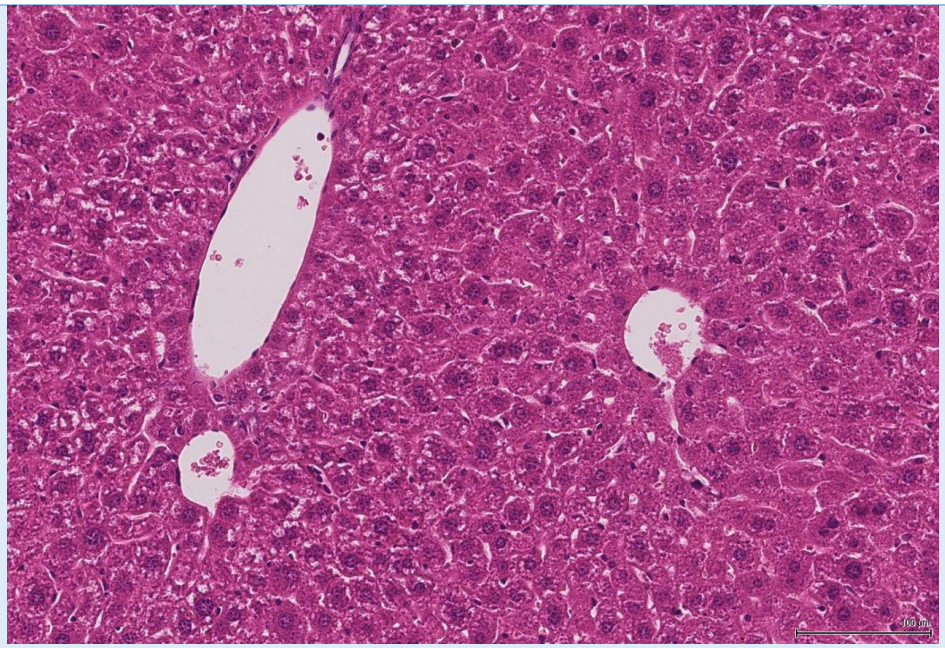
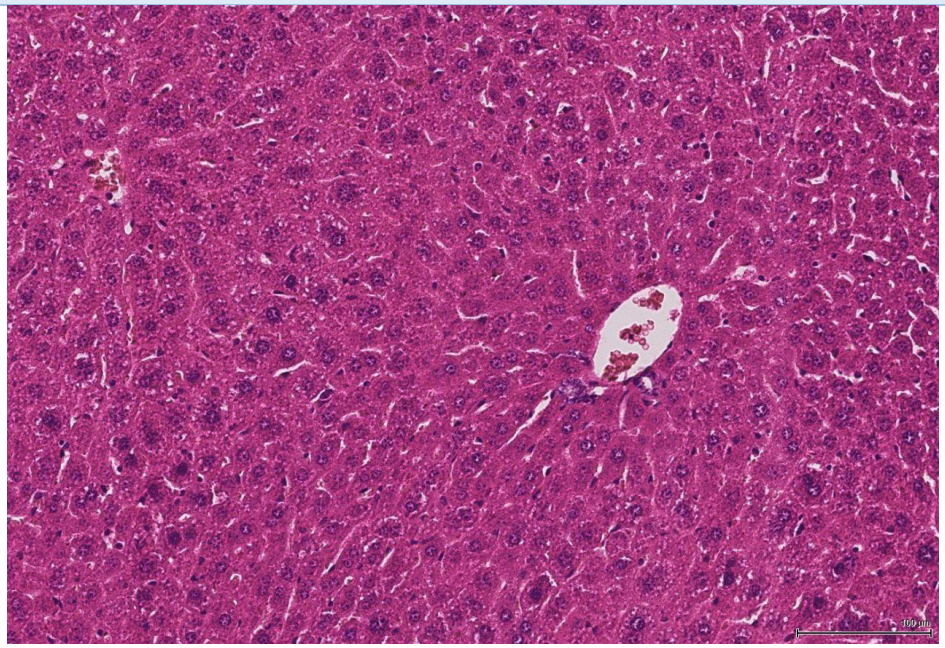
Table 24 - Mouse survival following ischaemia-reperfusion injury

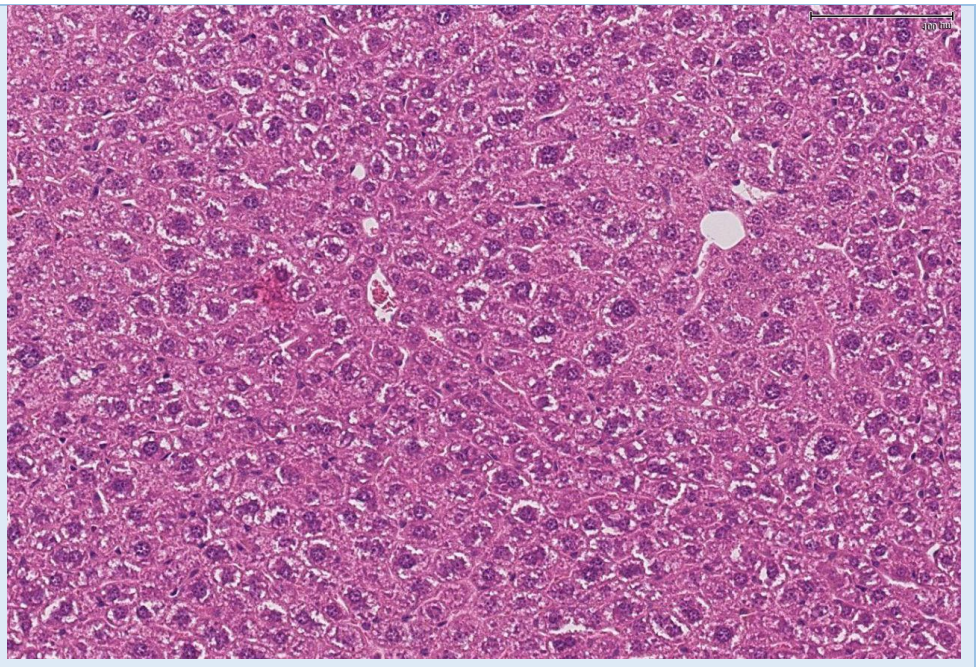
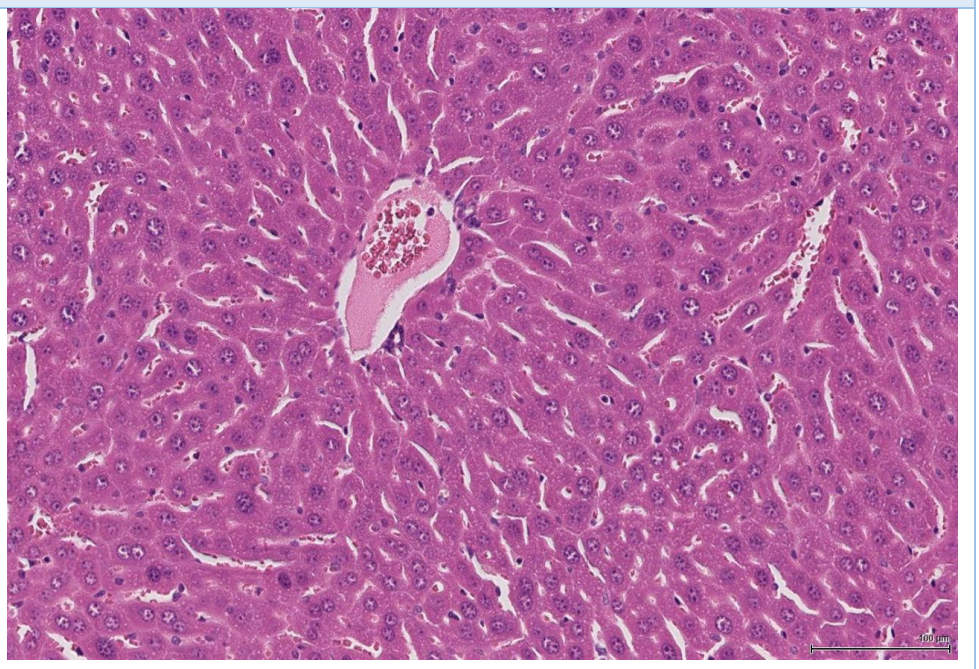
A total of 65 mice underwent laparotomy and ischaemia-reperfusion, of which 38 were wild type and 27 were knock-out. We aimed to achieve n=7 that survived to 7 days in each group. However, poor survival in the wild-type vehicle group precluded this.

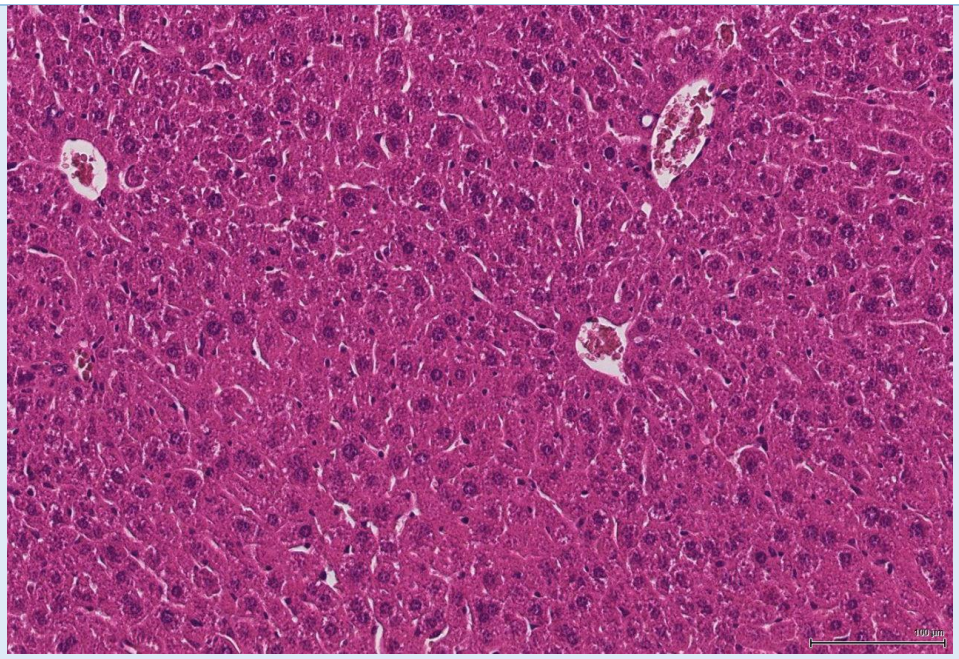
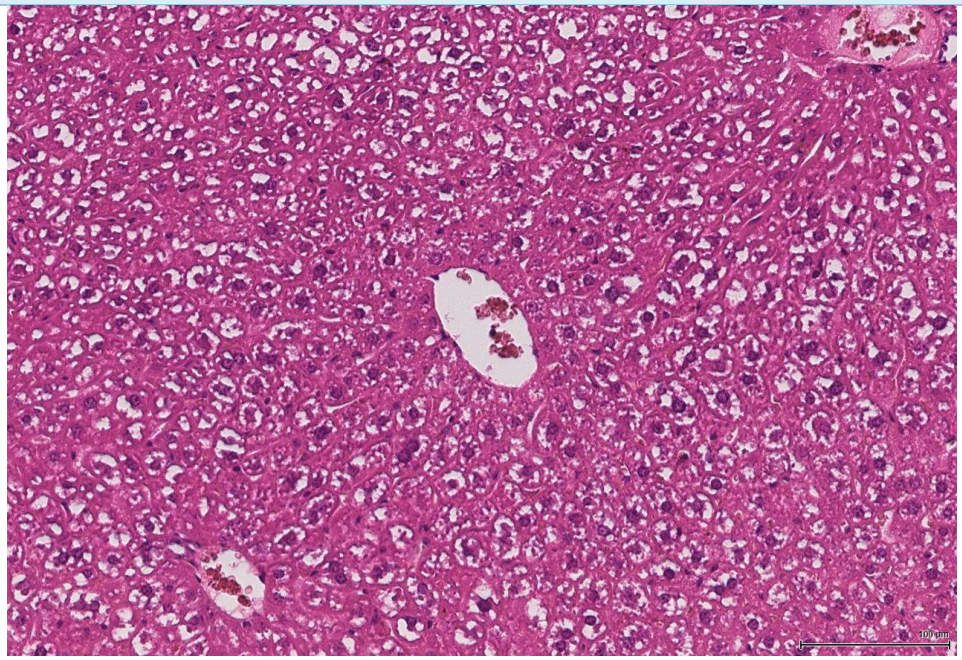
In the wild-type mice, survival to 7 days was significantly better in mice that received PCN compared with vehicle control. This PXR benefit was then lost in the knock-out mice. However, overall survival was better in knock-out mice, this was unexpected and is not fully explained. It is possible that other potentially detrimental effects of PXR are also being blocked.

The majority of deaths were due to culling by schedule 1 due to unsurvivable injury post-surgery. Almost all of these were within the first 24 hours post-operatively.

