Immuno-molecular profiling and modulation of donor livers during normothermic machine perfusion.

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A thesis submitted for the degree of

Doctor of Philosophy

Michaelmas 2022

Abstract

Normothermic machine perfusion (NMP) is an increasingly adopted preservation strategy in liver transplantation that facilitates organ assessment, safely prolongs preservation and is associated with reduced ischaemia reperfusion injury (IRI) related complications. However, these IRI-related benefits are less apparent in higher risk organs, which maintain a propensity to develop severe preservation injury despite NMP. The underlying mechanisms and contributory factors associated with this persistent injury are unclear, but may be related to molecular processes occurring during NMP or other preservation factors such as the length of cold ischaemia prior to NMP. This thesis aims to characterise the immunological and molecular processes occurring during NMP and devise interventions targeting these processes to reduce preservation injury. Through an evaluation of samples from clinical trials of human liver perfusion, I have demonstrated that ex situ reperfusion injury/inflammation (ERI) is an immunologically and molecularly distinct entity observed consistently across livers preserved with NMP. I have shown that the presence and length of prior static cold storage (pSCS) can impact the molecular signature of donor livers affecting key pathways and processes involved in mitochondrial function and cellular energetics. I have found that circulating damage associated molecular patterns (DAMPs), constitute an important element in ERI and alongside immune cells and inflammatory cytokines within the perfusate, propagate injury and inflammation ex situ, likely impacting the immunogenicity of the graft. I developed a large animal model to investigate these observations and explore possible modulatory interventions. Finally, I demonstrated that by mitigating ERI, ex situ graft function could be improved and preservation injury reduced in a porcine NMP model. This is achieved by the removal of highly pro-inflammatory nuclear DAMPs (neutrophil extracellular traps (NETs)/nucleosomes, free histones and cell free DNA) from the circulating perfusate during NMP with a specialised column.

Dedication

I am dedicating this thesis to Sinéad, my loving wife and unwavering confidant. Your love and support, through all of the early mornings, long nights and testing times, has been an inspiration. Our wonderful children, Oscar & Cecilia whom you have expertly shielded, have been a constant source of joy and relief, re-energising and motivating me through this journey.

To my parents, Caleb & Shingai Dengu, I am forever indebted. Your exemplary commitment to constant personal improvement has been a guiding light in my life and continues to inspire me. The sacrifices you made and obstacles you overcame to provide us with the opportunities to pursue our education are innumerable and for this we are eternally grateful. To Baba, I am most grateful for your example, of intellectual independence and ambition, a healthy disregard for convention and an unrivalled work ethic built upon person discipline. To Mumma, your confidence in me and constant support has been motivational and your intellectual curiosity exemplary. To my brothers, Pondai and Runyararo, your friendship and humour has sustained me through the most challenging of times, yet your restless ambition has driven me to strive for more.

Acknowledgments

I would like to thank my supervisor and mentor Professor Peter Friend for his wisdom, guidance, and forbearance. His ability to continually inspire and encourage me has been an unfailing source of motivation and our eclectic meetings a testament to his erudition and polymathic disposition. His confidence in me has been steadfast through the most challenging times and his humility has been consummate. My gratitude is also extended to Professor Paul Fairchild, who was willing to support and engage with me in the early and uncertain days at the start of this journey and he has continued to be source of wisdom and friendship.

I would like to thank Carlo Ceresa, Hussain Abbas, Ann Ogbemudia, Tamsyn Clark, Daniel Voyce and David Nasralla for their support, encouragement, laughter and friendship. Without you, this would not have been possible.

I have been fortunate to have the support of many friends and colleagues throughout this period, without whom I would not have been able to achieve the academic work presented in this thesis. Notably, I would like to thank Maria Letizia Lo Faro, Flavia Neri, Honglie Huang, James Hunter, James Fildes, Andrew Aswani and Professor Rutger Ploeg. Your academic contributions to my thesis are self-evident, but your personal commitment to this body of work has been tremendous and for that, I am truly grateful.

From the Nuffield Department of Surgery & Oxford Transplant centre, I would like to thank Julien Branchereau (Visiting Fellow from CHU de Nantes), Adam Thorne (now at the University of Groningen), Helen Stark, Fadi Issa, Joanna Hester, Maria Kaiser, Faysal El Gilani, Venkatesha Udupa, Kaithlyn Rozenberg, Sadr Shaheed, Marta Oliveira, Sotiris Mastoridis, Georg Ebeling, Alexander Sagar, Richard Dumbill, Annemarie Weißenbacher, Visesh Sankaran, John Milton and Sandrine Rendel. From the Dunn school of pathology, I thank Tim Davies (now at OXvax Ltd) and from the Target Discovery Institute, my thanks go to Professor Benedict Kessler and his

group. At the Institute of Biomedical Engineering, I would like to thank Professor Constantin Coussios & Luca Bau. From the Oxford Centre for Clinical Magnetic Resonance Research, Liam Young. At the Department of Clinical Biochemistry, my thanks go to Dr Alireza Morovat and Dr Tim James. I would like to thank the whole team at OrganOx Ltd. Who have provided me with technical support and numerous opportunities, in particular, Chris Morris and Dan Fower. At the Royal Free Hospital, I thank Professor Alberto Quaglia, at the University of Manchester, I would like to thank John Stone, Kavit Amin and William Critchley (now at the University of Leeds) and at the University of Liverpool, I thank Jeremy Schofield, Simon Abrams, Benjamin Chan and Professor Cheng-Hock Toh. I would also like to extend a special thanks to the entire staff at Long Compton Abattoir, whose patience, good humour and willingness to facilitate my project has been of incalculable value.

Finally, I would like to thank the Consortium for Organ Preservation in Europe, Quality in Organ Donation (QUOD), the donors and their families, without whom much of the work in this thesis would not have been possible. The bond between donors, recipients, researchers and clinicians in transplantation is unique and I am grateful to be a part of it.

The work presented in this thesis has been funded by the Oxford Transplant Foundation, Oxford Transplant Centre and Royal College of Surgeons of Edinburgh and delivered alongside commercial collaborators at OrganOx Ltd (Oxford, UK) & Santersus AG (Zurich, Switzerland).

Declaration

All of the work included in this thesis is my own unless otherwise stated. I was fortunate to have access to some of the most advanced molecular and immunological technology, working closely with collaborators at the Target Discovery Institute (Kessler Lab) and Transplant Immunology Research Group (TRIG) to analyse clinical samples. All pre-clinical liver perfusions were performed by myself with assistance from other research fellows (particularly Hussain Abbas and Tamsyn Clark). Specialised assays and analyses that were not possible or available in Oxford, were performed with the support of collaborators at other institutions such as the University of Manchester (in the Fildes lab) and the University of Liverpool (in the Toh lab).

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AUROC	Arear under the receiver operating characteristic curve
AKI	Acute Kidney Injury
ALD	Alcoholic liver disease
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BMI	Body mass index
CAMPs	Chromatin associated molecular patterns
CBD	Common bile duct
CCL13	C-C Motif Chemokine Ligand 13
CD	Cluster of differentiation (E.g.CD8, CD4)
cfDNA	Cell free DNA
cfRNA	Cell free RNA
CIT	Cold ischaemia time
COPE	Consortium for Organ Preservation in Europe
COR	Controlled oxygenated rewarming
CVA	Cerebrovascular accident
DAMPs	Danger-associated molecular patterns
DBD	Donation after brain-stem death
DC	Dendritic cell
DCD	donation after circulatory death

DEP	Differentially expressed protein
d-	
HOPE/HOPE	(dual) hypothermic oxygenated machine perfusion
DLI	Donor liver index
DRI	Donor risk index
EAD	Early allograft dysfunction
EDTA	Ethylenediaminetetraacetic acid
ERI	Ex situ reperfusion injury/inflammation
ET-DRI	Euro-transplant donor risk index
EV	Extra cellular vesicle
FDR	False discovery rate
FFPE	Formalin-fixed paraffin embedded
FOXP3	Forkhead box P3
GDA	Gastroduodenal artery
GGT	Gamma glutamyl transferase
H&E	Haematoxylin and eosin
HA	Hepatic artery
HAT	Hepatic artery thrombosis
HCC	Hepatocellular carcinoma
HMGB1	High mobility group box 1 protein
HOT Panel	Human Organ Transplant Panel
IC	Ischaemic cholangiopathy
IEOCOPD	Infective exacerbation of Chronic Obstructive Pulmonary disease
IFN-g	Interferon gamma

IL	Interleukin
INR	International normalised ratio
IRI	Ischaemia reperfusion injury
ITBL	Ischaemic type biliary lesions
IVC	Inferior vena cava
KC	Kupffer cell
LFQ	Label-free quantification
LSEC	Liver sinusoidal endothelial cells
MAP	Mean arterial pressure
MEAF	Model for early allograft dysfunction
MELD	Model for end-stage liver disease
MSC	Mesenchymal stem cells
mtcfDNA	Mitochondrial cell free DNA
MTPT	Mitochondrial permeability transition pore
NaCL	Sodium chloride
NAFLD	Non-alcoholic fatty liver disease
NAS	Non-anastomotic biliary strictures
NET	Neutrophil extracellular trap
NHSBT	NHS Blood and Transplant
NK	Natural Killer
NMP	Normothermic machine perfusion
NO	Nitric oxide
NRF2	Nuclear factor-erythroid 2-related factor 2
NRP	Normothermic regional perfusion
PAMPs	Pathogen Associated Molecular Patterns

PAS	Periodic acid Schiff
PCA	Principal Component analysis
PNF	primary non function
PRI	Preservation reperfusion injury
PRR	Pattern recognition receptor
PRS	Post reperfusion syndrome
pSCS-NMP	Prior Static cold storage normothermic machine perfusion
PV	Portal vein
RAGE	Receptor for Advanced Glycation Endproducts
RCT	Randomised controlled trial
RET	Reverse electron transport
ROS	Reactive oxygen species
RRT	Renal replacement therapy
SCD	Standard criteria donor
SCS	Static cold storage
TLR	Toll like receptors
TNF	Tumour necrosis factor
Tol-DC	Tolerogenic Dendritic cells
Treg	Regulatory T cells
UK	United Kingdom
UNOS	United Network for Organ Sharing
UW	University of Wisconsin solution
WIT	Warm ischaemia time

Introduction

"I cannot give any scientist of any age better advice than this: the intensity of the conviction that a hypothesis is true has no bearing on whether it is true or not."

— Peter B. Medawar, Advice To A Young Scientist

1.1 Background

Liver transplantation has charted an extraordinary journey from a quasi-experimental procedure to the globally accepted standard of care for patients with end-stage liver failure (1). The early experience of liver transplantation unforgivingly illuminated the magnitude of the challenge of achieving successful and reproducible clinical transplantation (2,3). The enigmatic combination of perilous technical, ethical and pathophysiological obstacles faced by the pioneers seemed barely surmountable at the time, but steadily and remarkably have been decoded and tamed, paving the way for the global acceptance of liver transplantation (2–5). From defining donors and modalities of 'death', constructing an acceptable ethical and legal framework to permit transplantation, to addressing the clinical service issues (such as organ preservation, allocation logistics, surgical and anatomical variation, immunological barriers, physiological derangement, infectious disease and post-transplant critical care management), transplantation has necessarily been a multidisciplinary endeavour from its inception (2,4,6). Burgeoning with new sub-disciplines and blurring the often-stifling traditional dividing lines of professional subspecialty designations, transplantation has charted its own course. A testament to the progress of the field is the inclusive mindset of the modern 'transplant professional'.

The unequivocal success of liver transplantation has brought with it challenges. At this juncture in the evolution of transplantation, we still face a steady increase in demand owing to expanding indications (7), yet we remain constrained by limited donor organ supply (8). Despite major

scientific breakthroughs in the last decade that have significantly reduced the burden of a previously leading indication for liver transplant (Hepatitis C Virus infection) through the use of direct-acting antivirals (9), we face yet more liver disease heralded by the explosion of non-alcoholic fatty liver disease (NAFLD) (10). NAFLD is fast becoming the leading indication for liver transplant in North America and poses novel surgical and medical challenges to the field (11). Furthermore, 'transplant oncology' is emerging as an important liver transplant indication (7), whose expansion will place further strain on the ability to provide transplant services. Consequently, we have a continued increase in demand for liver transplantation in the populations with access to it, and an enormous burden of untreated liver disease globally, in those without (10).