	Group	
Sham Surgery	Wild Type Vehicle	
	Wild Type PCN	

	Knock-out Vehicle	
	Knock-out PCN	

Hepatic IRI	Wild Type Vehicle	
	Wild Type PCN	

	Knock-out Vehicle	
	Knock-out PCN	

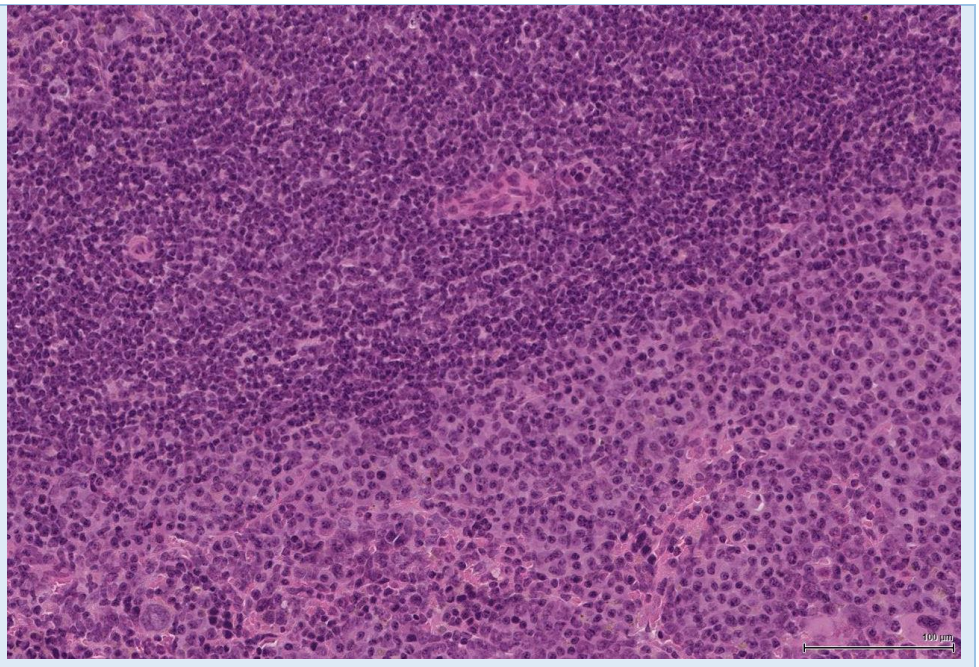
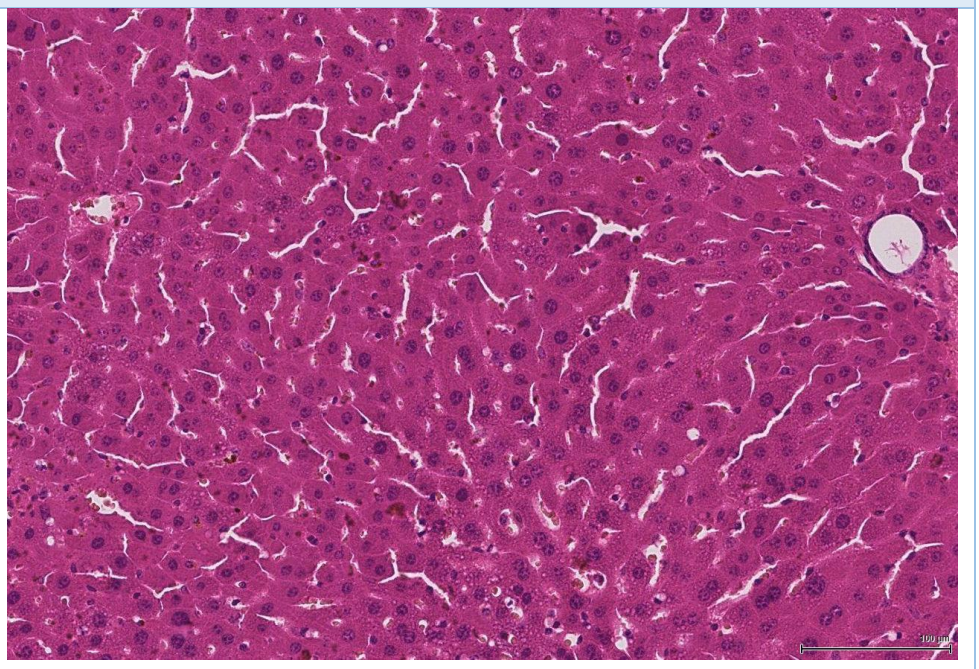
Died	Wild Type Vehicle	
	Wild Type PCN	

Figure 83 - Histopathology of mouse livers stained in H&E in each group

Histological analysis of the livers retrieved from mice at the end of the study show that the sham surgery group, as expected do not show significant pathology within their livers. The knock-out mice do show a higher rate of cellular injury, and this may account for their higher mortality rate (independent of PCN). The livers subjected to ischaemic reperfusion injury, demonstrate higher levels of inflammation and cellular injury. There is no histological evidence of PCN being protective against IRI. Analysis of livers of mice that sustained unsurvivable injuries show widespread inflammation and hepatocellular death.

8.2.2.3 Liver enzyme analysis

8.2.2.3.1 ALT

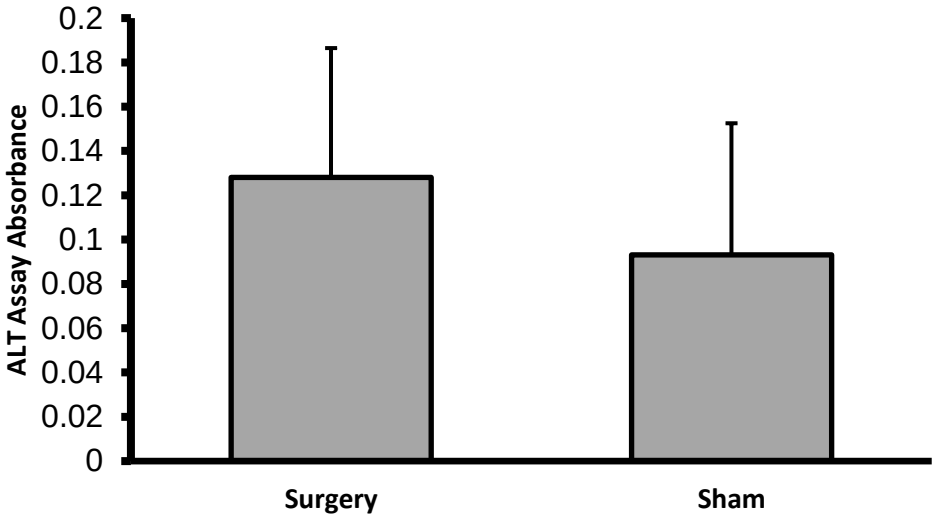


Figure 84 - Tail vein bleed serum ALT levels on first post-operative day. Showing mean +/- S.D.

A small rise in day 1 ALT was observed in the surgery group when compared with the sham surgery group, although this failed to reach statistical significance. It is likely our numbers were insufficient to demonstrate this difference.

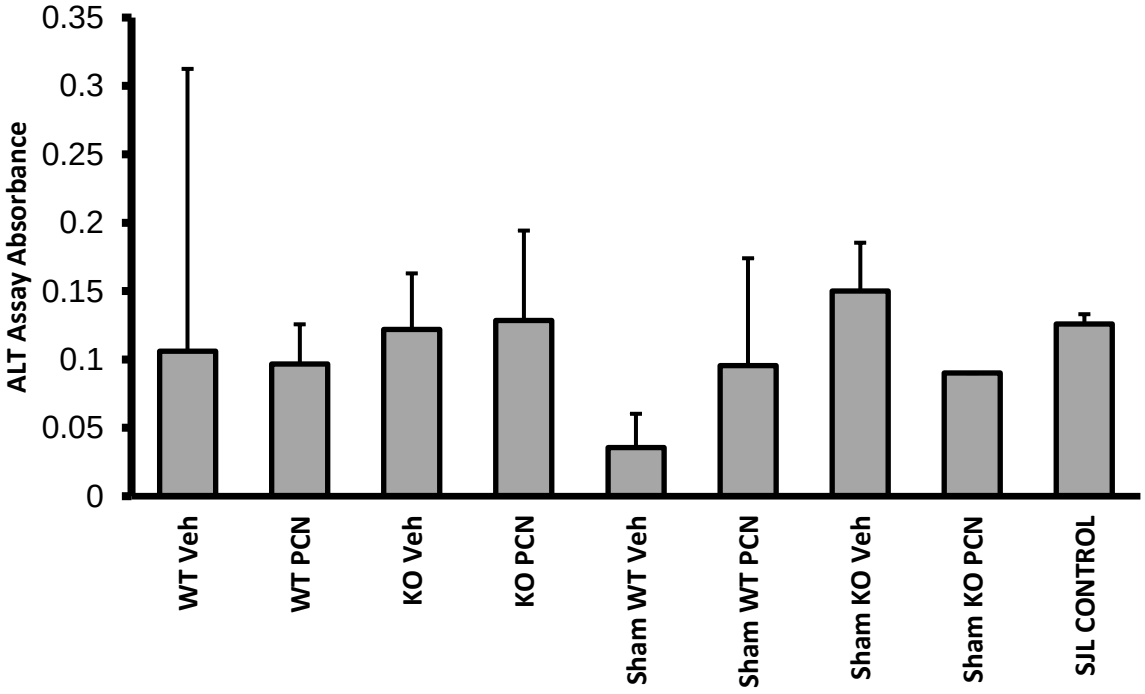


Figure 85 - Tail vein bleed serum ALT levels in each study group, showing mean +/- S.D. , WT = Wild Type, Veh = Vehicle, KO = Knock-out

8.2.2.3.2 ALP

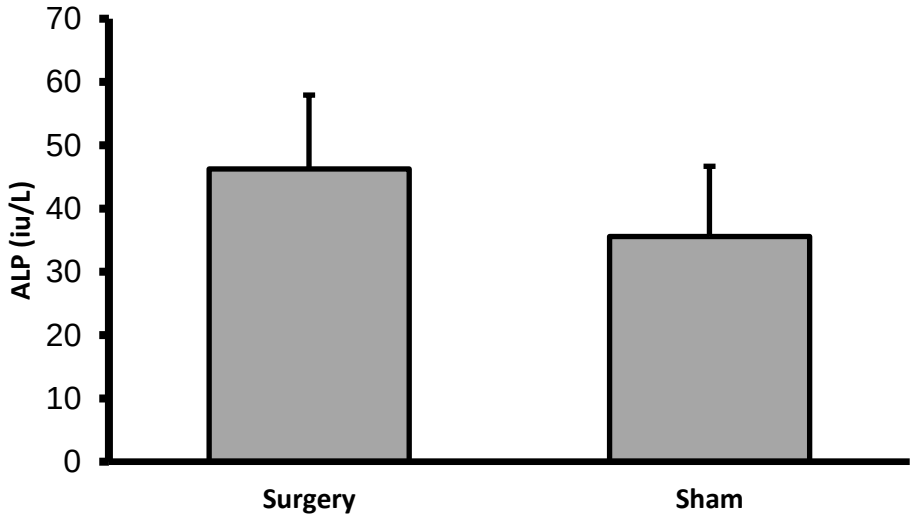


Figure 86 - Tail vein bleed alkaline phosphatase (ALP) levels on first post-operative day. Showing mean +/- S.D.

A small rise in day 1 ALP was observed in the surgery group when compared with the sham surgery group, although this failed to reach statistical significance. It is likely our numbers were insufficient to demonstrate this difference.

8.2.2.3 PXR Activation

Liver samples retrieved after schedule 1 were used for PCR. PCR demonstrates that in wild-type mice that were treated with PCN, CYP3A11 was induced, but not in those that received vehicle controls and knock-out mice did not demonstrate CYP3A11 induction regardless of whether treated with PCN or vehicle control. This confirms that PXR was activated as intended.

8.2.3 Discussion

The surgical and anaesthetic technique proved to be effective. There was a slight rise in serum ALT and ALP in the mice that had IRI compared with the sham surgery group, although our numbers were insufficient to demonstrate statistical significance.

Within the wild-type mouse group, there was a significantly improved survival to 7 days in mice that received a PXR activator compared to the vehicle control and this benefit was lost in mice that had the PXR gene knocked out. The cause for improved overall survival in knock-out mice is not fully understood, but other potential targets of PXR may be responsible such as anti-inflammatory actions.

Overall, mortality (particularly in wild-type vehicle group) was higher than expected although this did not exceed severity as mice were culled when unsurvivable injury had occurred. It may be that in hindsight, 30 minutes of total liver ischaemia was an excessive insult on the mice and this may have influenced our results.

Our results support to some extent the findings of Amer *et al*. [106] that PCN is beneficial to rodents sustaining liver ischaemia-reperfusion injury. As the survival benefit was lost in the knock-out mice this could suggest that

the effects are dependent on the PXR. However, given the high mortality rate and the unexplained increase in mortality in the knock-out mice, this would need to be investigated further.

Our next step was to verify the actions of PXR on human hepatocytes prior to introducing its use on human livers.

8.3 PXR activation in Human Hepatocytes

8.3.1 Introduction

Our results in mouse ischaemia-reperfusion as well as our group's previous results in a rat model have supported the beneficial role of PXR activation in livers undergoing IRI. Prior to translation to a whole-liver model, we aimed to demonstrate target activation in human hepatocytes in-vitro.

The Cytochrome 3A subfamily of P450 monooxygenases (CYP3A) account for, on average, 50% of the total P450 in the human liver, and participate in the metabolism of up to 50% of drugs currently in use[160]. Mostly, CYP3A4 is induced by activation of PXR and/or Constitutive Androstane Receptors (CAR). There are four CYP3A genes in humans – CYP3A4, CYP3A5, CYP3A7 and CYP3A43, although CYP3A7 is expressed in fetal livers [161, 162]
Demonstrating inducibility of CYP3A in human hepatocytes would support translation of PXR activation into human tissue. Trial PXR activation with different known PXR activators would also confirm the choice of drug and optimum dose. The objectives of this study were therefore to demonstrate safety at different doses and also efficacy in inducing CYP3A.

8.3.2 Hepatocyte Isolation and Culture

Whole human livers, that were deemed unsuitable for transplantation, and therefore offered for research were used for hepatocyte isolation. Livers underwent normothermic machine perfusion as per our standard NMP protocol to optimise them prior to hepatocyte isolation.

For successful hepatocyte isolation, the liver must be digested as quickly as possible in normothermic conditions to minimise cell death. The increased time required for digestion of a whole human liver leads to decreased cell yield. Therefore livers were split whilst on perfusion. Segments II & III were identified and dissected maintaining haemostasis as far as possible. The right hepatic artery and the segmental artery supplying segment I were both ligated, leaving the cannulated common hepatic artery in continuity with segments II & III for isolation. The same was performed on portal venous anatomy. The central and right hepatic veins were also ligated, allowing the left hepatic vein to continue draining into the cannulated IVC. After ligation of vessels, effectively an extended right hepatectomy with caudate was performed. This setup allows for fully cannulated perfusion of the left lateral segment of the liver in isolation.

The same circuit, components, heater-cooler and pump were then utilised for hepatocyte isolation. This is a setup that to our knowledge has not been attempted before for this purpose. The circuit was drained and washed with 2L NaCl 0.9%.

Maintaining the liver and perfusate at 37°C the following components were added in succession.

Perfusate 1

1000 mls Earle's Balanced Salt Solution (EBSS, without Ca^{2+} and Mg^{2+}) with ethylene glycol-bis(oxyethylenenitrilo)tetraacetic acid (EGTA) to final concentration of 0.5mM

Perfusate 2

1000 mls EDTA (without Ca^{2+} and Mg^{2+})

Perfusate 3

1000 mls EDTA (without Ca^{2+} and Mg^{2+}) with 1 ml iM $CaCl_2$ and 300 mg Collagenase A (added just before use)

Run perfusate 1 at total flow rate of ~150ml/min with effluent to waste then add perfusate 2 and continue same rate, also running to waste. Perfusate 3 is then added and recirculated for 25-30 minutes until the consistency of the liver becomes soft enough to be passed through a coarse sieve.