1.2 Deceased Donors

Transplantation is ultimately dependent on the supply of suitable donor organs, which has been the rate-limiting factor for transplant activity and shaped an ethical dimension to the field that has existed since its inception. Major efforts to increase the donor pool have been employed, ranging from different definitions of death (12) and the use of living donors (13,14) to attempts at xenotransplantation (15), the latter representing an avenue which may yet have its day. Coupled with media campaigns and, to varying extents, legislative changes in many European countries (including the United Kingdom (16)), there has been a substantial increase in organ donation rates (8,17).

1.2.1 Donation after brainstem death (DBD)

In the UK, Europe and North America the predominant form of donation in deceased donor liver transplantation, has been from donation after brainstem death(DBD) (18,19). DBD donors have suffered an irreversible neurological injury that meets a set of criteria that determines that there is no neurological function within the brainstem(20). This is assessed by two intensive care doctors on two separate occasions who declare brainstem death and therefore allow the process of organ

donation and procurement to take place (20,21). DBD organs have comprised the vast majority of liver transplants historically and, although some may have high-risk features (addressed below), they have been considered less injured and 'lower-risk' grafts as they do not incur a warm ischaemic injury in the procurement process(22,23).

Despite the observed and commendable increase in potential donors, the challenge of expanding indications and increasing waiting lists has seen transplant activity stall, with activity having plateaued or even declined in the years immediately prior to the COVID-19 pandemic (Figure 0.2). This inability to grow activity despite higher donation rates has largely been due to the high-risk characteristics of the additional organs available for transplantation. Although living donation is an important, albeit numerically small, contributor to organ donation, it is still very much in its infancy in the UK and will not be featured in this thesis.

1.2.2 Donation after circulatory death (DCD)

DCD transplantation refers to donors whose death has been determined using cardio-respiratory criteria (12) and can be defined as either controlled (occurring following the withdrawal of life-sustaining care that is considered no longer in the best interest of the patient) or uncontrolled (in persons whose death occurs following sudden cardiac arrest and unsuccessful resuscitation) (18,24). The legal frameworks that govern this donation pathway are heterogenous, with different laws and protocols governing permissible pre- and peri-mortem interventions, methods of determination of death and duration of "no touch" periods(25). For the purposes of this thesis, DCD will refer to controlled Maastricht III/IV DCD, as is the norm in the UK (18,26).

DCD liver transplantation is inherently riskier with long-term data clearly demonstrating inferior outcomes in previous eras of transplantation (26–28). Therefore, despite more donors being available through greater numbers of DCD donors, the overall transplant activity has not increased to the same extent. This challenge has sharpened the focus of academic transplant professionals

on methods to better utilise the available donor pool, mitigate the associated risks and enable implanting surgeons to transplant these grafts with increased confidence (19,26,29,30).

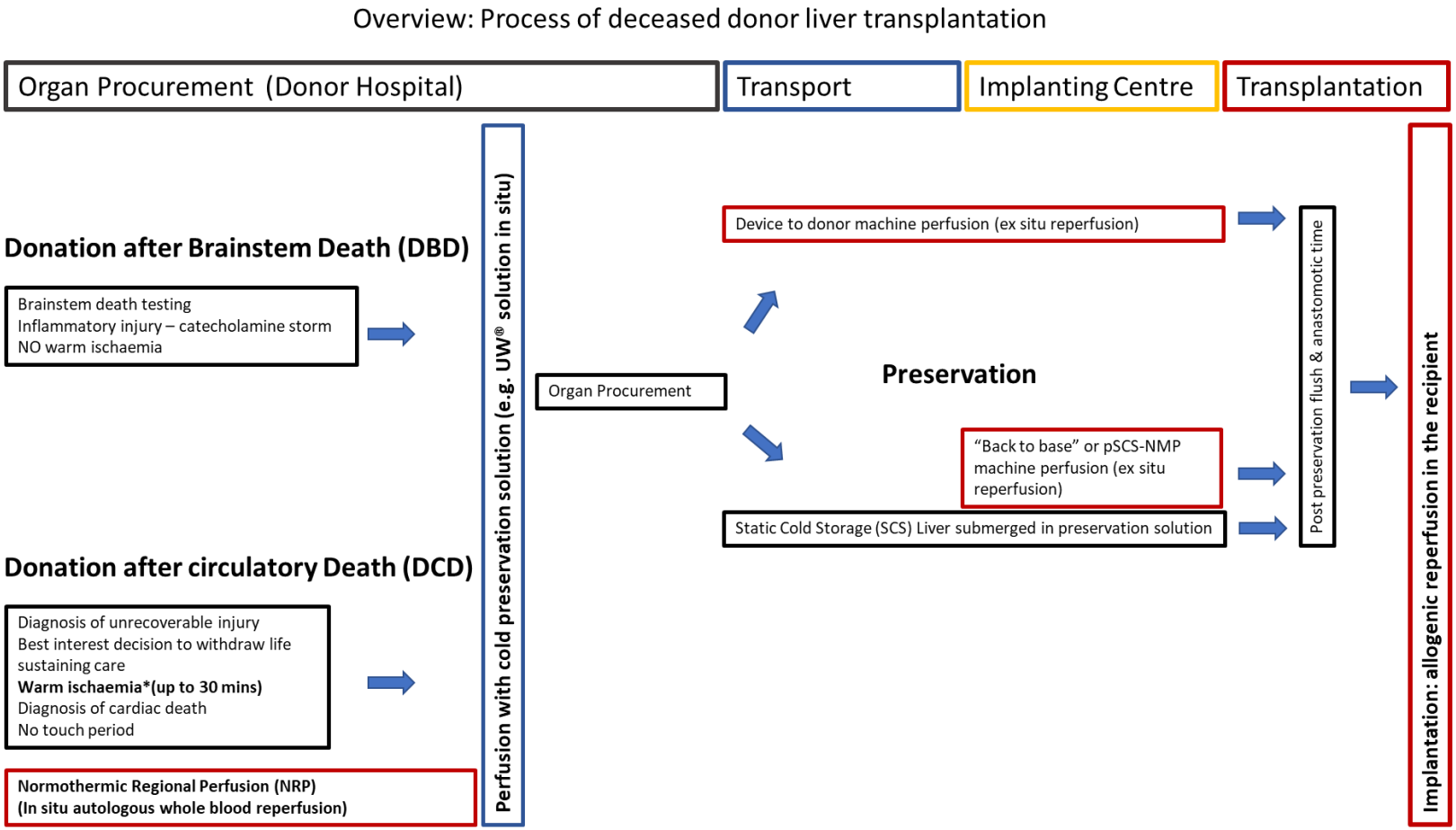


Figure 0.1: Overview of donation, preservation and transplantation process. Following the declaration of death in DCD grafts, in situ perfusion with normothermic regional perfusion (NRP) can be employed for organ recovery and assessment prior to cold perfusion. Cold flush, organs can either be initiated on a portable normothermic machine perfusion (NMP) device at the donor centre for transport and preservation un until immediately prior to implantation, or this can be initiated at the implanting centre after SCS for transplant (Back to Base). In both cases, the organs are flushed prior to implantation and there is a period of ischaemia associated with the anastomotic time at the end of preservation prior to reperfusion.

1.2.3 Extended criteria Donors (ECDs)

Beyond donor type (DBD vs DCD), a great proportion of additional donors are older, have more co-morbidities and have a higher degree of steatosis, dramatically reducing the number of these donor livers that can be deemed suitable for transplantation. Moreover, when these 'marginal' donor livers are accepted for transplant, they carry a significant risk of worse early and long-term outcomes (31,32). Other donor, recipient and preservation-related factors can also impact the suitability for transplantation of a graft, such as size mismatch, donor biochemistry, logistical considerations that impact preservation times and recipient factors such as modified end-stage liver disease score (MELD/UKMELD) or re-transplantation; all of which can alter the risk profile of a liver (26,33). This higher risk, previously referred to as 'marginal' cohort of livers is increasingly referred to as extended criteria donor (ECD) livers and they have been repeatedly shown to have inferior early and late post-transplant outcomes (26,32,34,35). A combination of poorer outcomes and increased complications that are often costly and resource-intensive (e.g. prolonged critical care stay, the requirement for renal replacement therapy (RRT), interventional radiology, endoscopy interventions and re-transplantation), have shaped the risk appetite of surgeons for accepting and transplanting these livers and resulted in a significant donor organ discard rate (36). At the centre of the consideration of whether a donor liver is suitable for transplantation, is a pathophysiological process that links donation, preservation and the recipient: ischaemia reperfusion injury (IRI). An understanding of IRI and its impact on liver grafts from the moment of donation to transplantation and beyond is critical if we are to unlock the enormous potential of the available donor organs and begin to meet the growing demand for transplantation.

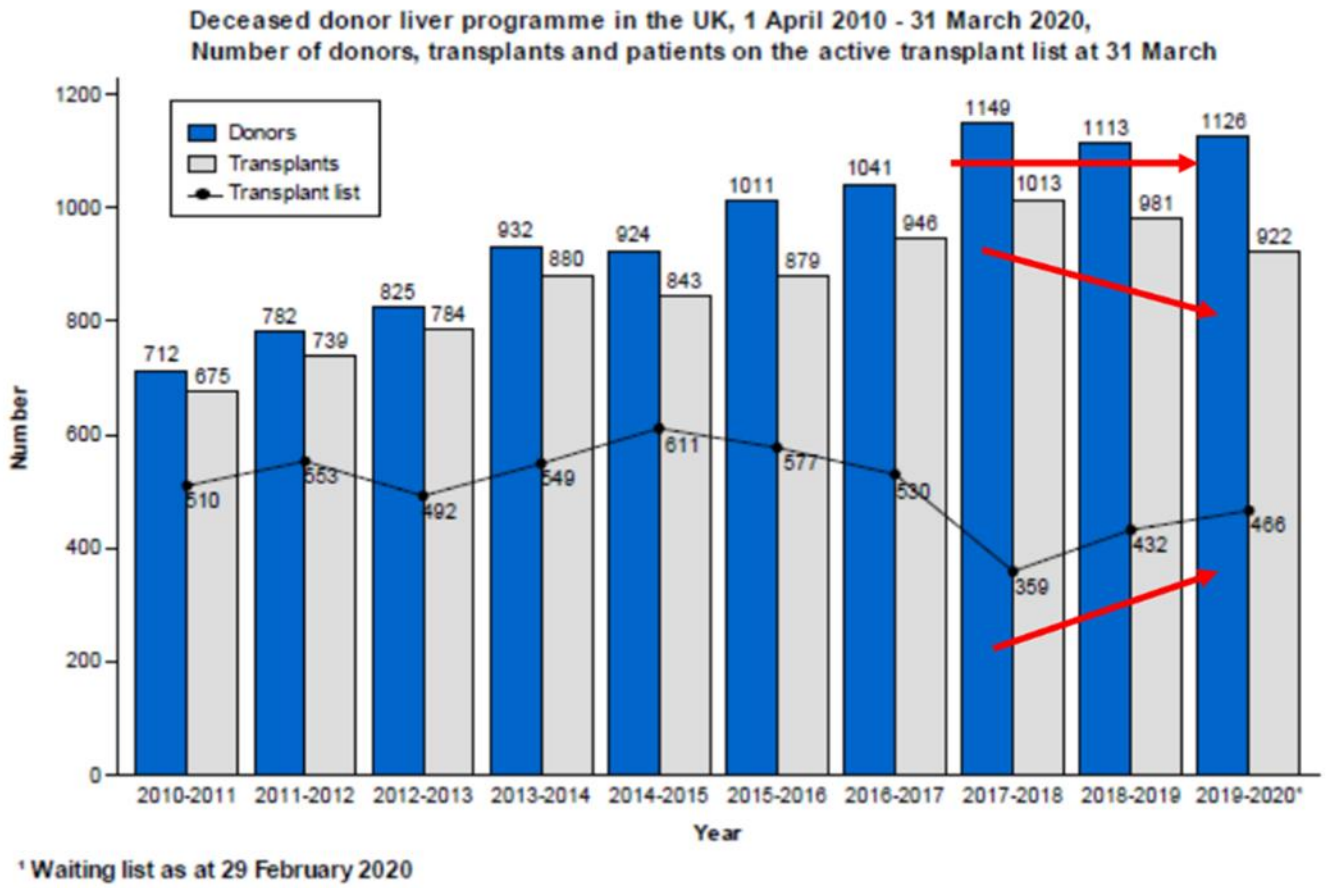


Figure 0.2: Pre- covid UK Liver transplant activity, waiting list and donor numbers. NHSBT Annual report 2019/20. Red arrows demonstrate stable donor numbers, falling transplant activity and rising waiting list numbers.