Decannulate the lateral segment of liver and break up into small pieces. Pass pieces through a coarse sieve then through a fine nylon filter into sterile container.

Centrifuge cells at 50g for 3 mins and resuspend gently in Williams' Medium E (WME) and check viability by trypan blue.

Hepatocytes are best cultured in WME. Initially this is supplemented with 1 µg/ml insulin (Sigma-Aldrich, I6634), x1 penicillin/streptomycin/glutamine (Thermo-Fisher Scientific, 10378016), 50 µg/ml Gentamycin (Thermo-Fisher Scientific 10570037) and 10% fetal calf serum (FCS) on collagen type-1 coated plates. After 2 hours, the medium is then replaced with the same formulation minus FCS. Cells were plated with a seeding density of 500,000 cells per well in a 6-well plate.

The hepatocyte cell yield from each left lateral segment isolated was between 1215 x 10^6 and 1560 x 10^6 cells, in the 3 livers isolated.

8.3.3 Results

Plated human hepatocytes were cultured in medium for 24 hours then treated with known cytochrome P450 activators (CYP) for a further 24 hours before harvest. Avasimibe, Rifampicin and Phenobarbitone are known potent CYP activators that act through the PXR receptor. Whereas beta-naphthoflavone (BNF) is a known CYP activator that acts on genes regulated by Aryl hydrocarbon receptors (AhR), e.g. CYP1A1.

After cell harvest, RT-qPCR was performed as per methods and fold change in CYP3A4, CYP3A5, CYP3A43 and CYP1A1 were noted.

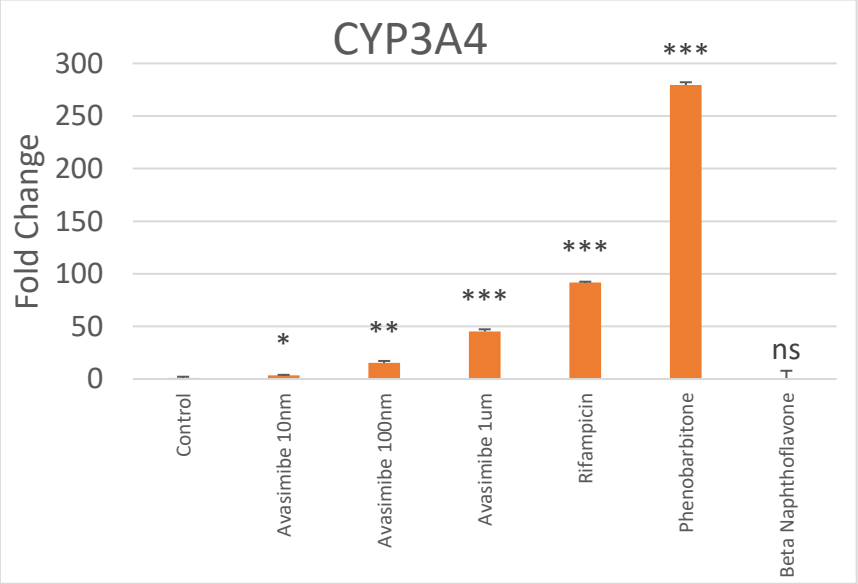


Figure 87 - CYP3A4 upregulation in human hepatocyte cell culture Showing mean +/- S.D.

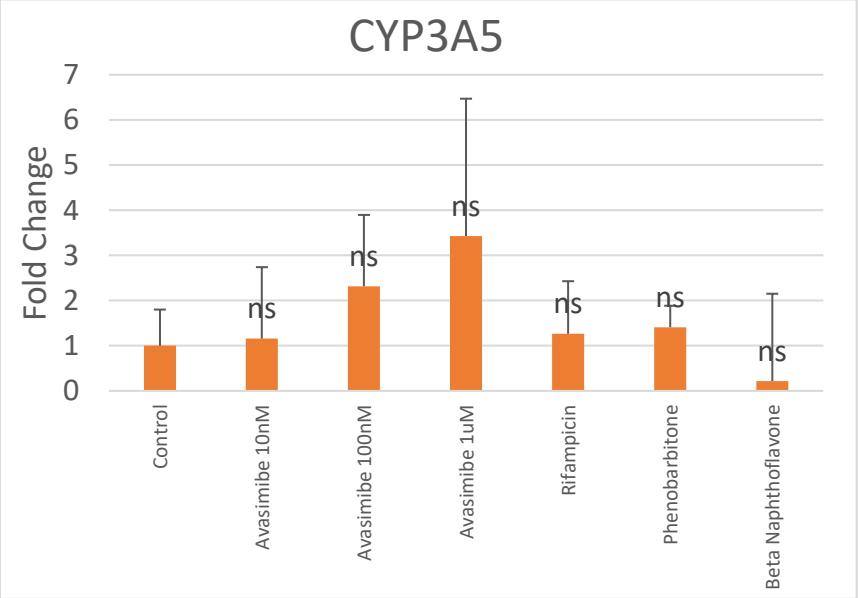


Figure 88 - CYP3A5 upregulation in human hepatocyte cell culture. Showing mean +/- S.D.

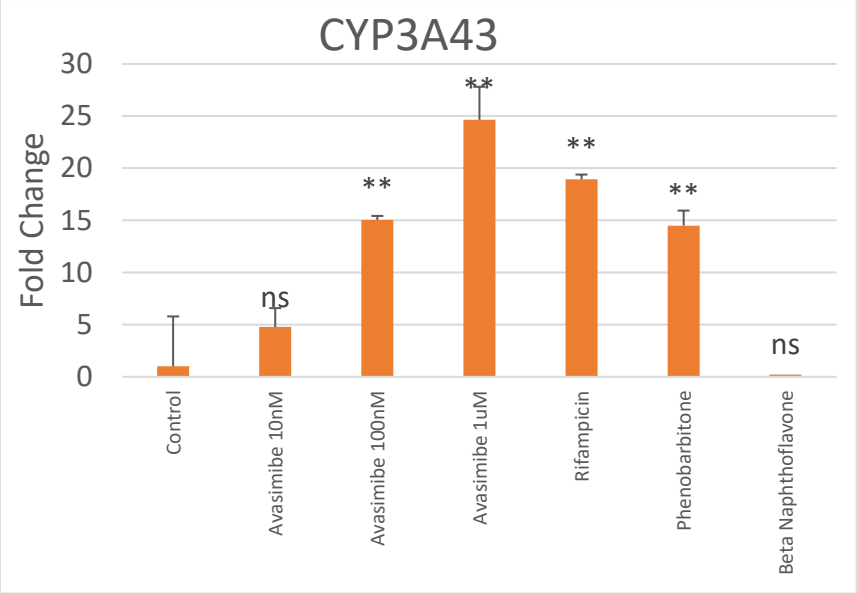


Figure 89 - CYP3A43 upregulation in human hepatocyte cell culture. Showing mean +/- S.D.

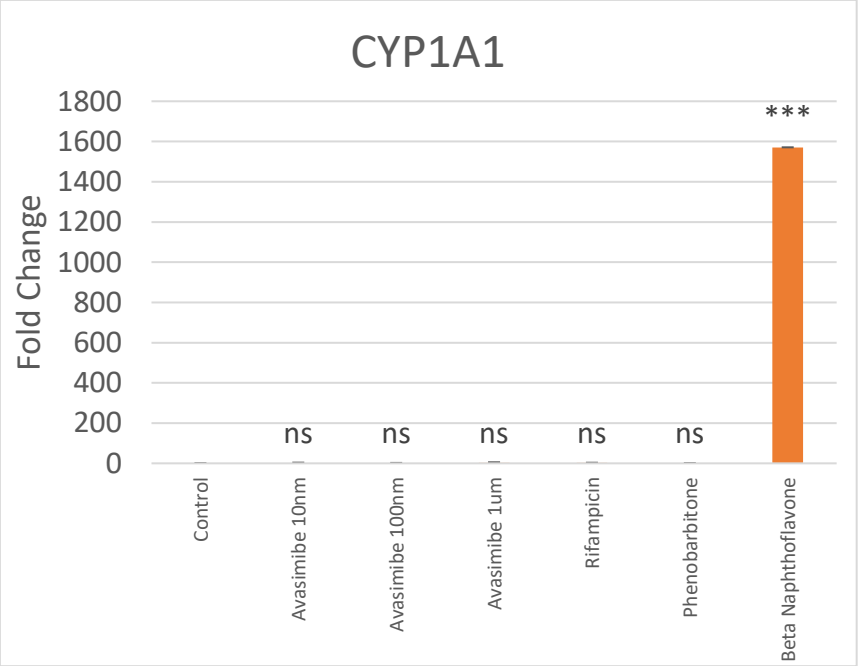


Figure 90 - CYP1A1 upregulation in human hepatocyte cell culture. Showing mean +/- S.D.

There is a consistent dose response induction for Avasimibe in expression PXR-regulated genes (CYP3A4, CYP3A5 and CYP3A43), with no measurable effect on AhR-regulated genes (CYP1A1). There was also a consistent and similar response for Rifampicin and Phenobarbitone, both known PXR activators. CYP3A5 was much less inducible by all PXR activators than CYP3A4 and CYP3A43, and did not reach statistical significance. In contrast, BNF showed negligible activation of PXR-regulated genes and a >1500 fold change in CYP1A1.

8.3.4 Discussion

We have demonstrated a very successful novel process for hepatocyte isolation from human livers with very high hepatocyte yield from each left lateral segment. To our knowledge an ex-vivo perfusion circuit has not previously been utilised for this purpose.

Treatment with PXR activators demonstrated successful target activation. This was particularly evident in CYP3A4 and CYP3A43.

8.4 Treatment during Ex-vivo Perfusion

8.4.1 Introduction

Ex-vivo liver perfusion in itself has great potential to recondition livers prior to implantation and potentially transform untransplantable livers into livers that are suitable for transplantation and therefore increasing the utility of the existing donor pool. We have already demonstrated clinically some of the benefits of perfusion alone, however there is much greater untapped potential in ex-vivo perfusion- if it can be utilised as a platform for the delivery of active treatments that can further improve livers, either by treating pre-existing conditions such as steatosis or by delivering treatments that may be protective to the liver during the insults it sustains through the transplantation process.

Due to physiological metabolic activity in normothermic perfusion, this is likely to be the preferred perfusion method if active treatments are to be delivered, although there is some remaining metabolic activity even in hypothermia.

Our current results, as well as previous studies have demonstrated evidence in rodent models of liver IRI that PXR activation is protective against IRI. We therefore aimed to translate our cell-line and rodent experience to human ex-vivo liver perfusion.

8.4.2 Materials and Methods

Discarded livers that were offered for transplant were accepted for this purpose. 5 consecutive liver offers (subject to staff availability) were perfused in accordance with our NMP protocol with the addition of Avasimibe.

Avasimibe (Sigma-Aldrich, PZ0190) 3mg dissolved in 50 µL dimethyl Sulfoxide (DMSO) was added to the perfusate at the start of perfusion (after initial biopsy), to give a final concentration of approximately 2µmol. NMP was performed for 6 hours with standard perfusate sampling. The elimination half-life of avasimibe ranges from 15-24 hours therefore no additional doses were required during the perfusion period[163].

32mm core biopsies were obtained at the start and end of perfusion and immediately preserved in RNAlater. These tissue biopsies were used for analysis of inducibility of PXR-regulated CYP enzymes, namely CYP3A4, CYP3A5 and CYP3A43. This was analysed in accordance with standard RT-qPCR methods.

Perfusate samples were analysed for levels of lactate, ALT, and a multiplex array of cytokines.

8.4.2.1 Multiplex Analysis

Multiples analysis was performed on the MESO QuickPlex SQ120 multiplex analyser using the same method described previously in Section 6.6.1 Methods.

8.4.2.2 Alanine Transaminase

An ALT activity colorimetric assay kit (Abcam ab105134) was used for assessment of ALT levels in perfusate samples, using the same method described previously in Section 6.5.1 Methods.

8.4.2.5 Lactate

Perfusate lactate was measured in real-time from samples taken from the return limb of the circuit. Samples were analysed using a Siemens RapidPoint 500 blood gas analyser, that is routinely self-calibrated.

8.4.3 Results

8.4.3.1 Cytochrome P450 activation during ex-vivo perfusion

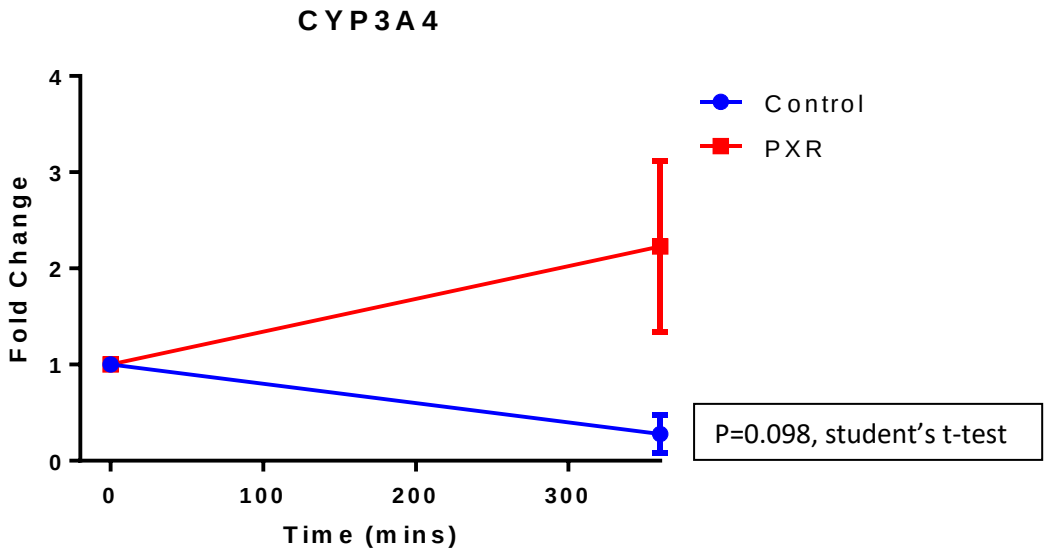


Figure 91 - CYP3A4 expression in control liver perfusions and livers with PXR-activator added to circuit Showing mean +/- S.D.

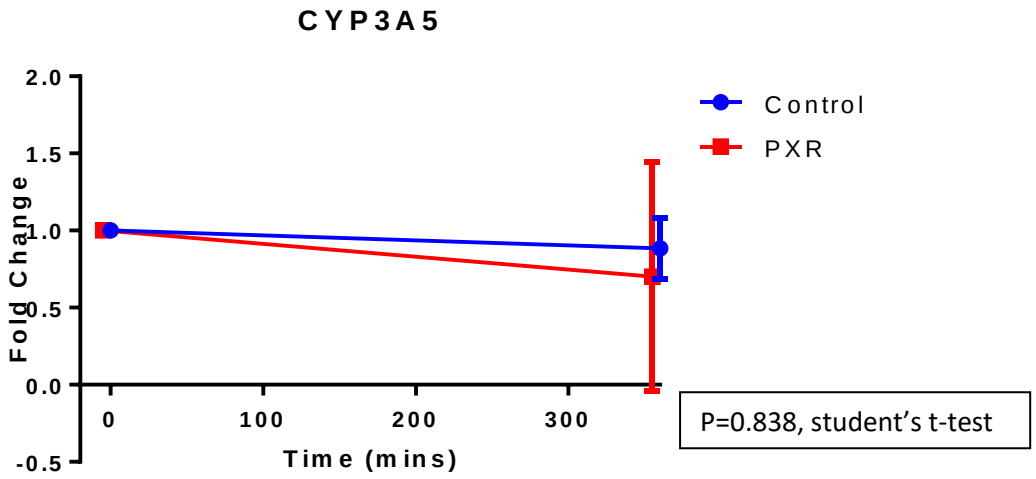


Figure 92 - CYP3A5 expression in control liver perfusions and livers with PXR-activator added to circuit. Showing mean +/- S.D.

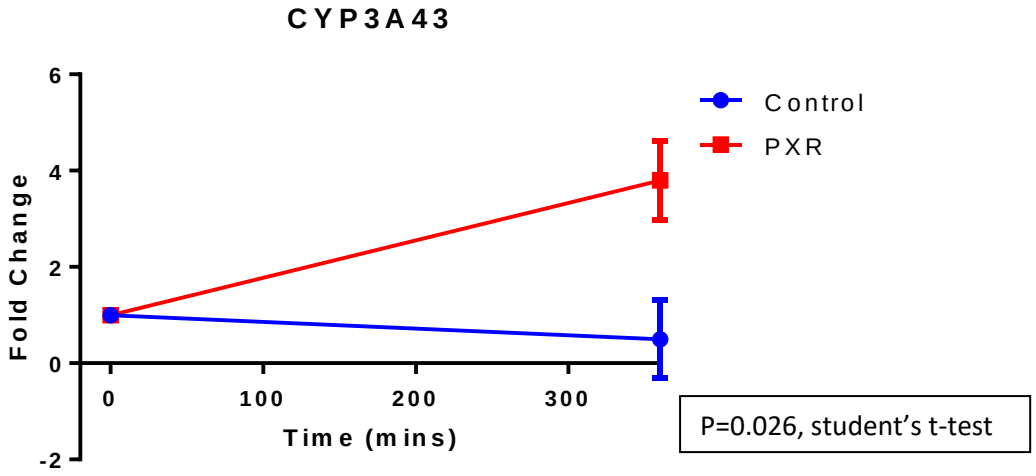
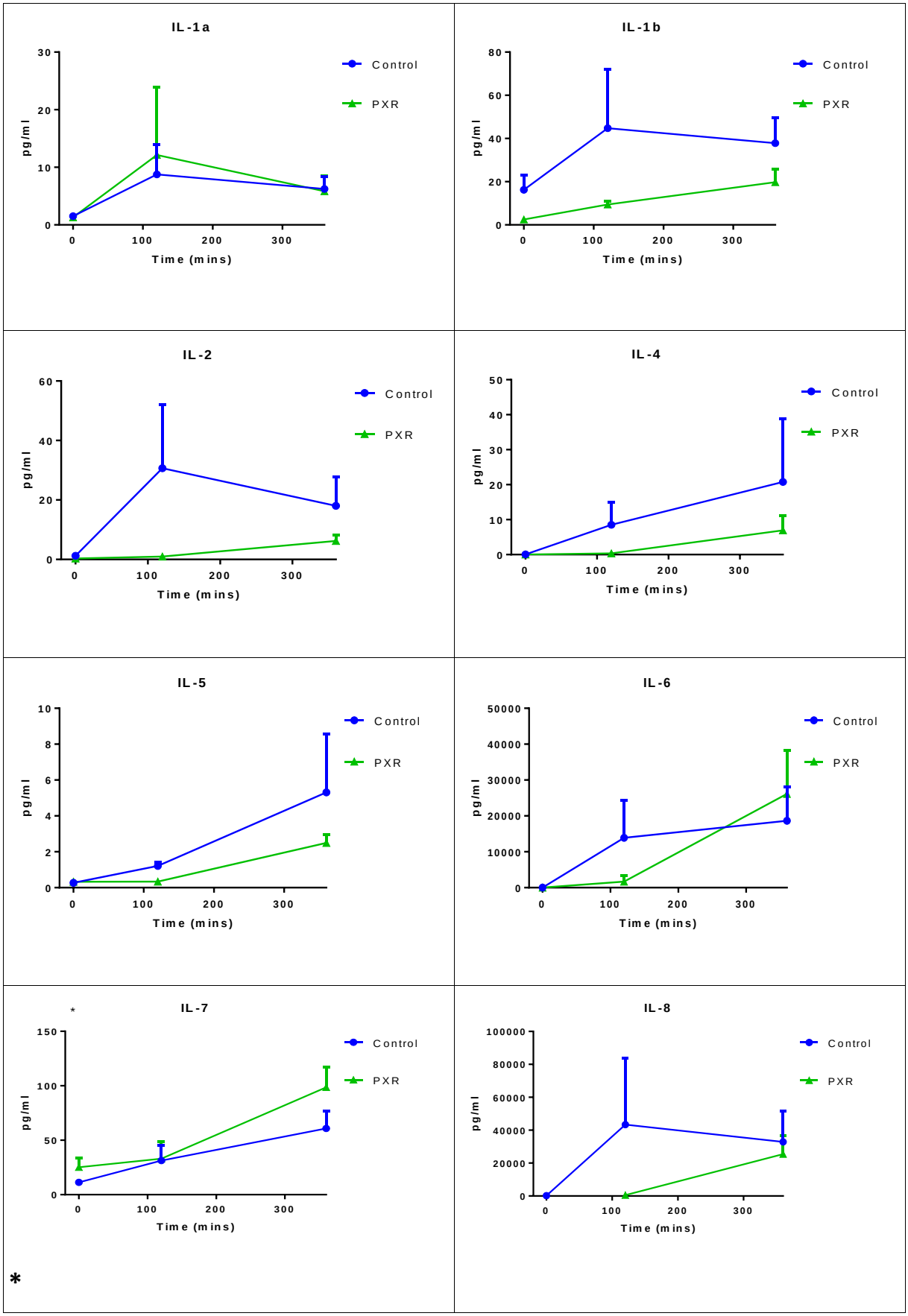


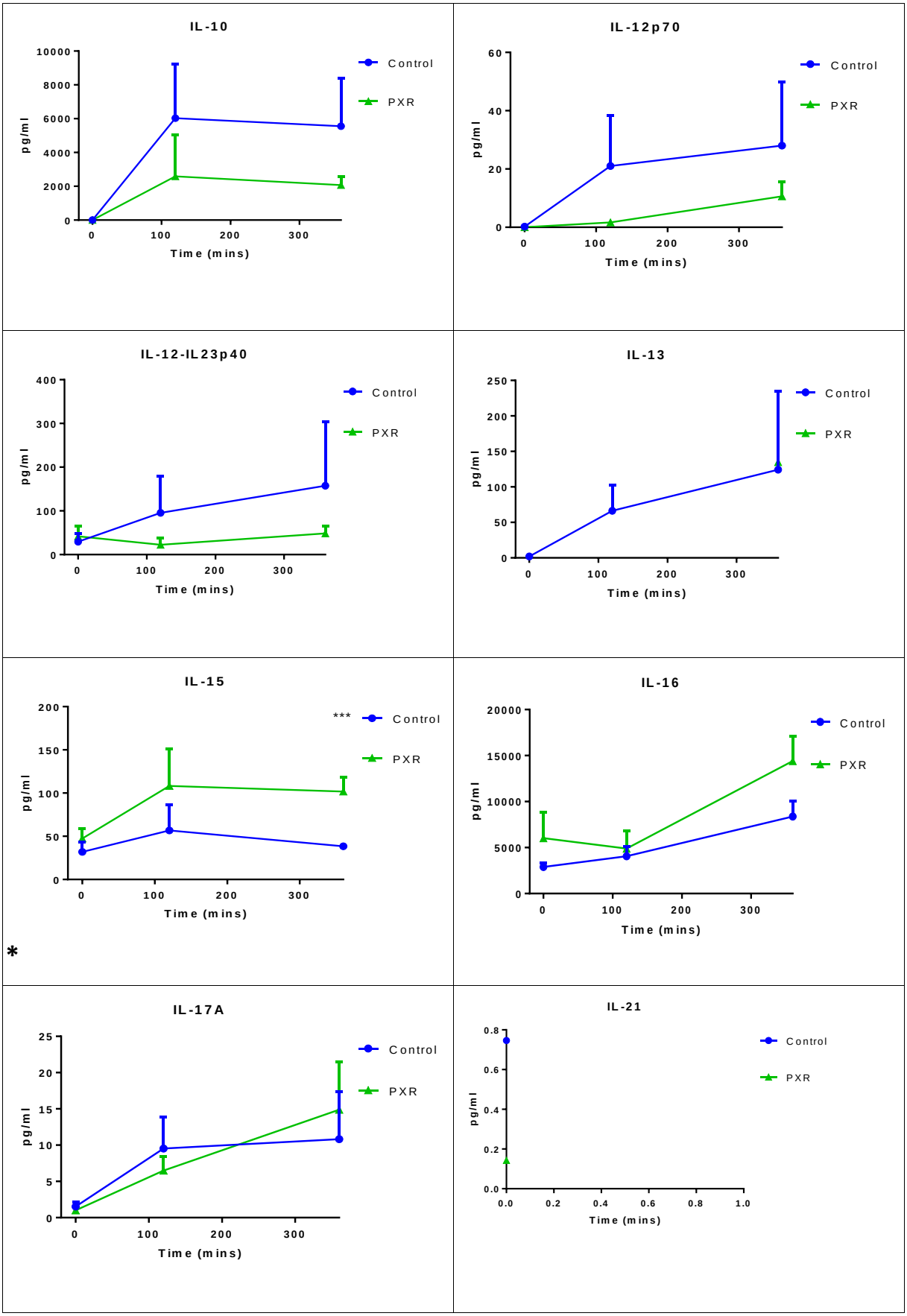
Figure 93 - CYP3A43 expression in control liver perfusions and livers with PXR-activator added to circuit. Showing mean +/- S.D.

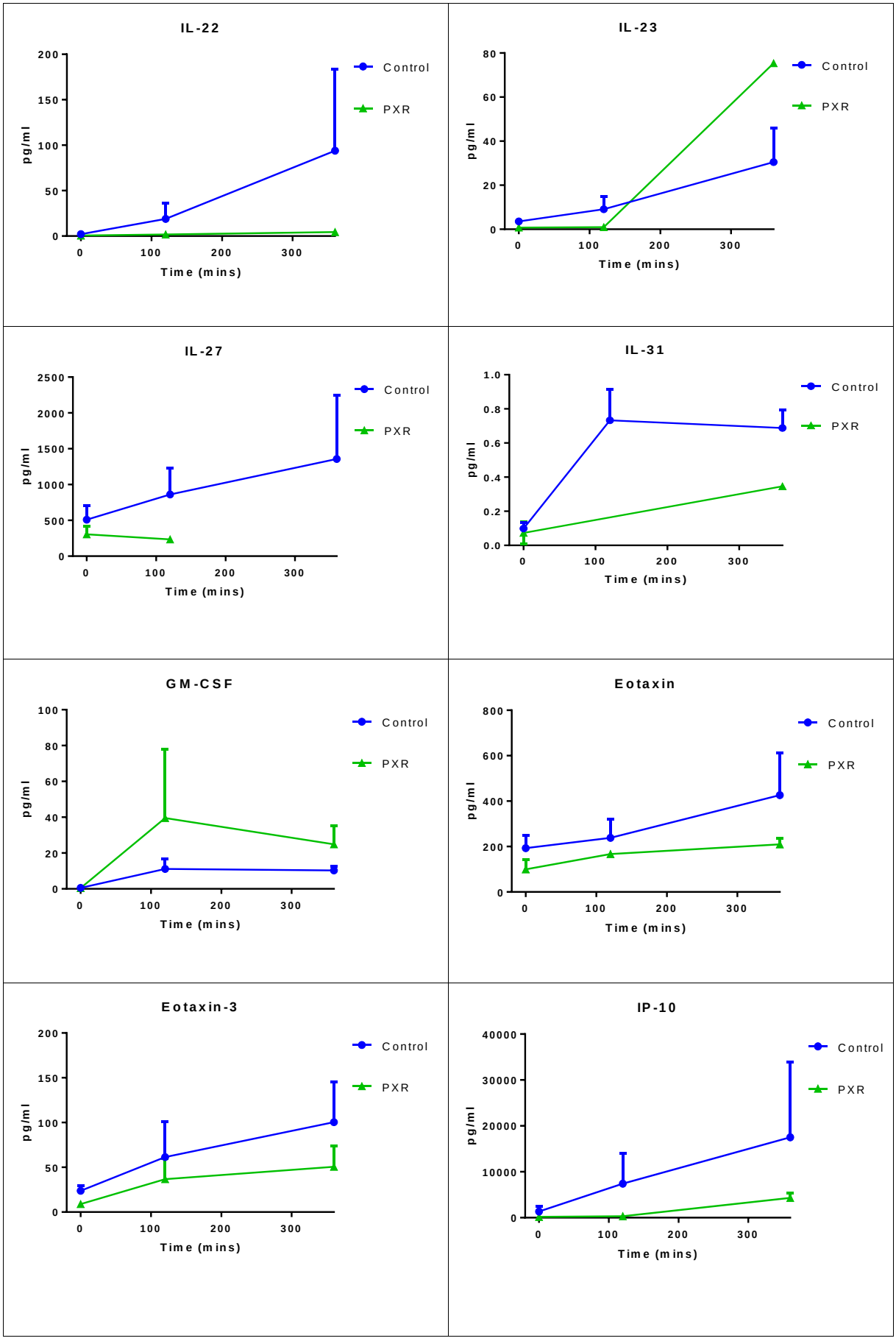
There is increased induction of CY3A4 and CYP3A43 over the course of perfusion by 2.2-fold and 3.8-fold respectively (p=0.098 and p=0.026 respectively, t-tests). However, CYP3A5 did not show inducibility by avasimibe in our ex-vivo perfusion model. This mirrors our results in-vitro to some extent, where CYP3A5 had a much lower fold-change than CYP3A4 and CYP3A43. This demonstrates that there is activation of the desired enzyme targets by introduction of a PXR activator to the perfusate during normothermic perfusion.