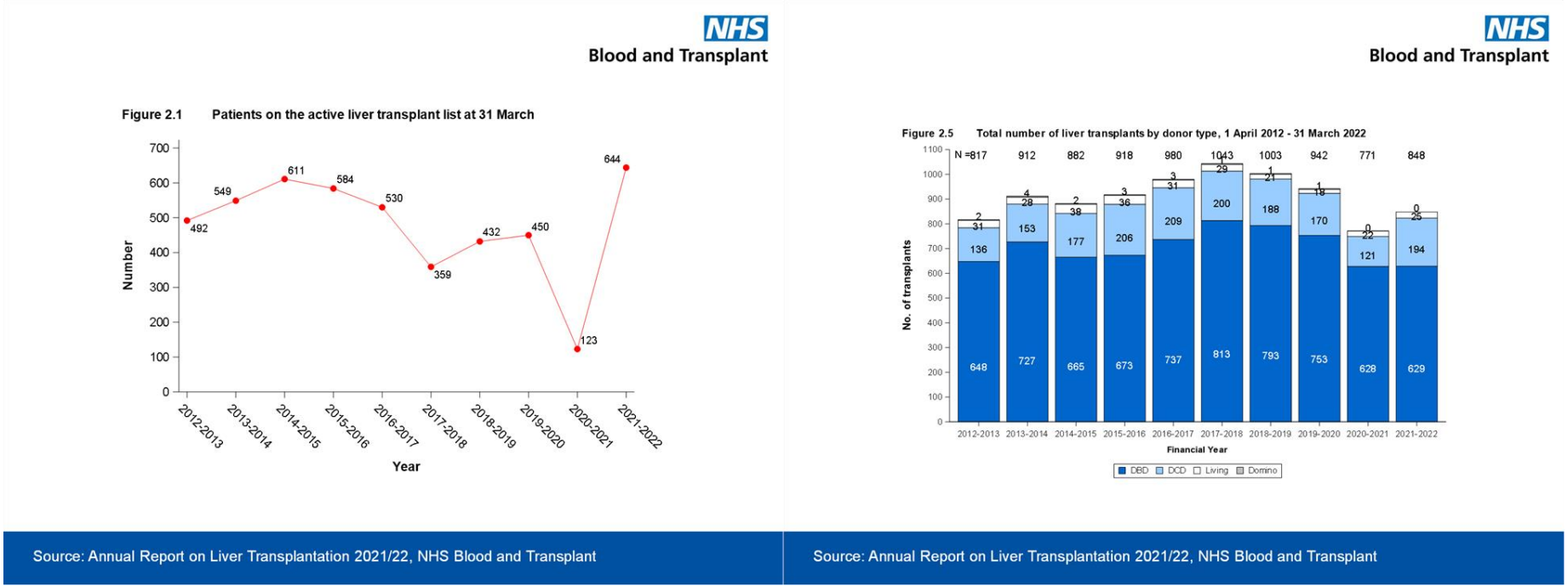


Figure 0.3: Post covid UK Liver transplant activity, waiting list and donor numbers. NHS BT Annual report 2021/22. LEFT: The waiting list in 2020/21 was affected by the COVID-19 pandemic and national protocols and interventions resulted in a sharp decline in listing for transplantation. RIGHT: the transplant activity fell sharply in 2020/21 due to the pandemic but was also on a downward trend dating back to a peak in 2018.

1.3 Ischaemia Reperfusion Injury

The fundamental reason for the higher risk profile of ECD grafts, as mentioned earlier, is the central process in liver transplantation of IRI, whose underlying pathophysiology is an amalgamation of several complex interactions between molecular and immunological processes resulting in an often-critical impact on transplant outcomes and the development of complications (37). A sensitivity of donor livers to this original adversary of transplantation has driven developments in preservation research and technological applications that have sought to mitigate the deleterious effects of IRI, in an attempt to improve post-transplant outcomes (38). The process of solid organ transplantation uniquely involves a number of essential stages: donation, storage and transport (preservation) and finally reperfusion, which collectively create the conditions that necessitate IRI and thus underpin the molecular events that result in the clinical manifestations of IRI (39) (Figure 0.1).

1.3.1 Ischaemia

IRI is initiated during the donation process, with the onset of ischaemia. The mitochondria within the graft, which are primarily responsible for energy production in the form of adenosine triphosphate (ATP) are starved of oxygen, impeding this and other functions such as cell signalling, DNA protection, synthetic function and maintaining the electrochemical integrity of the cell (40). Despite being rapidly cooled to 4 degrees, the metabolic demands of the graft quickly outstrip the aerobic energy supply available in this now hypoxic static state, forcing the liver to resort to anaerobic respiration to generate the requisite ATP. Mitochondrial hypoxia during ischaemia results in mitochondrial electron transport chain disruption and subsequent TCA cycle disruption resulting in the accumulation of metabolites such as NADH (complex 1) and succinate (Complex II) (41). This results in lactate production and intracellular acidosis with an accumulation

of intracellular hydrogen ions. In order to reverse this, the H+/Na+ exchanger works to expel incoming H+ ions thereby increasing intracellular Na+. Consequently, cellular oedema ensues, directly damaging the cells. Concurrently, as the ATP:ADP ratio falls, there is reduced activity of the ATP-dependent Na+/K+ pump and a further increase in intracellular Na+, worsening cellular oedema and accelerating the vicious cycle that ultimately causes cell death with membrane disruption. Furthermore, hepatocellular oedema results in the narrowing of the sinusoidal lumen and reduced microcirculatory flow capacity upon restoration of blood flow, all of which exacerbate the insult upon reperfusion (42). Additionally, calcium homeostasis is disturbed, as it relies on ATP-dependent processes to maintain a low intracellular Ca2+ concentration. Consequently, rapid ATP depletion can result in intracellular Ca+ overload, mitochondrial permeability transition pore (MPTP) formation, mitochondrial dysfunction and cell death during the reperfusion phase (37,38,43–45).

1.3.2 Reperfusion

Paradoxically, the re-introduction of oxygen upon restoration of blood flow to ischaemic tissue initiates an injurious cascade. The rapid metabolism of anaerobically accumulated metabolites (such as succinate) via a dysregulated electron transport chain, produces bursts of reactive oxygen species (ROS) that mediate the core drivers of injury via damage-associated molecular pattern (DAMP) signalling through Toll-like receptor (TLR) activation and innate immune responses (37,46).

The basis of this injury is multifaceted; however, this key pathological event of a rapid burst of ROS from the mitochondrial respiratory chain is critical (44). ROS produced on reperfusion quickly overwhelm the normally protective anti-oxidant system of the liver, which can buffer brief periods of hypoxia under physiological circumstances and thus limit the detrimental effects of the

associated ROS production. In solid organ reperfusion, vast amounts of ROS are generated and therefore invariably cause pathological oxidative stress, which is an elemental constituent of IRI (44). As mentioned before, the oxidation of accumulated succinate (accrued during ischaemia) is a key driver of graft injury via reverse electron transport (RET) at mitochondrial complex 1 and induction of MTPT, which together with mitochondrial damage lead to cell death (47). Mitochondrial DAMPs released from damaged mitochondria and cell death, alongside metabolites such as succinate and other chromatin fragments (Histones, cfDNA, nucleosomes) enter the circulation and amplify the injury by initiating inflammatory innate immune responses (48). ROS are also directly toxic to endothelial cells and hepatocytes, inducing the release of cellular nuclear DAMPs such as High mobility group box 1 protein (HMGB-1) activating downstream pathways like NF-kappa-B and Nuclear factor erythroid 2-related factor (NRF2) leading to pro-inflammatory cytokine production (e.g. TNF-a, IL-1a, IL-6, IL-8) (49). This combination of ROS bursts, DAMP release and cytokine production drives IRI, propagating microcirculatory disruption and recruiting immune cells (39,49–52).

1.3.3 Injury

The downstream effects of ROS generation are the initiation of a self-amplifying process of inflammation and injury driven by the cellular and molecular events highlighted in (Figure **0.4**) and further amplified by innate immune activation (53). The vascular endothelium of the liver, within the sinusoids, is lined by specialised non-parenchymal cells called liver sinusoidal endothelial cells (LSECs). They play a critical role in regulating the microenvironment within the liver, contributing to antigen presentation and the creation of a tolerogenic milieu, but are also critical in modulating the response to IRI (54). LSEC dysfunction triggered by ROS produced during reperfusion causes microcirculatory failure as it results in the activation of adhesion molecules (VCAM-1, ICAM) and

reduces nitric oxide (NO) production, leaving unopposed vasoconstriction due to an imbalance in vasoactive molecules (NO and Thromboxane A2)(42,55). Furthermore, activated endothelial cells increase the production of pro-inflammatory cytokines and activate complement which in combination with increased expression of adhesion molecules recruits acute inflammatory immune cells that add to the injury. (42,54)

Under normal physiological conditions, Kupffer cells (KCs), which are resident macrophages within the liver, patrol the liver acquiring/sampling the multiple antigens that are exposed to the liver via the portal system, scavenging them and presenting them to T-cells in a tolerogenic manner (56,57). The uniquely tolerogenic microenvironment of the liver is dependent on this system, alongside regulatory T cells, LSECs and other immune cells that predominantly express a tolerogenic phenotype and produce anti-inflammatory cytokines such as IL-10 to create a tolerogenic milieu (58). Although this microenvironment can withstand the large antigen load passing through the liver from the gastrointestinal circulation without constantly initiating inappropriate pro-inflammatory immune responses (59,60), in circumstances such as IRI, this can be overcome and the inflammatory state can prevail (53).