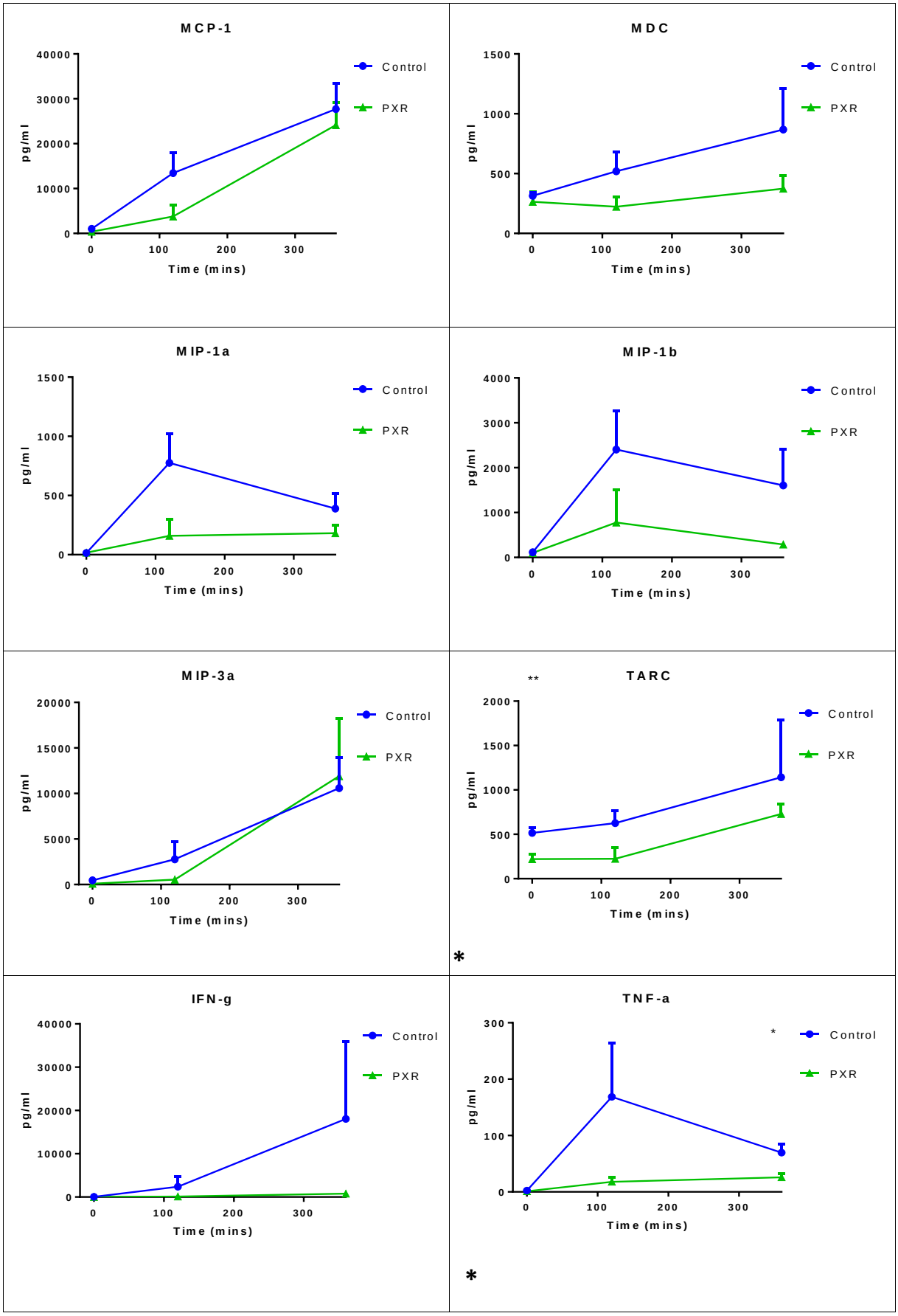
8.4.3.2 Perfusate Analysis

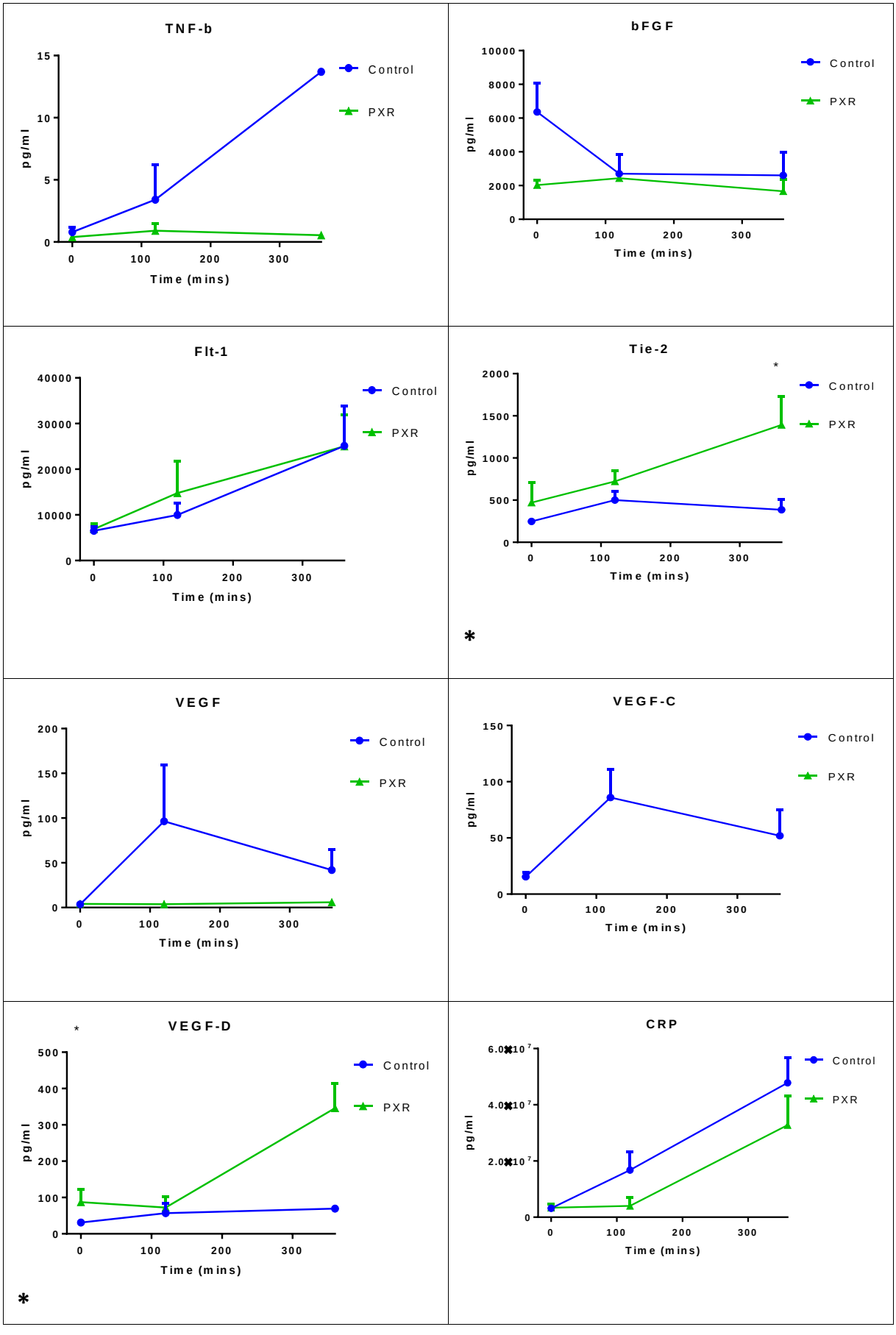
Perfusate samples from livers that were treated with Avasimibe were compared to a control group that underwent perfusion under the same protocol without the addition of Avasimibe.


IL-1a
pg/ml
Time (mins)
Control
PXR
IL-1b
IL-2
IL-4
IL-5
IL-6
IL-7
IL-8
*
*


IL-10
pg/ml
Time (mins)
Control
PXR
IL-12p70
IL-12-IL23p40
IL-13
IL-15
***
*
IL-16
IL-17A
IL-21


IL-22
pg/ml
200
150
100
50
0
0
100
200
300
Time (mins)
Control
PXR
IL-23
pg/ml
80
60
40
20
0
0
100
200
300
Time (mins)
Control
PXR
IL-27
pg/ml
2500
2000
1500
1000
500
0
0
100
200
300
Time (mins)
Control
PXR
IL-31
pg/ml
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
Time (mins)
Control
PXR
GM-CSF
pg/ml
100
80
60
40
20
0
0
100
200
300
Time (mins)
Control
PXR
Eotaxin
pg/ml
800
600
400
200
0
0
100
200
300
Time (mins)
Control
PXR
Eotaxin-3
pg/ml
200
150
100
50
0
0
100
200
300
Time (mins)
Control
PXR
IP-10
pg/ml
40000
30000
20000
10000
0
0
100
200
300
Time (mins)
Control
PXR


MCP-1
pg/ml
40000
30000
20000
10000
0
0
100
200
300
Time (mins)
Control
PXR
MDC
pg/ml
1500
1000
500
0
Time (mins)
MIP-1a
pg/ml
1500
1000
500
0
Time (mins)
MIP-1b
pg/ml
4000
3000
2000
1000
0
Time (mins)
MIP-3a
pg/ml
20000
15000
10000
5000
0
Time (mins)
**
TARC
pg/ml
2000
1500
1000
500
0
Time (mins)
*
IFN-g
pg/ml
40000
30000
20000
10000
0
Time (mins)
TNF-a
pg/ml
300
200
100
0
Time (mins)
*
*


TNF-b
pg/ml
Time (mins)
Control
PXR
bFGF
pg/ml
Time (mins)
Control
PXR
Flt-1
pg/ml
Time (mins)
Control
PXR
Tie-2
pg/ml
Time (mins)
Control
PXR
*
VEGF
pg/ml
Time (mins)
Control
PXR
VEGF-C
pg/ml
Time (mins)
Control
PXR
VEGF-D
pg/ml
Time (mins)
Control
PXR
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CRP
pg/ml
Time (mins)
Control
PXR

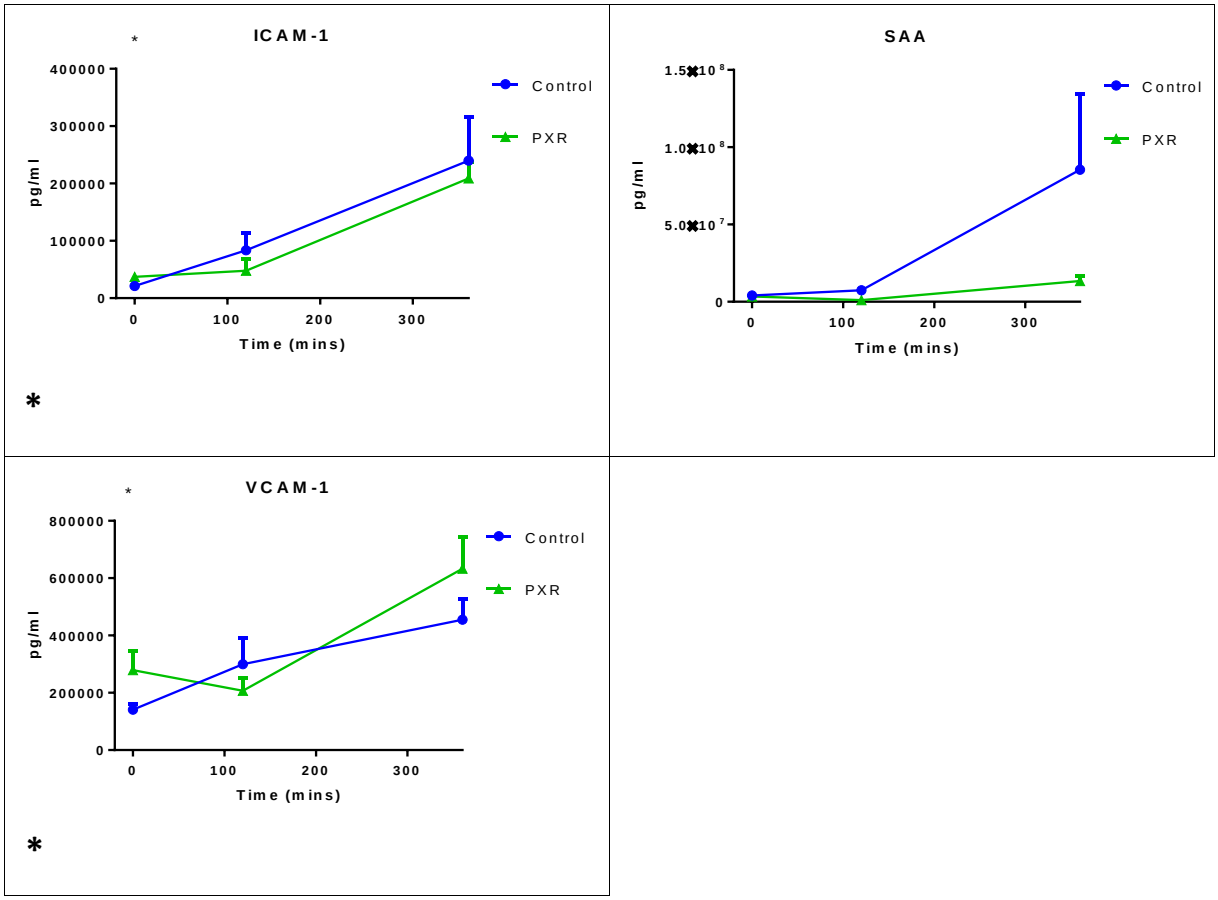

Figure 94 - Perfusate chemokine and cytokine levels in control liver perfusions and livers with PXR-activator added to normothermic perfusion

Cytokines IL-7 and IL-15 that are involved in regulating the immune response were significantly higher in the PXR group, IL-7 at the start of perfusion, and IL-15 was significant at the end time-point. However, TARC was significantly elevated in the control group at time-0 time-point.

Markers of vascular injury, ICAM-1 and VCAM-1 were significantly higher in PXR group at the start of perfusion, but over the course of perfusion this seemed to reverse so that markers of vascular injury were lower in the PXR group at the end of perfusion.

VEGF-D and TIE-2, angiogenesis markers were both significantly elevated in the PXR group, increasingly as time on perfusion increased.

It is known that activation of PXR attenuates NF-κB signals leading to decreased expression of some pro-inflammatory cytokines[153]. This appeared to be the case for IL-1β and Tumour necrosis factor alpha (TNF-α).

Overall there are much fewer significant results than previous multiplex comparisons of hypothermic clinical versus discard livers and normothermic livers with and without extended ischaemia. This supports the hypothesis that during the 6 hours of NMP, PXR activation has not delivered significant beneficial or deleterious effects measurable by cytokine levels in this time period.

8.4.3.3 Lactate Levels

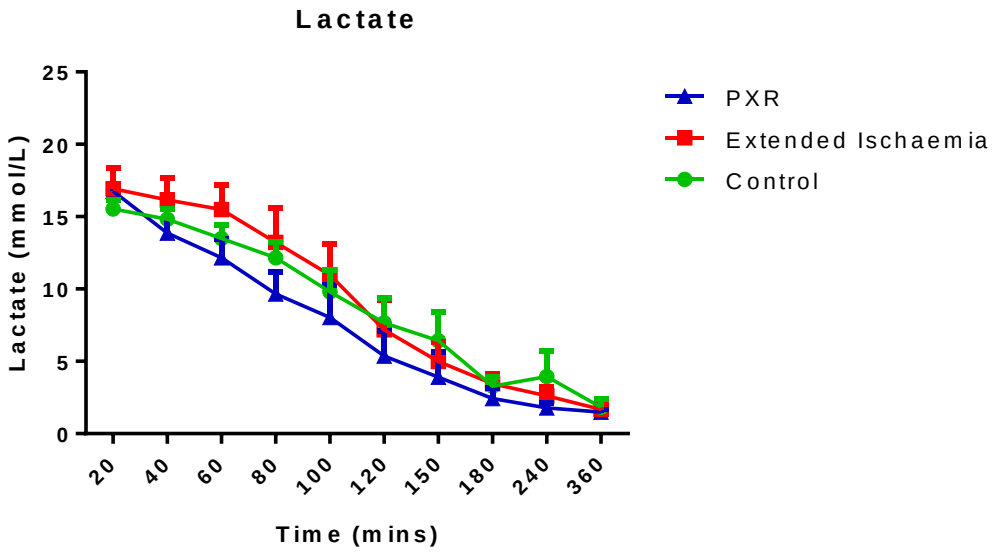

Figure 95 - Perfusate lactate levels (mmol/L) over time with and without addition of PXR-activator to normothermic perfusion

There was no significant difference in lactate levels between the 3 groups at any time-point as shown in Figure 95.

8.4.3.4 ALT Levels

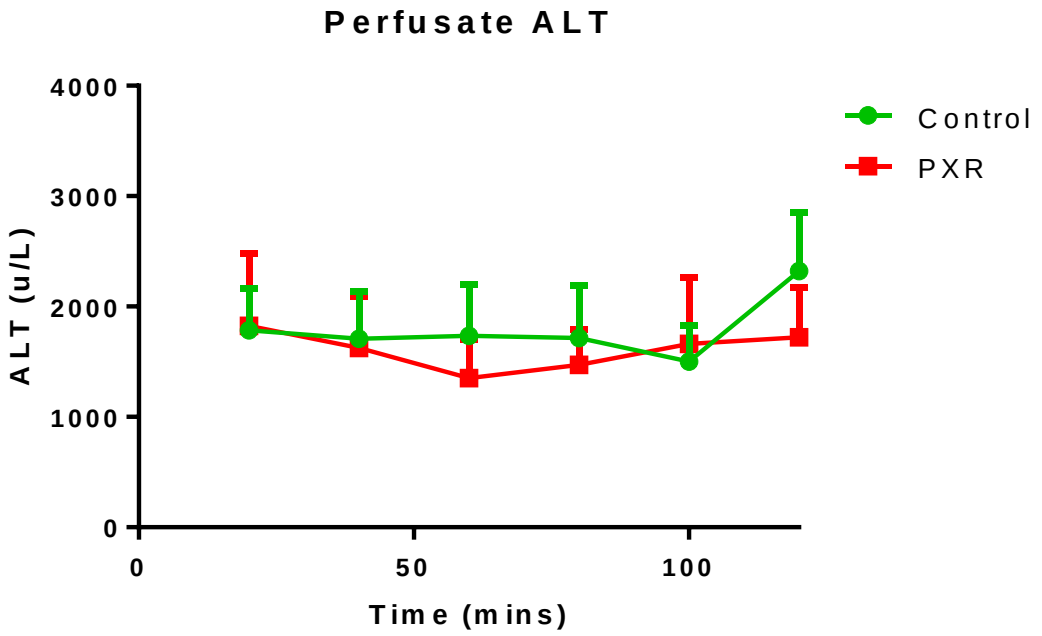

Figure 96 - Perfusate ALT levels (u/L) over time with and without addition of PCR-activator to normothermic perfusion

Similarly, there were no significant differences in ALT levels between control and PXR-activated livers at any time point as shown in Figure 96

8.4.4 Discussion

There is vast potential for therapeutic drug delivery during NMP with the potential to improve livers further than perfusion alone can achieve. The protective effects of PXR activation on IRI in our rodent model, led to PXR activation being selected as a potential therapy in NMP.

Our results demonstrate feasibility and safety of PXR activation during NMP. The perfusion dynamics were unaffected by therapeutic intervention. Perfusate measurements including ALT and lactate showed no difference compared to our control liver perfusions. Our wide multiplex panel showed few significant differences, some favouring the control group and some favouring the PXR group. Overall, during the 6-hour period of perfusion there were no significant detrimental effects to the livers. Most significantly, IL-1β and TNF-α, are known to be reduced by PXR activation through co-inhibition of the NF-κB pathway, and was evident in our results.

However, within the 6-hour period, we were able to demonstrate upregulation of CYP3A4 and CYP3A43 within tissue biopsies. Although this does not prove the potential benefits of PXR activation, it does demonstrate successful utilisation of NMP as a platform for drug delivery, and in doing so opens up many doors for potential therapeutic agents.

Although measurable benefits of PXR activation were not demonstrable within this model, we believe there still could be potential protective benefits of PXR activation in this setting. However, any potential benefits to the recipient of receiving a liver that has been actively treated with a PXR activator during NMP may only be seen at a much later time point. For example, any benefits in reducing ischaemic cholangiopathy, would likely only become apparent months after transplantation.

Future work could include perfusing livers for much longer time periods to assess for any measurable effect, and/or transplanting PXR-treated livers in a large animal model, prior to considering translating PXR activation during NMP with Avasimibe to clinical practice.

8.5 Discussion

There is ongoing need for livers for transplantation, and widespread improved utilisation of marginal livers has contributed to a fall in the liver transplant waiting list in recent years [9]. Strategies to utilise extended criteria donors, without any additional therapy, comes with increased risk to the recipient, both in short and long-term. Normothermic machine perfusion alone has been shown to benefit livers prior to transplantation, however the potential for active therapeutic treatments for livers by using NMP as a platform for drug delivery has yet to be fully investigated and exploited, but the potential is very exciting.

The CYP3A subfamily of cytochrome P450 plays an important and wide-ranging role in the regulation of liver metabolism and PXR activation is critical to its regulation. Previous studies have demonstrated protective effects of PXR activation in a rodent IRI model[106]. Our mouse model confirms the benefits of PXR activation in terms of survival to 7-days post IRI insult, and also demonstrates that this benefit is lost in mice that have the

PXR gene knocked-out. Our results do however also show improved overall survival in knock-out mice. An explanation for this has yet to be fully elucidated, however the wide-ranging potential targets of PXR receptors means that further effects of PXR knock-out are possible.

A novel equipment setup for hepatocyte isolation has been developed with very promising results. Our cell yield is higher than that reported in the literature[164-166] particularly for the size of liver perfused and even more so considering these livers are by definition sub-optimal as they have been declined for transplantation. These human hepatocytes were used to demonstrate induction of the CYP3A subfamily of P450. Avasimibe, rifampicin and phenobarbitone, all known PXR activators, demonstrated induction of CYP3A in-vitro.

Avasimibe was selected for translation to our human NMP model. The addition of avasimibe to the perfusate in NMP successfully induced CYP3A4 and CYP3A43 during the limited 6 hours of perfusion. There were no significant measurable detrimental effects to any of the livers, thus demonstrating the safety of the setup and avasimibe in this context. Our results demonstrate the successful use of NMP as a potential method for delivering therapeutic agents to livers prior to transplantation. Whilst the full benefits of PXR activation could not be established within the limited time-frame of perfusion, further investigation may yet continue to support its protective role in IRI and there is potential to investigate the long-term benefits to recipients from receiving avasimibe-treated livers.

Chapter 9. Discussion

Liver transplantation is a relatively modern surgical procedure and has seen improving outcomes over the last few decades, particularly with improvements in immunosuppression. The resulting improvement in recipient survival has opened up the option of liver transplantation to a wider population. Subsequently, the waiting list in the UK and around the world for liver transplantation had been increasing steadily, with a significant waiting list mortality. One could therefore argue that it has become a victim of its own success. In order to meet the growing demand for transplantation, either the number of donors must increase, or there must be increased utilisation of existing donors. In particular, currently only 36% of livers from DCD donors that meet organ donation criteria end up being transplanted[9]. In recent years, there has been increased utilisation of marginal livers, which has led to a subsequent fall in the UK liver transplant waiting list. However, increased utilisation of marginal livers (particularly DCD) comes at a cost. Well documented inferior outcomes from DCD liver transplantation have held back successful utilisation of DCD livers, in particular higher rates of EAD and long term biliary complications.

In order to improve marginal liver utilisation, this project had two broad aims: to improve graft quality by developing ex-vivo perfusion in our centre, and find novel ways to test liver viability in real-time to aid objective graft selection for transplantation.

Ex-vivo organ perfusion is not a new concept, but there is renewed interest and excitement in the techniques due to the modern issues highlighted above. Recent experience in ex-vivo perfusion falls broadly into two categories: hypothermic and normothermic perfusion. To date there are no studies published comparing the two modalities and there is no consensus on the optimal method.

Hypothermic oxygenated perfusion (HOPE) in various forms has been successfully performed clinically in a small number of centres internationally [25, 31-33, 44, 68]. Guarrera *et al*. first published a clinical series, followed by the Dutkowski group and most recently the Porte group. Guarrera and Porte both use a dual perfusion setup with the hepatic artery and portal vein cannulated, whereas Dutkowski has achieved good results with portal-only perfusion. Although, only cannulating the portal circulation simplifies the perfusion procedure, we felt dual perfusion theoretically would have greater potential for global perfusion and help to clear the arterial microcirculation as well as portal. We chose to oxygenate our perfusion with medical air, which none of the three centres currently do. This allows us to control the pO_2 of perfusate without over-oxygenating, as a result of some published deleterious effects of over-oxygenation [28]. The Medtronic setup we developed has a significant cost advantage over the LiverAssist setup used by Dutkowski and Porte. The Guarrera group also use a Medtronic setup but with a different circuit design. Much like Dutkowski and Porte, we chose to pressure-limit our perfusion rather than have a fixed flow as the Guarrera group published as this would provide less endothelial stress in a high-resistance scenario.

A stepwise introduction of multiple perfusion modalities was strategically planned. Our existing experience with hypothermic and normothermic kidney perfusion was beneficial in first establishing liver perfusion. Indeed, we adapted the Hosgood-Nicholson setup to deliver both types of liver perfusion. Initial liver perfusion

experience was gained by performing hypothermic perfusion on discarded livers first, before translating to clinical practice once our protocol and expertise had developed. Once clinical hypothermic perfusion started, experience in normothermic perfusion of discarded livers started contemporaneously. This allowed us to gain experience in clinical hypothermic perfusion whilst also developing our normothermic perfusion protocol. By first performing hypothermic perfusion (an inherently safer technique) before graduating to normothermic perfusion, this allowed our experience and expertise to develop in a systematic and safe manner. As such, no technical failures were experienced in any liver perfusions, and there were no graft losses due to perfusion errors.

Our clinical hypothermic perfusion study to date has resulted in 10 transplants. High risk transplants were chosen as it was felt these were the cases that stood to gain most from reconditioning. As such, all 10 transplants had significant donor and in some cases recipient risk factors, as described in Section 5.4 Results. A local control cohort of transplants since 2010 that would have fulfilled our inclusion criteria into the HOPE study was analysed and compared to the HOPE recipient outcomes. The control cohort was similar to the study group in all aspects except MELD scores. The study group had significantly higher MELD scores reflecting the fact these were high-risk transplants.

Considering the donor and recipient risk factors, the study group of 10 recipients have had very good outcomes. There has been a single retransplantation, which was due to rejection. Most importantly, within the 10 transplants there has not been a single case of clinically-significant ischaemic cholangiopathy, with a median follow up of 14 months. This compares favourably with the control cohort, where 9 out of 24 recipients developed ischaemic cholangiopathy, all of whom had received DCD livers. Our sample size was insufficient to demonstrate significance, but nevertheless find this result encouraging. Given that ischaemic cholangiopathy was present exclusively in our previous DCD transplants, it could be argued that perfusion should be reserved only for DCD livers.

Between the two groups, there was no difference in length of ITU, overall hospital stay, or usage of blood products. None of the 10 HOPE transplants experienced significant reperfusion syndrome, but the rates in the control group were also quite low. The median peak ALT was lower in the HOPE group, and there were lower rates of AKI in the HOPE group, but neither were significant. In contrast, the bile leak rate and HAS rate were higher in the HOPE group, although again not significant.