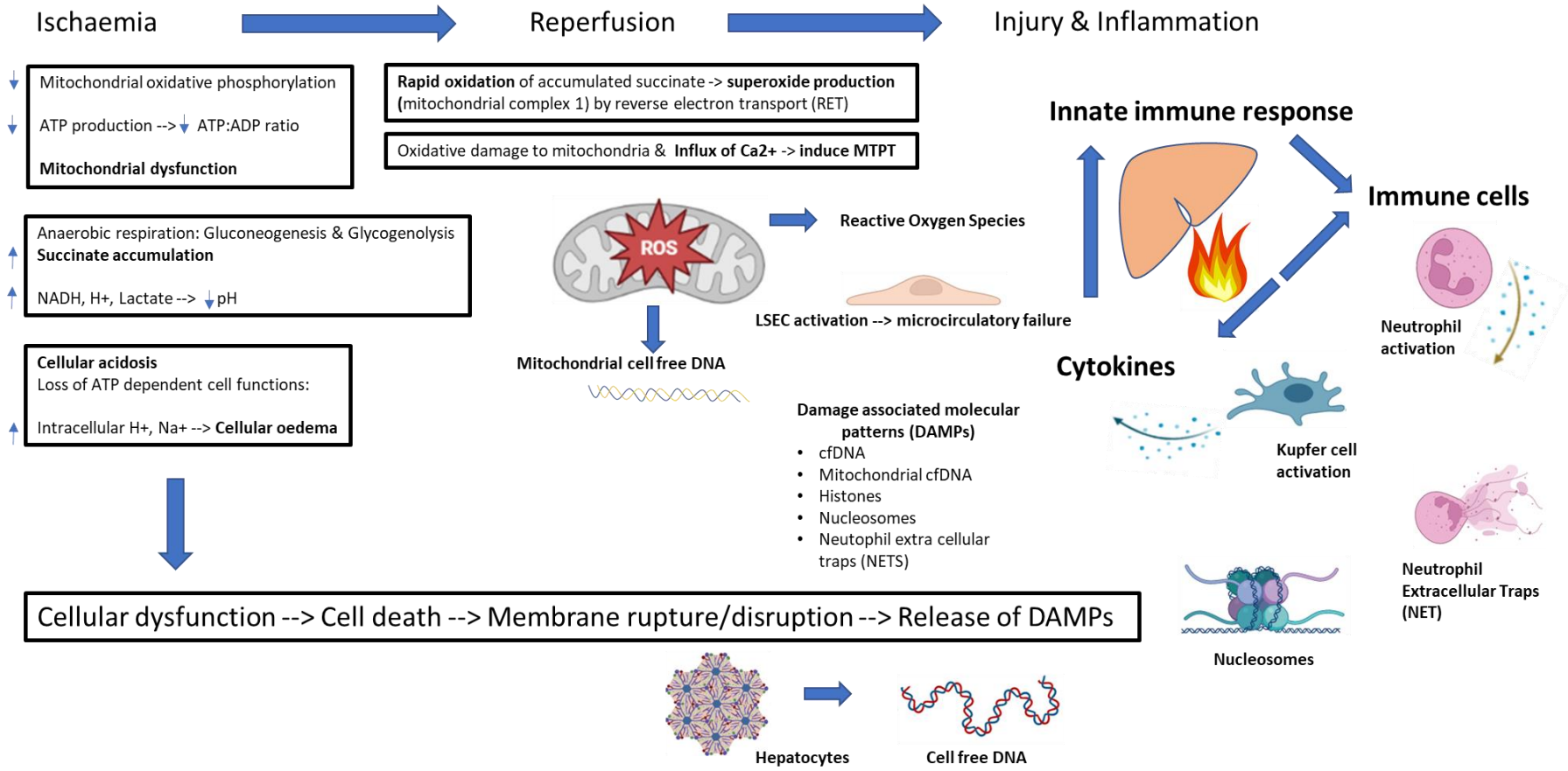


Figure 0.4: Overview of Ischaemia Reperfusion Injury. Ischaemia results in rapid ATP reduction and mitochondrial dysfunction, with cellular oedema and disruption due to loss of ATP dependent cellular functions. Upon reperfusion ROS are generated via RET from mitochondria cause damage to the graft via directly damaging endothelial cells and hepatocytes, inducing the release of cellular and mitochondrial DAMPs and activating downstream pathways like NF-kappa-B and leading to cytokine pro-inflammatory cytokine production. This combination of ROS bursts, DAMP release and cytokine production drives IRI, propagating microcirculatory disruption and recruiting immune cells such as Kupffer cells and Neutrophils that further amplify the injury through cytokine release, NETosis and inflammatory signalling. *(This illustration was composed with the assistance of Biorender)*

1.4 Damage-associated molecular patterns and IRI

DAMPs are key propagators of the inflammatory process that ensues upon reperfusion (outlined above). DAMPs interact with pattern-recognition receptors (PRRs) to trigger innate immune responses and inflammation (61,62). DAMPs encompass a broad range of molecules that typically originate from an intracellular source (nucleus, cytoplasm or mitochondria) and have the capacity to initiate pathways that lead to inflammatory responses (63). A subset of these molecules originate from the nucleus, referred to as 'nuclear DAMPs' (64) which I will use throughout the thesis or a recently coined term 'Chromatin associated molecular patterns' (CAMPs) (65) and these molecules can be particularly injurious in liver IRI.

1.4.1 Nuclear DAMPS

Chromatin is a structural complex of DNA, with basic (and positively charged) proteins associated with acidic (and negatively charged) DNA. The positively charged histones combined with the negatively charged DNA form a core unit called a 'nucleosome' that collectively are the basis of chromosomes. Nucleosomes have four pairs of histone proteins (H2A, H2B, H3 and H4) in an octamer wrapped with 147 bp of DNA, which have H1 (linker) histone proteins at the DNA tails (65). This structural arrangement is designed to facilitate the compaction of DNA within the nucleus but also has an important role in genome stability, gene transcription and multiple cell functions reliant on gene transcription. Chemical modifications of chromatin (or epigenetic marks) can alter its structure and expose different elements of the DNA to enzymes and transcription factors resulting in altered gene expression (66). Nucleosomes, but also free nucleic acids and proteins such as cfDNA, free histones, mitochondrial DNA, cfRNAs, HMGB-1 and extracellular traps (ETs) are nuclear DAMPS when released into the circulation following, typically unprogrammed, injury or death of cells (50,67,68). These molecules bind to TLRs (4,2,7 and 9),

RAGE (Receptor for Advanced Glycation Endproducts) and other proinflammatory PRRs to activate innate immune response (69–71). The presence of these nuclear DAMPs in the circulation, in the extracellular compartment, is well recognised as a biomarker of injury and inflammation but is now increasingly seen as a key propagator of this injury by amplifying the signal (47,61).

1.4.2 Neutrophils & NETosis

Circulating neutrophils are recruited to the site of injury, and through activated endothelial cells, adhere and transmigrate into the sub-sinusoidal space via integrin αMβ2 (Mac1)-dependent adhesion to endothelial ICAM-1 expressed on LSEC following reperfusion and guided by KC derived chemokines and a chemotactic gradient from the site of injury (72–74) Neutrophils aggravate injury by promoting inflammatory responses and in the context of IRI release neutrophil extracellular traps or NETs (extracellular DNA decorated with histones and granular proteins) that have been implicated in propagating the liver injury further (75). The process of NETosis has only relatively recently been described in detail (76), but denotes a distinct set of neutrophil cell death pathways which differ from caspase-dependent apoptosis in which apoptotic bodies are formed from controlled cell dissolution in classic apoptosis, with no inflammatory sequelae. Instead, NETosis describes separate pathways (lytic and non-lytic) which ultimately result in cell membrane rupture and release of nuclear proteins and chromatin into the circulation. In the lytic process, nuclear de-lobulation is characteristic and immediately progresses to cellular polarisation and chromatin de-condensation as the plasma membrane ruptures releasing extracellular chromatin fragments. The non-lytic pathway, is not associated with cell death but characterised by the expulsion of decondensed chromatin with a web like DNA structure decorated with granular and other intracellular proteins into so called NETs that reside in the extracellular space. The net result, is an initiation of number of processes that exacerbate IRI such as thrombosis and exacerbation inflammation-driven injury within the graft (70,71,77)

1.5 Clinical manifestations of IRI

The clinical sequelae of IRI in liver transplant covers an entire spectrum ranging from transient haemodynamic instability upon reperfusion easily managed intraoperatively or mildly deranged biochemistry post operatively; to potentially fatal cardiac arrest upon reperfusion with severe post-reperfusion syndrome (PRS) or early graft failure as seen in primary non-function (PNF).

1.5.1 Intraoperative

The effects of IRI can be observed immediately, with intraoperative haemodynamic instability and biochemical derangement. This clinical entity, PRS, is a phenomenon that has been observed and recognised throughout the history of clinical transplantation but classified and defined by Hilmi & colleagues (78,79). The authors ascribed the observation of dysrhythmias, reduced mean arterial pressure and systemic vascular resistance, right heart strain (increased pulmonary artery pressure/pulmonary wedge pressure /central venous pressure), acidosis, hypothermia, hyperkalaemia coagulation dysfunction (hyperfibrinolysis) to this syndrome and this has formed an important part of stratifying magnitude of IRI following liver transplantation (78).

1.5.2 Post operative

When IRI is severe, the effects go beyond the intraoperative cardiovascular instability described above and cause graft dysfunction in the immediate postoperative period. When function eventually recovers, this is referred to as early allograft dysfunction (EAD). The definition of EAD has evolved over the years, the most widely accepted definition is a binary distinction between immediate graft function and EAD (Olthoff criteria, EAD is any one of: bilirubin level ≥10 mg/dL on day 7, an international normalized ratio(INR) ≥1.6 on day 7, and an alanine aminotransferase (ALT) or aspartate aminotransferase (AST) level >2000 IU/L within the first 7 days after LT (80)), this definition was widely adopted and validated in the MELD era of

transplantation(81) by Dr Kim Oltholff. This has recently shifted to continuous scores such as the Model for Early Allograft Function (MEAF) score (82,83) and the 'Liver Graft Assessment Following Transplantation' (L-GrAFT) (84,85). MEAF is a score ranging from 0 to 10, providing a 10-point scale to capture the spectrum of EAD and more accurately predict early outcomes (3month patient survival) based on increased hazard ratios, achieving superior assessment of transplant survival compared with the categorical/binary Oltholff classification of EAD. Most recently, the L-GrAFT model was developed, which incorporates 10 post-liver transplant days of serum AST levels, a marker of graft injury, and the INR and bilirubin levels as measures of graft synthetic and metabolic functions and provides an accurate predictive tool for 3-month graft failure. This was validated in the COPE liver NMP trial demonstrating that L-GrAFT$_7$ performed better than other predictive algorithms, with an area under the receiver operating characteristic curve (AUROC) of 0.78, significantly superior to binary EAD (AUROC 0.68, $p = 0.001$) and MEAF scores (AUROC 0.72, $p < 0.001$) (85).

In some cases, reperfusion injury causes graft dysfunction that doesn't resolve, resulting in PNF. This is a devastating scenario in which the liver fails to function sufficiently to sustain life, necessitating 'super urgent' re-transplant to avoid early postoperative mortality (86,87). This clinical scenario is often complex and multifactorial, but the importance of IRI in this is well established and this has significantly influenced donor acceptance practices by implanting surgeons (88). Also devastating, is the occurrence of coagulation dysfunction in the context IRI and the consequent prothrombotic state which ensues, potentially contributing to the development of hepatic artery thrombosis (HAT), which also requires early re-transplantation as graft loss is inevitable (89–91).

Furthermore, IRI is implicated in the pathophysiology of non-anastomotic biliary strictures (NAS) also commonly referred to as ischaemic type biliary lesions (ITBL) or ischaemic cholangiopathy (IC) (35,92). This clinical entity is specifically troublesome in the context of DCD liver

transplantation and is one of the key issues limiting the use of DCD livers. Patients that go on to develop biliary complications can require repeated endoscopic interventions, re-operation and, often re-transplantation, prolonging admission and carrying substantial morbidity for the patient (35,93).

IRI can also be manifest through its impact on other organ systems, typically the kidney, requiring patients to be on renal replacement therapy post-transplant (94). The aetiology of post-transplant acute kidney injury is multifactorial and complex, with IRI often being a contributory (but not sole) factor (95). What is clear, is that in patients who do go on to develop AKI, long-term outcomes are worse (95), thus efforts to minimise the contribution of IRI remain a major area of focus in the field.

1.5.3 Immunological, oncological and longer-term consequences of IRI

The immunologic consequences of IRI are important considerations owing to their direct impact on graft outcomes (96–98). Beyond the increased risk and impact of acute rejection on longer-term graft survival (99), the role of IRI in setting the immunogenicity of transplanted grafts may be crucial in determining the capacity of immunomodulation and tolerance induction within these grafts.

Early observations in experimental porcine liver transplantation by Sir Roy Calne found that the liver uniquely exhibited innate "tolerogenic properties" (100) sparking a transatlantic discussion on "Strange English Pigs" (101) and the unexpected immune properties of the liver observed in these experiments, which at the time were not reproducible in the USA, hence the Lancet editorial challenging these findings. Further experimental and subsequent clinical corroboration soon followed as it then became apparent that a proportion of human liver transplant recipients became 'spontaneously tolerant' of their liver grafts, whereby they had a functioning allograft in the absence of immunosuppression, typically stopped due to non-adherence (102). A formal

investigation of this clinical phenomenon was conducted in a series of systematic clinical immunosuppression withdrawal trials that produced a significant proportion of 'operationally tolerant' recipients with functioning grafts having been weaned off of immunosuppression (103,104). Although this remains an exciting area of research with huge promise, the patients currently eligible for enrolment in such trials constitute a very small subset of possible liver recipients (maximally ~15%) who meet stringent criteria for withdrawing immunosuppression (58). Suitability for immunosuppression withdrawal is largely dictated by the presence or absence of clinical/subclinical inflammation within the graft, a feature that may be related to the magnitude of IRI or its consequences (58,105). It is a reasonable hypothesis, therefore, that the impact of IRI on subclinical inflammation may have a critical role in determining the potential for 'operational tolerance' induction and the probability of successful immunosuppression weaning (58,105–108).

Finally, IRI has also been associated with longer-term adverse oncological outcomes such as the recurrence of hepatocellular carcinoma (HCC) (109,110). In a recent study of ischaemia-free transplantation conducted in China, Tang et al have shown reduced recurrence of HCC post-transplant when IRI is avoided altogether, providing impressive clinical data supporting this proposed mechanism of recurrence (111).

Beyond the morbidity associated with these clinical consequences, clinical IRI is additionally financially burdensome, as it increases the length of stay in critical care and hospital, together with the costs of additional procedures and associated healthcare spending (112).

1.5.4 Susceptibility to IRI: Extended Criteria Donor livers

The risk of developing severe IRI has conventionally been determined by some 'nonmodifiable' factors such as donor age, donor liver enzymes/biochemistry, DCD status and degree of steatosis; but also some modifiable factors such as the length of cold ischaemia, donor hepatectomy time, and anastomotic times (33,113–117). Grafts that have one or more of these features are often

referred to as ECDs, although there is no specific agreed definition of this term. Particular risk criteria that are relatively widely accepted include moderate/severe steatosis, advanced age, long cold ischaemic times, split or reduced size, from donors with elevated liver enzymes or high sodium and DCD livers (118–120).

1.6 Mitigating IRI

The influence of IRI on the fate of transplanted grafts is thus undeniable, making interventions to reduce its magnitude essential for safe transplantation and expansion of the donor pool (121,122). Many of the essential features and necessary logistical considerations of clinical transplantation relate to the central notion of addressing and managing IRI. An effective strategy to mitigate IRI is therefore fundamental to the entire process and outcome of transplantation, and includes: the technique of donor procurement to minimise warm ischemia; the development and use of specialised preservation solutions to maintain cellular integrity; the application of hypothermia to slow metabolism; the limits placed on preservation times to minimise ischaemic times; the emphasis on technical proficiency during implantation (19,117). All of these different aspects of transplantation directly relate to the pathophysiology of ischaemia reperfusion injury (Figure 0.4).

Throughout the history of liver transplantation, innovative technologies and strategies have been investigated and adopted to mitigate IRI (123). Ranging from relatively minor variations in traditional SCS with insufflation of oxygen to more radical approaches such as super cooling organs to sub-zero temperatures (124,125), different strategies have been trialed. However, despite initial optimism, many seemingly promising approaches have not translated to the clinic, even with encouraging pre-clinical or early clinical data (126). The major exception, however, has been the successful rise of machine perfusion technology (127–129). Machine perfusion is changing the

landscape of liver transplantation, with several high-quality clinical studies demonstrating that these technologies in situ and ex situ can effectively mitigate elements of IRI.

1.6.1 Machine perfusion technology

Machine perfusion technology encompasses a spectrum ranging from (in situ) normothermic regional perfusion (NRP), that occurs in the donor at the time of procurement (30,130,131) to ex situ applications such as normorthermic machine perfusion (NMP) and end ischaemic (dual) hypothermic oxygenated machine perfusion d-HOPE/HOPE (132–134) as well as any combination of technologies: D-HOPE-COR-NMP, (COR - controlled oxygenated rewarming) (135) and sequential NRP-NMP/d-HOPE (29). Adoption of these technologies in general has been variable, and some technologies, such as NRP, have been fully integrated into clinical transplantation in France and Spain with mandates for use in all DCD grafts, due to the exceptional results from multiple clinical studies of DCD liver transplantation (30,136). Yet, NRP remains unfunded in the United Kingdom (albeit the Scottish Government has committed to future funding) and infrequent in the USA (34.113,137). With respect to ex situ perfusion technologies, hypothermic machine perfusion applications, once limited by their evidence base, are now supported by more rigorous clinical data (134), and are being applied more widely. One significant hurdle with hypothermic preservation remains the lack of a validated multi-compartmental (i.e. biliary, hepatocellular, synthetic etc) functional assessment assay(s) to aid decision making on 'transplantability' during preservation (138). Normothermic machine perfusion (NMP), on the other hand, has achieved success partly on the basis of high-level evidence from randomised multicentre trials providing robust level I evidence (139,140), but largely because it enables organ transplantability to be assessed by measuring biochemical liver function in various ways. This thesis will focus on NMP of the liver.

1.7 Normothermic machine perfusion (NMP)

The concept of machine perfusion is not new; Alexis Carrell and Charles Lindbergh in the early 1930s established a glass chamber perfusion system able to support whole organs for prolonged periods (141,142). However, this complex system lacked an obvious direct application and failed to gain traction, falling out of favour shortly thereafter. In the 1950s the Gibbon heart-lung machine was the next major incarnation of the extracorporeal perfusion concept and went on to underpin the successful development of cardiothoracic surgery as a surgical discipline (143). This advancement coincided with the emergence of liver transplantation, which in the 1960s was an experimental procedure.

driven by enthusiastic groups, and a long way from mainstream clinical surgery. Although machine perfusion was used in some of the earliest liver transplants by Starzl et al (5), this fell from favour due to the development of successful static cold storage solutions (144) some research groups transiently explored versions of hypothermic ex situ machine perfusion for organ preservation (145,146) the development of specialised preservation solutions (147) and optimisation of transplant procurement and implantation techniques superseded these avenues of investigation and SCS in specialised solutions ultimately became and remain the standard of care in liver preservation for transplantation (148). Crucially, there has been a shift in the clinical challenges facing transplant clinicians outlined earlier in this chapter; related to mitigating preservation injury, increasing donor utilisation, and developing methods for organ donor evaluation in an era of higher-risk grafts (128,149). The most recent resurgence of machine perfusion for organ preservation has occurred amid rapid technological advancements, biomedical engineering developments, and an expansion in clinically relevant extracorporeal devices in cardiothoracic surgery, intensive care and nephrology, all of which have enabled its development. Therefore, the potential advantages of a perfusion device in terms of logistical optimisation, graft evaluation, and

thus efficient allocation and graft intervention and optimisation are manifest and have contributed to an explosion in the field (150–152).

1.7.1 Rationale of NMP

NMP maintains the liver in a quasi-physiological environment, ex situ, during preservation while being supported by vasoactive medications, nutrition, and protected with antimicrobials (128,150,152). This ex-situ environment permits cellular metabolism while the graft is being perfused at physiological temperatures, allowing organ assessment via assessment of each organ-specific cellular compartment (153,154). The development of NMP has occurred through multiple pre-clinical large animal studies that have initially established the technical feasibility but quickly progressed to demonstrate the superiority of NMP to traditional SCS preservation, purportedly achieving these results due to an ability to replenish ATP, avoid glycogen depletion and protect the mitochondria within the donor liver during preservation (155–159). This foundational large animal work, paved the way for a relatively smooth translation to human studies of NMP (150,160). An important concept in the design of NMP was the fact that ex situ reperfusion not only shortened the length of cold ischaemia but facilitated reperfusion (and thereby cell recovery) ex situ, in the absence of key elements of IRI such as platelets, complement and leukocytes, all of which are considered detrimental propagators of reperfusion injury in transplantation (91,161,162).

1.7.2 Translating NMP to the Clinical

In a crucial study, Ravikumar et al (152) showed that the observed benefits of reduced graft injury seen in porcine preclinical studies were also present in clinical human NMP, reporting on the first-in-human experience of cNMP. This study played a key role in the design of what would become the multicentre Consortium for Organ Preservation in Europe (COPE) randomised study (140). Although the pre-clinical models had provided critical mechanistic insights related to cellular energetics predominantly, the exact mechanisms and molecular processes underpinning the

benefits associated with human NMP have only more recently begun to be revealed (163,164) . Device to donor NMP (cNMP), where the device is taken to the donor hospital has provided, perhaps the most insightful data on the immune effects of NMP on human livers. In a study by Jassem & colleagues, 12 cNMP livers were compared with 27 SCS livers through transcriptomic tissue analysis using gene microarray of gene sets involved in key immunological processes. This revealed that 'pro-healing' and protective transcripts against oxidative stress such as nuclear factor erythroid 2-related factor 2 (NRF2) were upregulated in the NMP livers compared to SCS livers, which had proinflammatory expression patterns. NRF2, for example, is the master regulator of the antioxidant response and plays an important role in oxidative stress responses and has since been found to be important in liver IRI in multiple contexts, such as older donors (165) and steatotic livers undergoing NMP (166). This initial study has been hugely informative in generating key areas of interest for investigation and gaining a deeper understanding of the events occurring during NMP.

Notably, in the Jassem et al study, immunoprofiling of hepatic lymphocytes, the authors concluded that the Tregs: T-effector ratio within the graft was altered in favour of Tregs by NMP. This finding, is less clearly supported by the data presented. The method of isolation of cells, a cold flush and cell isolation process from perfusate, is an indirect measure and may not proportionally represent the tissue resident immune cell population in the way a homogenisation of tissue and flow cytometric analysis, as performed by (167) would. The methodology the authors adopted was adapted from prior work by the same group, which had suggested an acceptable degree of equivalence was achievable with this approach when compared to homogenisation (168). However, this was performed in the context of SCS deceased donor preservation and it is not clear if this approach holds when flushing an organ that has been perfused using NMP and whether the perfusate during perfusion would more representative than cold preservation fluid at the end of storage. As the full gamut of immune profiling techniques has become more accessible, greater

insights into the graft immune environment using more advanced molecular biology and immunological techniques might shed more light onto the immune compartment during NMP. But at present, the effect of NMP on the immune cell populations, molecules and cytokines in the liver and perfusate remains incompletely understood.

1.8 Clinical NMP

NMP has high level evidence to support its clinical application. Two large randomised multi-centre studies have been performed and reported similar findings in relation to the technology: improved early IRI related outcomes with NMP, improved graft utilisation and safe extension of organ preservation (139,140). However, neither study demonstrated a significant graft or patient survival benefit from the application of NMP. This finding was anticipated, given the exclusion criteria of donor livers (for both studies) the investigators only permitted the inclusion of livers that were standard criteria donor organs, already accepted for transplant, suitable for traditional preservation methods and procured with the intent to transplant (i.e. not considered to require viability assessment). This resulted in a homogenous group of livers with a high likelihood of excellent graft and patient survival. The studies were not designed nor powered to demonstrate a difference in these outcomes, albeit they were recorded as secondary outcomes. Instead, the trials were primarily focused on the IRI-related outcomes (e.g. EAD and peak AST within 1 week) which are validated surrogates of organ injury (80,169) and provided more specific information on the impact of preservation injury. Concerning the effect of NMP on IRI-related clinical outcomes, the evidence from the clinical trials is clearly in favour of NMP. In the COPE liver study, 220 livers were randomised to cNMP (121) or SCS (101), and the researchers observed a 50% reduction in peak AST (primary endpoint), a 63% reduction in PRS and 74% reduction in EAD (Oltoff criteria (80)). No statistically significant difference was observed in the radiological incidence of NAS, but the trials was not powered for this endpoint and the incidence of clinical events were low (one retransplant for NAS in each arm). Notably, NMP was also associated with the safe prolongation

of preservation time and greater organ utilisation (11.7% NMP vs 24.1% SCS discard rate), exemplifying the ancillary benefits of the technology not captured in the primary end point. With the recent publication of a similar study, with broadly similar results, in North America (139), cNMP is has the highest level of evidence to support its adoption in clinical transplantation when compared to all other technologies, which are also accruing clinical data (138). These landmark liver NMP studies have undoubtedly emboldened transplant surgeons and transplant professionals across the world, to adopt, develop and explore machine perfusion technologies in liver transplantation (127,151).