Our HOPE series has demonstrated safety and efficacy of our setup using cost-effective equipment. Our results demonstrate non-inferiority to our control cohort, but with some encouraging results. In particular, the absence of ischaemic cholangiopathy in the HOPE group is encouraging and will require further evaluation.

Our series of non-clinical normothermic perfusion of discarded livers has enabled us to refine our NMP protocol and enhance our perfusion experience before translating to clinical practice. As such we have perfused 20 livers with our NMP protocol. As well as developing our NMP setup, these perfusions allowed valuable work to be carried out on real-time viability testing and potential therapeutic applications.

The ability to objectively predict the post-transplant function of a liver in the recipient is the "holy grail" of successful expansion of utilisation of the current donor pool. Well documented inferior outcomes in marginal livers, particularly DCD livers, have hindered using this resource to its full potential. However, a significant proportion of available livers are currently not transplanted because of fears around PNF and biliary complications. Being able to accurately identify which of these organs will develop complications would allow safe expansion of the transplanted livers and better utilise this untapped resource. We have been able to perfuse a range of types of liver, from ones that were clearly untransplantable, to those that have been successfully transplanted following HOPE. A number of parameters have been analysed, from non-invasive markers such as vascular resistance, simple point-of-care tests such as blood gases, widely available lab tests such as liver transaminases, through to complex biomarker profiling through multiplex analysis and specialist imaging including contrast-enhanced ultrasound.

Our previous experience with ex-vivo kidney perfusion suggested increased vascular resistance is suggestive of poorer tissue viability and subsequently poorer function [110]. Current opinions on viability testing of livers undergoing NMP also suggest that flow rates should be taken into account [63, 73]. In our series, no significant difference in flow rates were observed in hypothermic perfusion between our 2 study groups suggesting it is of little prognostic value (Section 6.2 Perfusion Dynamics). In normothermic perfusion, HA flow also did not appear to be significant, although there was a trend towards better PV flow in better livers, but we did not demonstrate significance. It is probably therefore appropriate to conclude that flow rates, particularly PV flow rates in NMP should not be ignored, but are not necessarily of great importance in viability assessment.

The significance of lactate clearance during perfusion has been widely reported as being of crucial value in real-time assessment. Whilst it has been demonstrated that livers that fail to clear lactate may fail to function after transplantation [61], it has also been reported that livers that successfully clear lactate may still fail to function post transplantation. Nevertheless, proposed criteria for viability testing place significant emphasis on its importance [63, 73]. Whilst there is logic that the absence of lactate metabolism should be indicative of a poorly functioning liver, there is no evidence that lactate clearance necessarily indicates adequate function. Our results demonstrate that there appeared to be a useful discrepancy in hypothermic perfusion, however in normothermic perfusion all livers behaved almost identically. For the first time to our knowledge, we have demonstrated that even livers that are far beyond anyone's margin for transplantability still clear lactate during NMP (Section 6.3.2.2 Normothermic Perfusion). Published cut-off values for lactate are generally 2.5 mmol/L, and all our livers reached this threshold during NMP. This is a very important finding, as many centres are currently using lactate clearance as a measure of graft viability, and our results demonstrate this is falsely reassuring. This also fits with the series published by Watson *et al*. where the only death was in a patient whose liver had cleared lactate during NMP [28].

Volume of bile production has also been postulated as a marker of graft viability, however, the process of bile production is affected by many factors, which may or may not correlate with function – such as levels of glucose (although we did not demonstrate a correlation in our series). In both the COPE trial [61] and in Watson's series [28], there were livers that produced no bile but had adequate function in the recipient. In our

series, bile production was not significant enough to reliably quantify in hypothermic perfusion; however, in NMP we demonstrated that extended ischaemia rendered livers unable to produce bile. All livers in the extended-ischaemia group produced minimal or no bile, demonstrating a clear distinction between the groups. This places some importance on the volume of bile produced for viability assessment, although judging by published experience, the lack of bile production does not necessarily suggest poor function post-transplantation.

Alanine transaminase makes up part of standard liver function tests and is commonly used to monitor graft function after transplantation. As hepatocellular injury or death results in release of ALT from cells, its analysis during perfusion could provide useful information. In Watson's series, the PNF liver had significantly elevated levels of ALT during perfusion. In our hypothermic perfusion series, we demonstrated a trend towards lower perfusate ALT levels in the clinical livers compared with discard, but did not reach significance. In the clinical series, only a weak correlation was demonstrable between perfusate ALT and recipient ALT in the 7 days after transplant. In our NMP series, we found that prolonged ischaemia had no effect on perfusate levels of ALT during perfusion.

In the search for suitable biomarkers for viability assessment, we performed an extensive multiplex analysis on perfusate samples. In our hypothermic series, a total of 18 markers analysed demonstrated a statistically significant difference in levels between the clinical livers and discard livers. In particular, markers of vascular injury, angiogenesis markers and pro-inflammatory markers were higher in the discard group. Of these, ROC-curve analysis identified 10 markers that were highly significant and optimum cut-off values were identified (Section 6.6.2.1.2). It is unlikely that a single biomarker would ever be identified that in isolation could reliably predict post-transplant viability, but a combination of biomarkers could prove a much more powerful tool. By combining all 10 tests that were highly significant in our ROC analysis, we propose a potential scoring system which in our series resulted in a significant difference in scores between clinical and discard livers ($p<0.0001$). Even if only the 6 tests that were significant at the start of perfusion were used, our modified scoring system still achieved significance ($p<0.0001$). Similar analysis of perfusate in our NMP series was less successful in identifying potential biomarkers. This may well be representative of the fact that all livers we performed NMP on were 'discarded' untransplantable livers. Further analysis will need to be performed once perfusate samples can be obtained from NMP of clinical livers.

One of the simple theoretical advantages of any form of perfusion is the ability to clear the microcirculation of the liver. We have observational evidence of this, where livers that arrive with patchy perfusion have developed homogenous peripheral perfusion during both HOPE and NMP. We hypothesized that the inability of ex-vivo perfusion to open up peripheral microcirculation is indicative of a poor liver graft. The hypothesis is based on the principle that poor peripheral circulation that is not cleared by ex-vivo perfusion may be indicative of microemboli or thrombosis in situ in small vessels, and if not cleared by ex-vivo perfusion (either with acellular fluid or heavily heparinised blood), is unlikely to be cleared in-vivo. We managed to successfully utilise contrast-enhanced ultrasound to quantify this in a reproducible manner. As the absolute value for perfusion within an area of parenchyma that is analysed would display significant heterogeneity, our method

used a measure of perfusion within the lumen of large vessels to act as a reference for each liver. By comparing the level of perfusion within the peripheral parenchyma against luminal perfusion, any parenchymal deficit can be recorded. As such, we developed the dAUC measurement, where a high dAUC represents a large deficit in parenchymal perfusion relative to luminal perfusion. Using this method, we demonstrated that livers subjected to prolonged ischaemia lost the ability of NMP to reduce the dAUC. In summary, in control livers, NMP was able to remove the deficit in parenchymal perfusion within 6 hours, but in the extended ischaemia group there was no difference. This measure in isolation may not be entirely indicative of a functioning graft, but is a useful objective tool in the viability testing armamentarium.

Each of our parameters for viability testing was correlated against pathological grading of ischaemia-reperfusion injury, hepatocyte necrosis and levels of steatosis. The pathological grading was performed by a blinded pathologist. In each of the parameters, no significant correlation was found to any of the gradings. This may be due to insufficient numbers to demonstrate significance, alternatively either the parameters of viability testing or biopsy analysis are not indicative of function.

In summary of the experience obtained in real-time viability assessment of livers during ex-vivo perfusion, we were unable to find a single parameter or biomarker that is reliably indicative of function or viability. Combining multiple factors that represent vascular resistance (flow rates), peripheral microcirculation (CEUS), hepatocellular function and viability (lactate clearance, ALT), synthetic function (bile volume and composition), and further biomarkers (cytokines and chemokines), adds power to the assessment, whilst simultaneously adding complexity to the assessment process. With further experience and larger numbers, a multifactorial scoring system may be developed and validated to simplify this process.

In addition to the methods illustrated above for real-time assessment of livers, methods of steatosis quantification have also been explored. Hepatic steatosis is a well-recognised risk factor for post-transplant outcome, including an increased risk of PNF, which was first recognised over 25 years ago. We have demonstrated in our rodent cell-line that steatosis has a significant effect on viability when exposed to hypoxic conditions, thereby supporting the importance of steatosis assessment as a predictive value for a liver's ability to withstand ischaemia reperfusion. Although severe macrosteatosis is universally recognised as being beyond the limits of acceptable risk for transplantation, there is significant variation in opinions on the limits of transplantability in moderate steatosis. Indeed, the growing discrepancy between supply and demand for liver transplantation has led to increased utilisation of livers displaying levels of steatosis that a few years ago would have condemned a liver to being discarded. However, the lack of suitable point-of-care tests to accurately measure degrees of steatosis renders this decision-making process subjective, leading to wide variation in practice. Most commonly, livers are visually assessed by the retrieving and/or transplanting surgeon who will estimate steatosis based on appearance and texture. We investigated performing oil-red colorimetry on biopsy samples obtained, and validated this on a sample of mice livers known to be either normal or severely steatotic. Although colorimetric assessment did not prove as accurate as software-based analysis of a fully prepared and stained slide, it did provide some useful information. This was followed up by performing the same method of analysis on human perfused livers. Although there was some correlation between

colorimetric absorbance and pathological grading of steatosis, this did not reach significance. However, a high colorimetric absorbance had good positive predictive value for severe steatosis, even though the negative predictive value was poor. That is, if a high colorimetry value is obtained, one can safely assume there is severe steatosis, but a low value may not necessarily mean the absence of steatosis. In our series of perfused discarded livers, one liver had been declined from clinical use by the transplanting surgeon due to visual appearance of steatosis, but both colorimetric assessment and then pathological grading confirmed there was only mild steatosis.

In addition to the ability to accurately and reliably assess the function of a liver prior to transplantation, and thereby predict its post-transplant function, ex-vivo perfusion has further potential benefits. It has been shown in our own and other published series that perfusion alone is likely to be beneficial to livers either by reconditioning, or simply reducing the impact of ischaemia-reperfusion injury. The next step is to actively manipulate the organ to optimise it pharmacologically. As well as potentially utilising treatments already clinically available, as there are no concerns regarding non-liver systemic toxicity, further pharmacological opportunities may exist. We built upon existing experience of our group on the investigation of protective properties of Pregnane-X Receptor (PXR) activation. Previous experience demonstrated a reduction in periportal inflammation following IRI in rats exposed to PXR activation. The current study demonstrated that the survival benefit was not seen in PXR knock-out mice, suggesting that benefit may be PXR-dependent. This would support the findings of Amer *et al*. [106] that PCN is beneficial to rodents sustaining IRI. The overall survival in knock-out mice was better than wild-types, and the cause for this remains unknown. PXR activation was then assessed in cultured human hepatocytes, where a consistent induction by Avasimibe of PXR-regulated genes, and no measurable effect on AhR-regulated genes. To perform this analysis, a novel technique for hepatocyte isolation was used, to our knowledge the first time an NMP setup has been used for this purpose and resulted in a high hepatocyte yield from the left lateral segment.

The assessment of PXR activation for the reduction of IRI in livers for transplantation was then taken to the next step of utilising NMP as a platform for drug delivery. We have demonstrated that it's downstream target is upregulated within the 6 hours of perfusion. Whilst this does not prove the benefits of PXR in human liver transplantation, it demonstrates the successful use of perfusion as a platform for drug delivery, which in itself opens up many opportunities. It is possible that any advantages of PXR activation would only become apparent days, weeks or even months after transplantation therefore the lack of significant measurable benefit within 6 hours of perfusion does not necessarily diminish the potential benefits of PXR that will require further investigation.

9.1 Future Perspectives

Continuing our HOPE series will add power to our statistics, and I would hope that with increased numbers a statistical significance would be demonstrated in ischaemic cholangiopathy rates and in graft and patient survival. Our HOPE programme continues, and we will continue data collection with this objective.

Introducing NMP to clinical practice in our centre is something we have naturally worked up to and will imminently progress to, but this still leaves a fairly basic unanswered question of whether HOPE or NMP should be the preferred modality to explore further. Ultimately, a large multicentre randomised control study that includes both these arms will be needed to ascertain if there is an advantage to either.

Particularly in NMP, there are many aspects of real-time assessment that need to be investigated further, including analysing bile composition in closer detail.

This study proposes 2 potential scoring methods based on perfusate analysis of HOPE livers. We aim to investigate this further in a second cohort of livers to validate the scoring system. If it proves to be effective in predicting outcomes, then migrating this to a point-of-care test would be the next step. Existing work on other biomarkers within our institution demonstrates feasibility of this.

Arguably the largest area for further investigation is pharmacomodulation of the liver during NMP. We have demonstrated with PXR that drug activation is present, and therefore there is enormous potential to explore treatment options. Our perfusion team has recently demonstrated that multipotent adult progenitor cells (MAPCs), when added to a kidney NMP circuit demonstrated an improvement in clinically-relevant parameters and biomarkers associated with IRI. This could and should be investigated in NMP of livers also.

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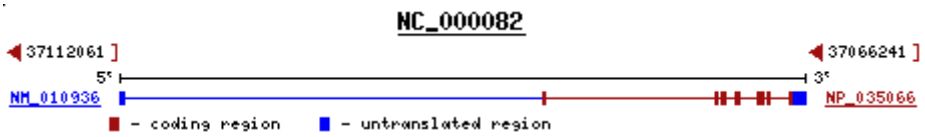
Appendix 1 - The mouse PXR gene

(minus strand) RefSeq below



cdna sequence:

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AAAAAAAAAAAAAAAAAAAAAAAAAAAA

orange sequence = translated sequence (underlined missing

Appendix 2 – HOPE Protocol

A2.1 Setting up for HOPE

The equipment will be stored on a trolley in the Institute of Transplantation (IoT) Perfusion Lab.