Although the results of the COPE liver study were unequivocal in terms of a reduction in IRI-related acute liver injury, there remained practical issues about how to deploy the technology, driven, in part, by logistical and technical considerations, but also by the structure of healthcare systems and funding models for novel devices such as these technologies (170,171). A possible solution to the inherent logistic challenge and complexity related to device to donor NMP, was the adoption of a 'back to base' approach whereby NMP is only initiated at the implanting centre after a standard procurement procedure and SCS for transport of the organ (170). This back to base or 'post SCS-NMP' (pSCS-NMP) approach has very quickly became the most acceptable approach to NMP within the UK, where National Organ Retrieval Service (NORS) teams perform organ procurement for all transplant centres, as opposed to individual units procuring their own grafts. The funding, training and delivery of this technology can therefore be based within the transplant centre obviating the requirement for national training of all NORS teams, universal adoption of protocols and avoiding the ambiguity regarding who bears the costs, as the implanting centre are able to control all of these factors. The key question however, was whether the clinical outcomes of pSCS-NMP were comparable to cNMP. To answer this, Ceresa and colleagues, recruited a cohort of (n = 31) NMP livers, using the same criteria as the recently completed COPE trial, from three UK transplant centres preserved with pSCS-NMP, and compared this to the UK

cohort of (n = 104) COPE NMP livers, to determine the extent to which prior SCS impacted clinical outcomes on NMP preserved livers (171). There were no differences in peak AST, MEAF or PRS with similar death graft and patient survival at 1yr. In parallel, a study was carried out in Canada (170) (n= 26) pSCS-NMP vs n = 17, which had similar findings: that pSCS-NMP had no significant negative impact on the outcomes of standard criteria donor organs preserved with NMP (170,171). These results were crucial, as they have shaped the practice of NMP in the UK, which is now almost exclusively pSCS-NMP. However, the extent to which these two differing modalities of NMP are truly similar, particularly on a molecular level, remains unclear. In the UK study, the risk profile of livers was slightly higher (risker) than in the original COPE liver study (DRI 1.87 (1.06-3.20) pSCS-NMP vs 1.45(0.78-6.35 NMP, p = 0.06) although this did not meet statistical significance. This may have impacted the graft survival which at 1 yr. was 84% pSCS-NMP vs 94% NMP (p=0.08), suggesting that the application of NMP may not alter the risk profile of grafts despite providing safe, prolonged preservation. It is important to note that this was a small study (n=30), the difference was not statistically significant and that factors unrelated to preservation could also account for the observed differences. However, this trend has become a more pressing concern as the risk profile of donor grafts being preserved with pSCS-NMP has altered substantially in recent years, with an increasingly complex and a high-risk cohort of livers being preserved in this way. An emerging picture of higher-risk livers, that maintain their propensity to develop preservation reperfusion injury despite NMP is increasingly apparent (Table 0.1). This has forced a revaluation of the mechanistic processes involved in NMP as well as the impact of pSCS, to better understand what is occurring during pSCS-NMP, that leads to preservation injury.

1.8.1 Clinical ECD NMP

In view of the excellent results with SCS in standard criteria donor livers and the resource implications associated with NMP, a focused approach is needed on the livers that are likely to gain the greatest benefit from NMP. The largest effect of NMP in terms of graft injury reduction

in the COPE liver study was observed in DCD livers (which had a two-thirds reduction in peak AST): this has since influenced how UK transplant units have employed NMP. Coupled with the logistical benefits of pSCS-NMP, the situation has arisen whereby the highest-risk grafts are preserved with pSCS-NMP, thereby effectively moving beyond the conditions that were studied in the original randomised clinical studies.

Furthermore, as clinical adoption has steadily increased, study designs have shifted from safety evaluation and standard criteria graft preservation to 'viability assessment' or transplantability of ECD grafts including those that would otherwise be declined for transplant. This cohort of livers represents the frontier of translational transplant preservation, as the majority of additionally available donor grafts yielded from rising donation rates are from older, more co-morbid and more steatotic donors, making their sensitivity to IRI greater and the requirement for improved preservation imperative.

These shifts towards higher-risk donor organs as well as the use of pSCS-NMP have been evaluated in a number of non-randomised studies (172–174). The results of these studies have been valuable, showing that organ utilisation has been significantly improved, PNF has been reduced and preservation extended with equivalent graft and patient survival to historic cohorts of ECD livers preserved with SCS. However, the IRI-related outcomes have been surprisingly disappointing (Table 0.1). ECDs have failed to show the improvements in IRI-related clinical outcomes observed in cNMP, instead being comparable or even worse than matched SCS livers and thus still carry significant risks. The mechanistic analysis of the impact of NMP on ECD livers after pSCS-NMP is yet to be done. Moreover, the extent to which the mechanisms observed in cNMP in standard criteria livers can be applied to marginal or extended criteria donor (ECD) livers (i.e older donors, donation after circulatory death (DCD) or steatotic livers) that undergo pSCS-NMP, is uncertain.

There is evidence, therefore, that the donor risk profile of grafts is not sufficiently altered such that ECD livers preserved with 'back to base' or pSCS-NMP, can go on to develop clinical manifestations of severe IRI. A deeper understanding of the underlying mechanisms of this modality of preservation and its impact on graft preservation injury must be a priority if we are to develop strategies to overcome these complications and safely increase the utilisation of higher risk grafts.

Table 0.1: Ischaemia reperfusion injury related outcomes from clinical normothermic machine perfusion studies:

Study & groups	NMP Preservation	EAD (SCS: group 1, group 2)	AKI/RRT (SCS group)	Ischaemic Type Biliary Lesions	Other findings: PRS, ATCMR,	Other findings: HAT
Watson et al 2018 47 liver perfusions 22 transplanted	pSCS-NMP	1/22 EAD 1/22 PNF	-	4/22 ITBL -> 3 re-transplants		
Mergental et al 2020 31 livers perfused 22 transplanted	pSCS-NMP	7(31.8%) vs 4 (9.1%) NMP vs SCS	4(18.2%) vs 11 (25%) NMP vs SCS	4 (18.2%) vs 1(2.3%) NMP vs SCS		
Reiling et al 2020 10 livers perfused 10 transplanted	pSCS-NMP	5 (50%) single arm study	2 (20%) (NA) single arm study		1 (10%) biliary anastomotic stricture	
Hann et al 2022 SCS1 (n = 31) SCS2 (n = 25) NMP (n = 26)	pSCS-NMP	12/31(39%) PNF 1/33 9/25 (36%) PNF 0/25 12/26 (46%) PNF 0/26	11/31 (35%) 9/25 (36%) 15/26 (57%)	4/31 (13%) 2/25 (8%) 1/26 (4%)	**Acute TCMR** **9/31(24%)** **6/25(29%)** **16/26(62%)**	**HAT** **1/31 (3%** **2/25 (8%)** **3/26 (12%)**
COPE Liver NMP (n =121) SCS (n = 101)	Continuous NMP (SCD)	12/121 (10.1%) vs 29/101 (29.9%) PNF 1/121 vs 0/101	25/121(21%) vs18/101 (18%)	1/121 vs 1/101 Both retransplanted	15(12%) vs 32 (33%)	
Back to Base MNP (31)	pSCS-NMP (SCD)	4/31(13%)	5//31 (16%)	No NAS	PRS: 3/31 10%	

Methods

This chapter outlines the methods commonly used in the thesis as well as some detailed information on the methods specific to individual chapters.

1.9 Normothermic machine perfusion device

Normothermic liver perfusions included within this thesis were all conducted using the OrganOx metra (2nd Generation) (OrganOx Ltd, Oxford, UK) NMP device. This commercially available device is both CE-marked (for up to 24 h) and FDA approved (for up to 12 hours) for the normothermic preservation of donor livers (140,152)

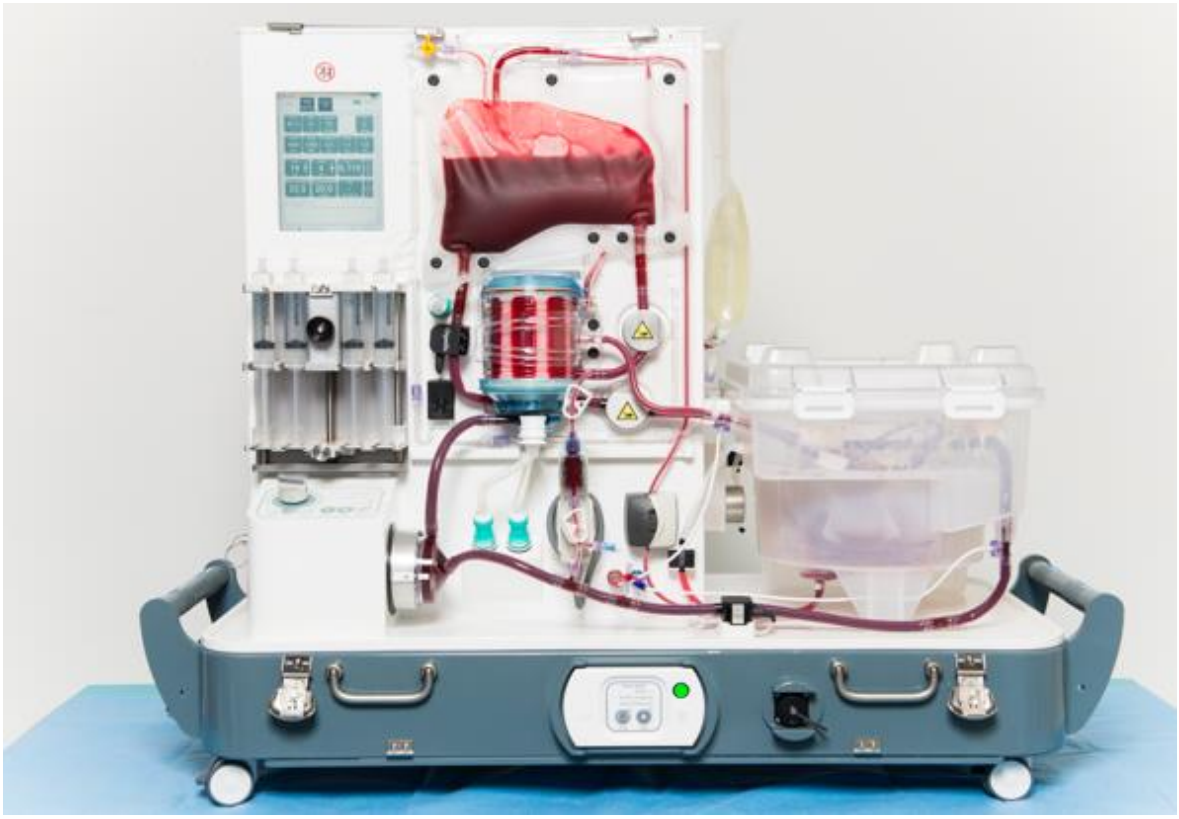


Figure 0.1: OrganOx Metra device. Second generation Organ Ox metra (OrganOx Ltd, Oxford, UK)

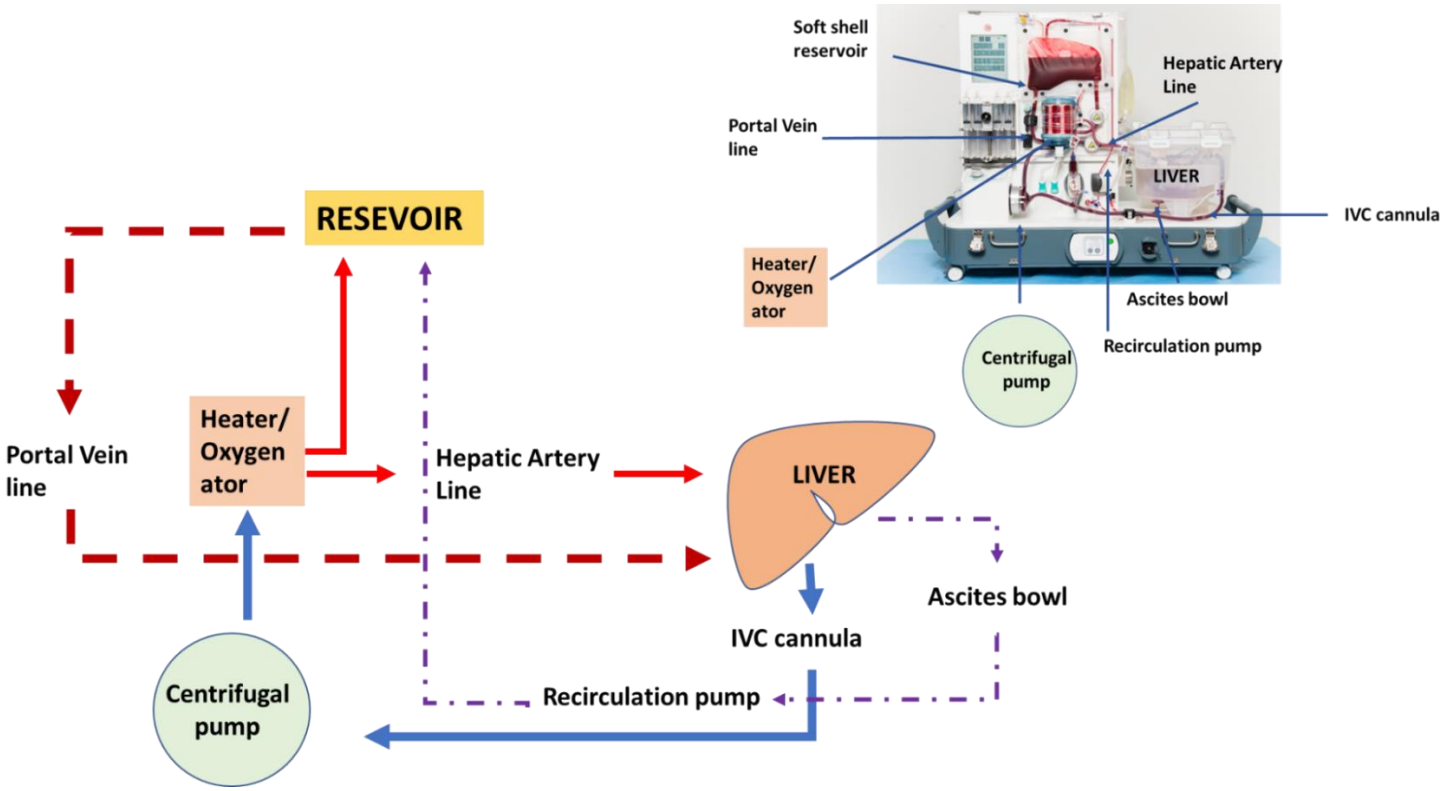


Figure 0.2: OrganOx metra circuit. Perfusate leaves the liver via the IVC cannula, passes through a flow sensor, before reaching the centrifugal pump. Perfusate is then pumped to the heater and oxygen concentrator (from air), warming the perfusate to 37˚C. The oxygenator is programmed to achieve a pO2 between 12-18kPa. Perfusate exiting the heater/oxygenator is then divided between a line delivering directly to the liver through the hepatic artery (a low flow, high pressure system) and a line going to the soft-shell portal reservoir. This line has a pinch valve, which is used to regulate the arterial pressure. For the purposes of continuous blood gas analysis and monitoring using the Terumo (shunt sensor), a shunt from the (hepatic) arterial, to IVC line passes through a blood gas analysis sensor which provides real-time values for pO2, pCO2 and pH. From the soft-shell reservoir, perfusate passes through a flow sensor on the portal line, to the portal vein line and canula, which is a high flow, low pressure system, with a pressure dictated by the height of the fluid level within the reservoir relative to the liver. The portal line has a pinch valve, which provides safety by preventing air from entering the liver. If the portal reservoir fluid level falls, (e.g. due to excess bleeding from the liver into the ascites bowl), any air detected in the portal flow sensor causes the pinch valve to close fully, therefore, preventing the entry of air to the liver and allowing the reservoir to refill. Blood collected in the ascites bowl from liver is recirculated from the bottom of the liver bowl to the reservoir via a roller pump. This re-circulates perfusate at a rate of 50ml/min. Bile is collected in a separate chamber within the liver bowl, passing through a sensor to record output in ml/h automatically but can also be manually collected out-with the flow sensor.

1.10 Perfusate composition

The circulating perfusate was composed of a red cell-based oxygen carrier, colloid, medications and nutritional supplementation. The composition of perfusate varied according to the experimental set-up in the porcine experiments, however in the human liver perfusions was standardised. Human liver NMP, packed red cells were donor/recipient-matched supplemented with 500 ml of Gelaspan (B Braun, Sheffield, UK), a colloid that was added to the perfusate normalise the haematocrit and osmolarity in human liver perfusions. Porcine liver NMP, leuko-depleted autologous blood or allogenic whole blood that was donor/recipient ABO matched (the latter was used for simulated transplant reperfusions) was used.

Boluses: were added to the reservoir during priming of the device

- 10% calcium gluconate (B Braun), 10mls to correct the binding of citrate to calcium

- 10,000 IU unfractionated heparin sodium (Wockhardt UK Ltd, Wrexham, UK) prevents thrombosis

- 750mg of cefuroxime (Flynn Pharma Ltd, Dublin, Ireland) or 1g Meropenem (Pfizer, Kent, UK) in the circuit and protect from bacterial overgrowth, respectively *(in porcine perfusions, Meropenem 500mg was given)

- a bolus of 30ml 8.4% sodium bicarbonate (BBraun) was administered to normalise the perfusate pH prior to connection of the liver (given at 37˚C).

Continuous infusions: All solutions were made up to 30ml with 0.9% sodium chloride (B Braun) and delivered via an integrated automated pump at a rate of 1ml/h. Four continuous infusions were administered:

- 25,000 IU unfractionated heparin sodium (Wockhardt UK Ltd) to prevent thrombosis during perfusion.

- 200 units insulin (Actrapid) (Novo Nordisk, West Sussex, UK) to assist with perfusate glucose control.

- 4.5g sodium taurocholate (bile salts) (OrganOx Ltd) to compensate for loss of exogenous bile salts.

- 0.5mg epoprostonol sodium (FlolanÒ) (Glaxo, Middlesex, UK) to optimise microcirculation.

For nutritional purposes, an infusion of total parenteral nutrition (TPN) (Nutriflex Special, B Braun) was infused at a rate of 4ml/h when the perfusate glucose level fell below 10mmol/L as a source of glucose and amino acids for liver maintenance.

1.11 Monitoring during NMP

The device was set up and primmed to meet optimal operating conditions: temperature (37° C), pO2 (12-18 kPa), pCO2 (4-6 kPa) and pH (7.25 – 7.35). During perfusion, the graphical user interface (GUI) provides real-time perfusion parameters and these data are recorded and stored on the device and can be downloaded for further analysis.

1.12 Preparing the liver "Back-table"

Human livers were retrieved by the designated National Organ Retrieval Service team, having been initially accepted for clinical transplantation. During deceased donor multi-organ retrieval livers were cold flushed in situ with University of Wisconsin (UW) (Bridge to Life, London, UK) solution. Following donor hepatectomy, livers were either prepared and cannulated at the donor hospital and immediately placed on the metra ("continuous NMP") or transported on ice submerged in cold 4°C UW solution to the implanting centre and only initiated on NMP on arrival, in what is known as "back to base" NMP. Livers were prepared as described by Makowka et al (175)and cannulated, primed and connected to the OrganOx as previously described by Ravikumar et (152). Once the back-table preparation of the organ was completed, including the identification and reconstruction of aberrant anatomy where necessary, the graft was cannulated in preparation for perfusion. Briefly, a purse-string suture was placed at the infra-hepatic IVC using 2.0 prolene (Ethicon) and a 28 Fr bespoke cannula (Sorin, Gloucester, UK) inserted and secured in place. A 20 Fr cannula (Sorin) was secured in the portal vein and 10 Fr paediatric aortic cannula (Sorin) was secured into the coeliac trunk patch using 2.0 vicryl ties (Ethicon). Common bile duct cannulation was achieved with a 12-18 Fr T-tube (Summit Medical, Cheltenham, UK) provided within the OrganOx surgeon's pack and secured with 2.0 vicryl (Ethicon). The fully cannulated liver was then primed with 0.5-1 L of Gelaspan (B Braun), via the portal and then arterial cannulae ensuring no air remained in the liver/cannulae. Once cannulated and primed, the liver was connected to the metra and perfusion commenced. The prepared and cannulated liver is shown in Figure 0.3.

The technique of porcine liver procurement, back table preparation and cannulation is covered in detail in Chapter 6.

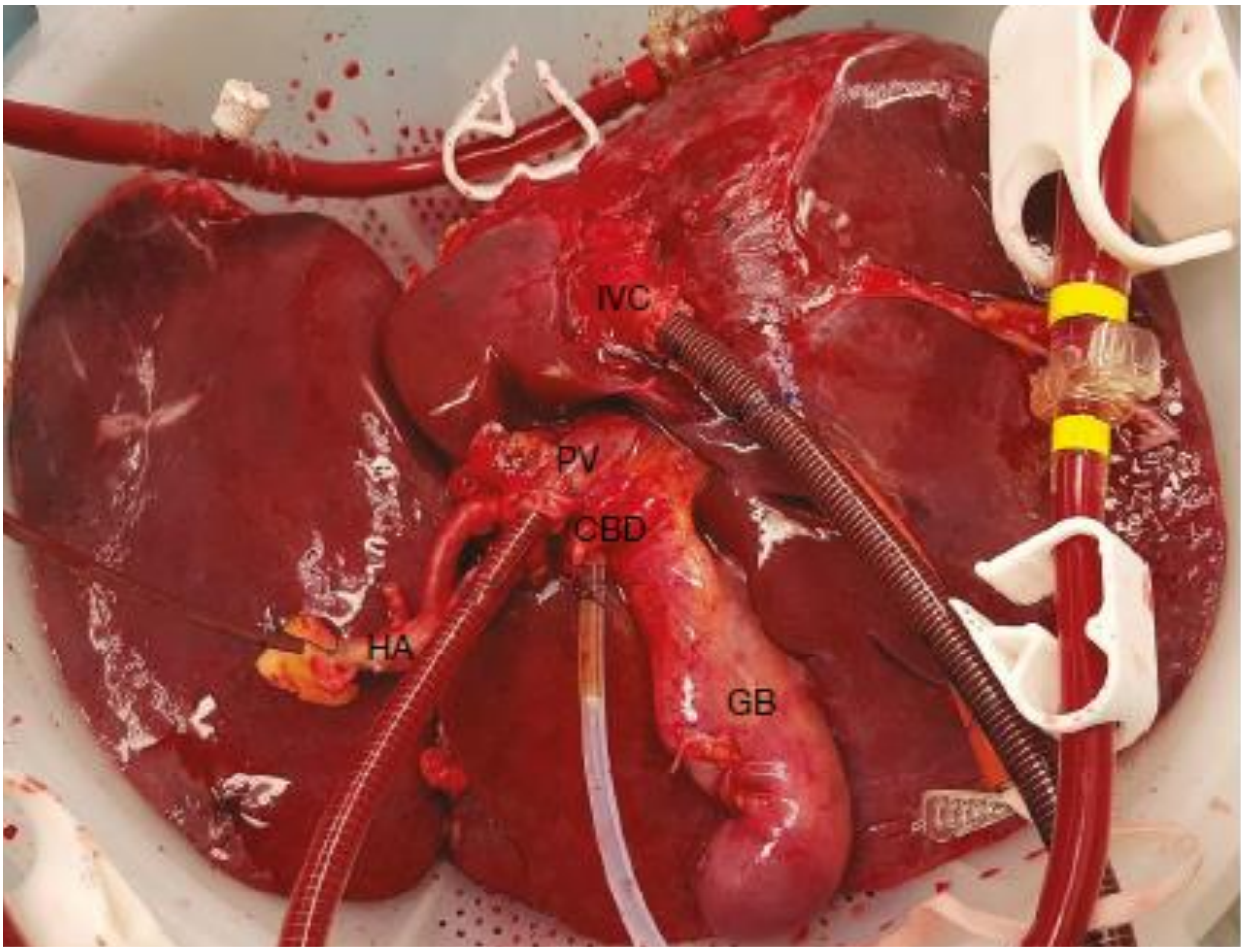


Figure 0.3: Image of cannulated human liver ready for the commencement of NMP. The inferior vena cava (IVC), portal vein (PV), hepatic artery (HA) and common bile duct (CBD) are all cannulated. The gallbladder (GB) is also present and has been closed. Image adapted from (140)

1.13 Clinical Studies, Tissue samples

Liver tissue samples analysed in this thesis were collected during two previous prospective clinical trials of NMP (140,171). These studies are summarised below, specifying the timing of sampling, sample handling and storage.

1.13.1 Consortium on Organ Preservation in Europe Liver NMP Trial: Multicentre randomized controlled trial of continuous NMP vs SCS.

The COPE liver trial (ISRCTN 39731134) was a phase III randomised trial conducted across 7 different European transplant centres which transplanted randomised donor livers to continuous NMP (n=121) or SCS only (n =101). In this study, the OrganOx metra® (1st Generation device) (OrganOx Ltd, Oxford, UK) was transported to donor centres. Following standard organ procurement, (DBD procurement or Maastricht III/IV DCD rapid cannulation technique), livers were placed on the device immediately after back table preparation and machine set-up (as previously described(Ravikumar et al., 2016c)). This approach minimised cold ischaemia times and maximised the proportion of preservation time spent on the device. In this study, samples were taken at the time of procurement on the back bench following removal of the graft and after cold flush (LT1), at the end of preservation (SCS or NMP, LT2) and following reperfusion (LT3). For the purposes of this thesis the NMP arm of this study will be referred to as continuous NMP or 'cNMP'.

1.13.2 'Back to Base' Study: transient static cold storage prior to normothermic machine perfusion.