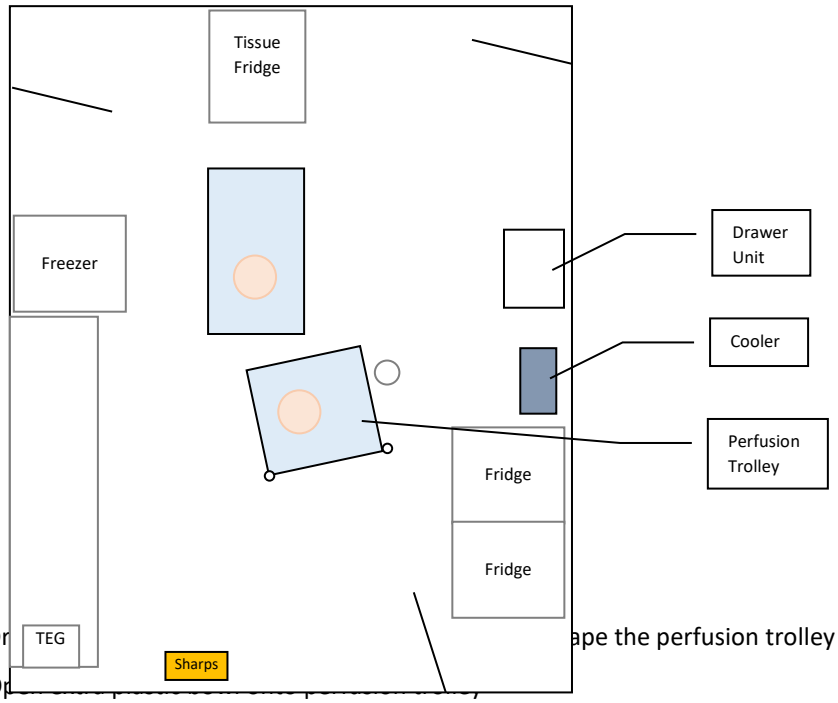
Equipment required

- Metal trolley with BioMedicus Cardiopulmonary Bypass pump
- Attached to the trolley should be:
    - Clamp for reservoir
    - Pump
    - Flow sensor x2
    - Temperature probe x2
- Hirtz cooler on it's trolley
- Box containing sealed sterile circuit
- 3 x 1L bags of Belzer Machine Perfusion Solution
- Equipment box containing:
    - Clamps
    - Cannulae
    - Pinch valves
    - Syringes
    - Drugs

Setting up in theatre

1. Set up in perfusion room to Theatre 18. Otherwise, theatre layout and setup as per standard liver transplant
2. Set up Perfusion Room as per layout below.

3.



4. Dr ape the perfusion trolley also
5. Op
6. Place pump and cooler on their respective trolleys adjacent to this
7. Plug everything in to sockets adjacent to 2 fridges
8. Switch on cooler and set to 5°C. Note it may be on safety lock and need to be increased to 5.1°C then back to 5°C to release safety lock
9. Connect oxygen tubing to **Medical Air** theatre supply and lay tubing on the floor out of the way
10. Hand sterile clamps, syringes, and cannulae to scrubbed surgeon on back table
11. Open sterile circuit, surgeon to remove from sterile box and place on draped trolley
12. Perfusion surgeon to hand off reservoir and pump head through the back of the trolley (between drip stands), but retain the tubing holder, connectors, venous line (blue tip) and arterial lines (red tip x 2)
13. Attach reservoir to clamp on metal pump trolley (must be as low as possible), ensuring no kinks in tubing
14. Attach pump head to magnetic pump
15. Attach flow sensors (small tube to blue sensor)
16. Connect air supply and open to 3L/min
17. Attach pinch valves just distal to Y-split
18. Clamp reservoir outlet
19. Fill reservoir with 3L Belzer's Machine Perfusion Solution (MPS)
20. Clamp distal to pump head and release clamp proximal to it to prime and de-air pump head.

21. Clamp HA arterial line (just distal to Y-junction) and unclamp pump outlet to prime oxygenator/heat-exchanger
22. Switch on BioMedicus Console
23. Ask perfusion surgeon to use vented 3/8" connector to connect sterile ends of PV and IVC lines
24. Start the pump at low rpm and use pump to prime venous side of circuit
25. Connect lines from cooler to the oxygenator and switch on the cooler and set to 5°C
26. Connect temperature probe to connector on oxygenator outlet and reservoir inlet
27. Set up pressure lines (x2) ready to connect to cannulae and connect to P1 and P2 on back of console. P1 for HA, P2 for PV.
28. Draw up medications to add to circuit (NAC, dexamethasone, Benzyl Penicillin)

Open liver from transport box

1. Perform back table dissection as standard
2. Cannulate suprahepatic IVC and tie in (purse-string with 2-0 Prolene)
3. Suture infrahepatic IVC
4. Cannulate portal vein (3/8" tubing) and tie in (purse-string)
5. Cut off connector on end of PV cannula
6. Cannulate hepatic artery (1/4" tubing) either with lifeport cannula or 12Fr straight cannula and tie in
7. Prime cannulae with UW and clamp (no air)
8. Tie off any leaking branches
9. Stop the pump
10. Surgeon to clamp and separate the connector in the 3/8" line leaving the connector on the PV side
11. Connect the cannulae (vented connector to PV, no connector end to IVC, ¼" line to HA) using syringe to de-air
12. Connect pressure lines to HA and PV cannulae. Use syringe to fill pressure line and remove air. Open 3-way tap to air and zero (x2)
13. Zero flow sensors (x2)

A2.2 Hypothermic Oxygenated Perfusion Procedure

1. Use pinch valve to clamp down on PV
2. Unclamp IVC line
3. Start the pump at 500rpm

4. Unclamp HA line
5. Adjust rpm until HA pressure is 25mmHg
6. Slowly open PV pinch valve
7. Adjust RPM and pinch valve to achieve PV pressure 2-3mmHg and HA pressure 25mmHg
8. The pinch valve may need to be readjusted to keep the differential pressures correct
9. It is important not to exceed these pressures as sheer stress will occur
10. Aim for total flow of 0.667ml/kg/min but do not exceed pressures
11. Maintain perfusate temperate 8-10°c
12. Use infra-red thermometer to check liver surface temperature
13. Continue to pump, maintaining these parameters for 2 hours or until transplanting surgeon is ready to implant
14. Record temperature, pressure, flow and arterial and venous gases every 20 minutes.

A2.3 Removing from pump

1. Turn pump dial to 0
2. Untie and remove IVC cannula first
3. Untie and remove other cannulae
4. Transfer liver in bowl to recipient for implantation

A2.4 Storage

1. Discard of sterile tubing and reservoir
2. Wipe down all equipment
3. Restock and return equipment to IoT perfusion lab

A2.5 – Equipment box contents

1. Line Clamps x 6
2. Cannulae arterial and venous x2 each
3. Multiple blood gas syringes
4. Multiple insulin (1ml) syringes
5. Pinch valves

A2.6 – Medications to add to circuit

- N-acetylcysteine 600mg
- Dexamethasone 6.6mg
- Benzylpenicillin 600mg

Appendix 3 – ENVP Protocol

A3.1 Setting up for NMP

When notified of liver transplant, the research fellow on call will go to Freeman theatre 18 to set up the equipment.

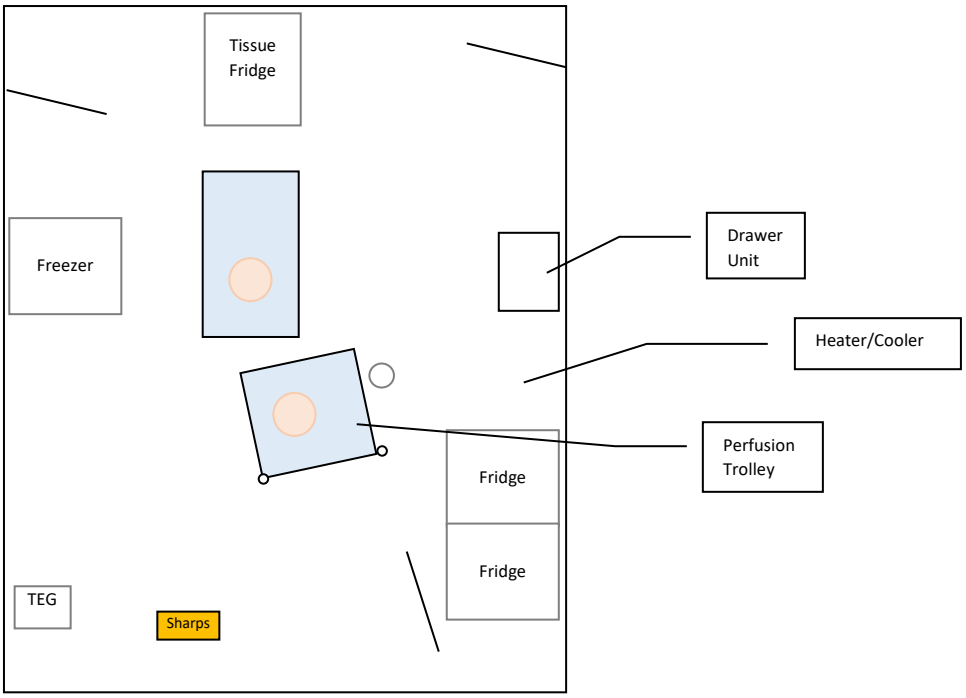
The equipment will be stored on a trolley in the Institute of Transplantation (IoT) Perfusion Lab.

Equipment to take from IoT Perfusion Lab

- Metal trolley with BioMedicus pump
- Attached to the trolley should be:
    - Clamp for reservoir
    - Pump
    - Flow sensor x2
    - Temperature probe x2
- Hirtz cooler on it's trolley
- Box containing sealed sterile circuit
- 3 x 1L bags of Belzer Machine Perfusion Solution
- Equipment box containing:
    - Clamps
    - Cannulae
    - Pinch valves
    - Syringes
    - Drugs

Setting up in theatre

29. Set up in perfusion room to Theatre 18. Otherwise, theatre layout and setup as per standard liver transplant
30. Set up Perfusion Room as per layout below.



31. Drape a trolley for back table work as standard and drape the perfusion trolley also.
32. Open extra plastic bowl onto perfusion trolley
33. Place pump and cooler on their respective trolleys adjacent to this
34. Plug everything in to sockets adjacent to 2 fridges
35. Switch on Cooler and set to 39°C. Note it may be on safety lock and need to be decreased to 38.9°C then back to 39°C to release safety lock
36. Connect Oxygen tubing to **Medical Air** theatre supply and lay tubing on the floor out of the way
37. Hand sterile clamps, syringes, and cannulae to scrubbed surgeon on back table
38. Open sterile circuit, surgeon to remove from sterile box and place on draped trolley
39. Perfusion surgeon to hand off reservoir and pump head through the back of the trolley (between drip stands), but retain the tubing holder, connectors, venous line (blue tip) and arterial lines (red tip x 2)
40. Attach reservoir to clamp on metal pump trolley (must be as low as possible), ensuring no kinks in tubing

41. Attach pump head to magnetic pump
42. Attach flow sensors (small tube to blue sensor)
43. Connect air supply and open to 3L/min
44. Attach pinch valves just distal to Y-split
45. Clamp reservoir outlet
46. Add to Reservoir: (in this order)

    1. 500ml Isoplex
    2. 10,000 units Heparin
    3. 3 units RBC
    4. 30ml Sodium Bicarbonate 8.4%
    5. Cefuroxime
    6. 10ml 10% Calcium Gluconate

47. Clamp distal to pump head and release clamp proximal to it to prime and de-air pump head.
48. Clamp HA arterial line (just distal to Y-junction) and unclamp pump outlet to prime oxygenator/heat-exchanger
49. Switch on BioMedicus Console
50. Ask perfusion surgeon to use vented 3/8" connector to connect sterile ends of PV and IVC lines
51. Start the pump at low rpm and use pump to prime venous side of circuit
52. Connect lines from cooler to the oxygenator and switch on the cooler and set to 5°c
53. Connect temperature probe to connector on oxygenator outlet and reservoir inlet
54. Set up pressure lines (x2) ready to connect to cannulae and connect to P1 and P2 on back of console. P1 for HA, P2 for PV.
55. Draw up and start infusions as follows:

    1. Actrapid
       Add 200 units (2ml) to 100ml NaCl 0.9%
       Run at 3.5ml/hr
    2. Heparin
       Add 10000 units heparin to 100ml NaCl 0.9%
       Run at 10ml/hr
    3. Cernevit
       Reconstitute full vial of cernevit in 10ml water and add full volume to 500ml Synthamin 9
       Run at 20ml/hr

4. Flolan

   Reconstitute using provided vials.

   Remove 16ml of reconstituted solution & add to 100ml NaCl 0.9%

   Run at 4ml/hr

Open liver from transport box

14. Perform back table dissection as standard
15. Cannulate suprahepatic IVC and tie in (purse-string with 2-0 Prolene)
16. Suture infrahepatic IVC
17. Cannulate portal vein (3/8" tubing) and tie in (purse-string)
18. Cut off connector on end of PV cannula
19. Cannulate hepatic artery (1/4" tubing) either with lifeport cannula or 12Fr straight cannula and tie in
20. Prime cannulae with NaCl 0.9% and clamp (no air)
21. Tie off any leaking branches
22. Stop the pump
23. Surgeon to clamp and separate the connector in the 3/8" line leaving the connector on the PV side
24. Connect the cannulae (vented connector to PV, no connector end to IVC, ¼" line to HA) using syringe to de-air
25. Connect pressure lines to HA and PV cannulae. Use syringe to fill pressure line and remove air. Open 3-way tap to air and zero (x2)
26. Zero flow sensors (x2)

A3.2 Normothermic Perfusion

15. Use pinch valve to clamp down on PV
16. Unclamp IVC line
17. Start the pump at 500rpm
18. Unclamp HA line
19. Adjust RPM until HA pressure is 75mmHg
20. Slowly open PV pinch valve
21. Adjust RPM and pinch valve to achieve PV pressure 4-5mmHg and HA pressure 75mmHg
22. The pinch valve may need to be readjusted to keep the differential pressures correct
23. It is important not to exceed these pressures as sheer stress will occur
24. Aim for total flow of 0.667ml/kg/min but do not exceed pressures
25. Maintain perfusate temperate 37°c
26. Use infra-red thermometer to check liver surface temperature

27. Continue to pump, maintaining these parameters for 2 hours or until transplanting surgeon is ready to implant
28. Record temperature, pressure, flow and arterial and venous gases every 20 minutes.

A3.3 Removing from pump

5. Turn pump dial to 0
6. Clamp proximal to HA and PV cannulae
7. Start infusion of cold UW solution
8. Untie and remove IVC cannula first
9. Untie and remove other cannulae
10. Transfer liver in bowl to recipient for implantation

Storage

4. Discard of sterile tubing and reservoir
5. Wipe down all equipment
6. Restock and return equipment to IoT perfusion lab

Appendix 4 – Technical specifications of circuit design