The 'back to base' samples were collected from a study conducted across three U.K liver transplant centres, Kings College Hospital, (London), Royal Free Hospital (London) and Addenbrookes Hospital (Cambridge) (NCT03176433) between May 4th and July 18th 2017 (171) using the same enrolment

(inclusion and exclusion criteria) as the previous randomised trial (COPE). Whole livers from DBD and DCD (Maastricht category III donors) from adults were procured in standard fashion, following NHSBT National Organ Retrieval Service protocols. The livers were transported to the implanting centre stored in UW solution and under SCS conditions. NMP using the OrganOx metra®(1st Generation device)(OrganOx Ltd, Oxford, UK) was initiated as previously described (152). 30 of the 51 livers recruited were ultimately transplanted and therefore included in this study, with 21 recruited livers not meeting the study inclusion criteria. The outcomes of the 30 livers were compared to the UK NMP component of the continuous NMP arm (n= 104) and the SCS arm (n=182) of the COPE liver trial(171). The study protocol required transplanted livers to undergo a minimum of 3 h and maximum of 8 h SCS prior to initiation of NMP. Liver tissue samples were taken at the end of SCS (i.e prior to NMP, LT1 timepoint), at the end of NMP but prior to implantation (LT2 timepoint) and finally after reperfusion in the recipient, just before skin closure (LT3). For the purposes of this thesis, samples collected from this study will be referred to as 'pSCS-NMP' samples.

The trial was approved by the London Dulwich research ethics committee (16/LO/2196) and registered (NCT03176433).

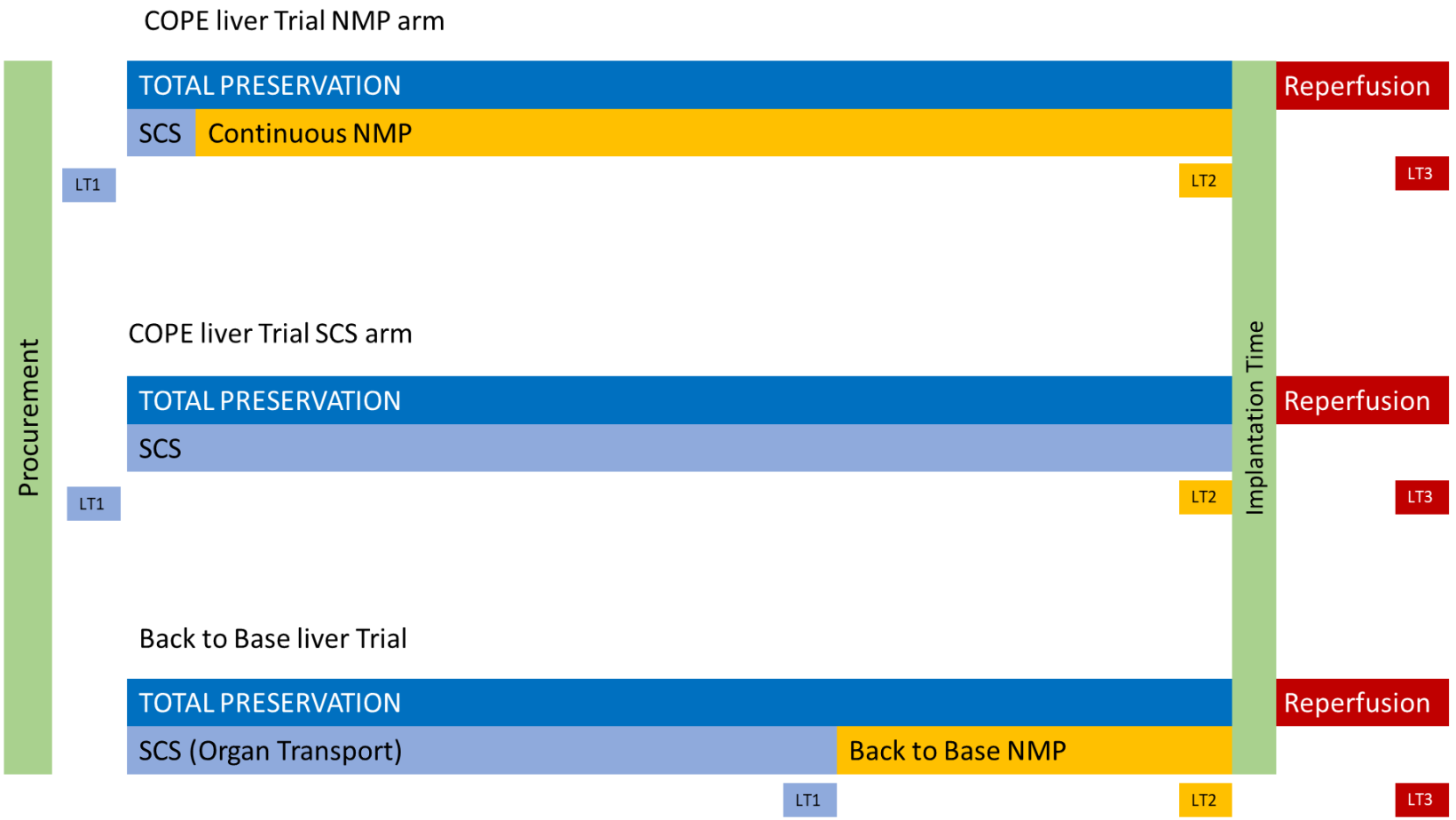


Figure 0.4: Overview of clinical studies, liver tissue samples & timepoints. (Top) COPE liver trial continuous normothermic machine perfusion arm (cNMP), (Middle) COPE liver trial static cold storage arm (SCS). Liver tissue (LT) samples were obtained at different times during preservation: LT1 = at procurement, LT2 = end of preservation (after NMP/SCS), LT3 = following reperfusion in the recipient prior to skin closure. (Bottom) Back to base study (pSCS-NMP), where samples were obtained at slightly different timepoints, LT1= end of SCS, LT2 = end of NMP, LT3 = following reperfusion in the recipient, prior to skin closure. COPE = consortium for organ preservation europe, NMP = normothermic machine perfusion, SCS = static cold storage, pSCS-NMP = prior static cold storage normorthermic machine perfusion.

1.13.3 Liver Tissue: Acquisition, handling & storage

Liver tissue (LT) was obtained using a Biopince™Full Core Biopsy Instrument (Argon Medical Devices, Texas, USA) using a 33mm throw length (yielding an approximately 29mm specimen length) or a small wedge biopsy taken depending on surgeons' preference. These samples were divided in half with a scalpel, with half of the tissue sample being immediately snap frozen in liquid nitrogen for 1-2mins and then transferred to -80 ˚C storage. The other half was stored in formalin at 4 ˚C for future histopathological assessment. These samples were collected as part of a biorepository established by the COPE PIs and accessed via an application to this group. Samples with a minimum of 2/3 timepoints were included, resulting in 57 samples in total from 23 livers. Sample handling was performed according to exactly the same protocols in both the back to base and COPE liver studies ensuring technical comparability of samples for subsequent analysis.

1.14 Proteomic Analysis:

Ion mobility spectrometry (IMS) combined with liquid chromatography (LC) and tandem mass spectrometry (MS/MS) were used to perform shotgun proteomics.

1.14.1 Mass Spectrometry Instrument: timsTOF Pro

timsTOF Pro instrument was used to analysis of all samples, it is a time-of-flight (TOF) mass spectrometer utilizing a trapped ion mobility spectrometry (TIMS) device operated with the parallel accumulation-serial fragmentation (PASEF®) scan mode. For all experiments, the timsTOF Pro was operated in PASEF mode.

1.14.2 Sample preparation

Tissue homogenization

Frozen liver tissue samples were kept on dry ice, weighed and cut (where possible) into two parts, approximately 0.5cm length was used for proteome analysis, and the rest of the tissue was kept frozen for future (Q-PCR or Western blotting) validation studies. Samples were lysed using 300ul of RIPA lysis buffer (Pierce) containing protease inhibitors (Roche) and phospho-protease inhibitor (Sigma) per10mg of tissue and homogenized in a beads beater (Precellys).

Tissue samples were added to tubes containing zirconia beads and RIPA lysis buffer and loaded on the beads beater. They were homogenized for 2 cycles (5000Hz, 20 sec), with cooling on dry ice between cycles. They were then centrifuged at 10,000g for 5 min at 4°C and the supernatant collected into new Eppendorf tubes. Protein concentration was determined by BCA assay and 50 ug of protein were aliquoted for subsequent reduction, alkylation and SMART digestion. The rest was frozen and stored with for validation experiments.

Reduction, alkylation and protein digestion

50μg of protein were transferred in a new tube and adjusted to a final volume of 100ul with 100mM Triethylamonium bicarbonate (TEAB) (ThermoFisher). The samples were denatured by adding 5ul of 200mM tris(2-carboxyethyl) phosphine (TCEP) (Sigma-Aldrich), followed by incubation at 55°C for 1 hour. For alkylation, a fresh 375mM solution of Iodoacetamide (IAA) was prepared. 5ul of the 375mM IAA were added to the samples and incubated for 30mins, also protected from light and at room temperature. The samples were then topped up to a volume of 200ul using 90ul of 100mM TEAB before adding 600ul of Methanol, then 150ul of Chloroform and being vortexed for 30 seconds. 450 ul of sterile water was then added prior to a further vortex for 30 seconds and centrifugation at max speed (17000g) at room temperature for 5 mins.

The upper aqueous phase was then pipetted off without disrupting the precipitate at the interface. 450 ul of Methanol was added to the sample containing the organic phase, and vortexed for 30 seconds prior to further centrifugation at max speed (17,000g) at room temperature for 2 mins. The supernatant was removed and the lid left open to allow the pellet to dry. Precipitated proteins were then resuspended with 50ul of 100mM TEAB before using the SMART Digest ™ Kit.

SMART Digest ™ Kit

50 ug of protein was loaded into a SMART digestion tube (Thermo Scientific™) containing 150ul of proprietary SMART digestion buffer containing immobilised trypsin beads. Proteins were digested by incubation at 70°C, at 1400 rpm for 3 hours on a heat shaker (Eppendorf Themomixer®). After digestion, the tubes were spun at 2500g for 5 minutes and the supernatant containing the tryptic peptides was separated from beads pellet and collected.

Desalting samples: SOLAμ™ Solid Phase Extraction Plates (Thermo Scientific™)

Samples were desalted using SOLAμ™ Solid Phase Extraction Plates (Thermo Scientific™). This was done by adding 200ul of 100% acetonitrile to the SOLAu SPE plates on 96-well vacuum and applying the vacuum to allow the acetonitrile (ACN) to pass through the cartridges to condition the columns. Cartridges were then equilibrated by pipetting 200ul of 0.1% trifluoroacetic acid (TFA) (in water) to each well. Vacuum was applied and the effluent discarded. 200ul of digested peptides were then loaded to the plates without touching the beads and acidified by adding 200ul of 1% TFA (the plate was gently tapped to mix samples). The vacuum was applied to allow peptides to pass through and bind to the C18 packed in the cartridges; then the cartridges were washed by pipetting 500ul of 0.1% formic acid (FA) in sterile water to each well, before applying the vacuum again. Collection plates were placed underneath the cartridge and the peptides were eluted by adding 50ul of "buffer B" (65% ACN, 0.1%

FA). This was then repeated using additional 100ul of buffer B. The eluted peptides were dried using a vacuum concentrator (Speed-Vac, Eppendorf) and resuspended in 50 ul of "buffer A" consisting of 98% MilliQ-H_2O, 2% acetonitrile (ACN) and 0.1% formic acid (FA). The samples were stored at − 80 °C in preparation for mass spectrometry analysis.

1.14.3 QC/Protein IDs

To optimise the timsTOF Pro protocol and to perform an assessment on the quality of the sample digestion, a master pool was created by mixing aliquots of all samples used in the analysis. The master pool was separated into 100 fractions by performing high pH fractionation. 60 fractions, containing the majority of the peptides, were then selected and run on the timsTOF Pro to create a reference peptide library. 6,185 proteins were identified in the fraction library. In the QC, 7 samples from the pool were also run alongside the 60 master pool fractions The peptide library allowed for better identification of proteins in our samples, as peptides could be matched by comparison between sample and library

1.14.4 LC-MS/MS

After being dried and resuspended and following QC, samples were loaded to Evosep (Evosep, Odense Denmark) tips for timsTOF analyses at PASEF mode. Acquired MS data was analysed by MaxQuant, MSFRagger and Perseus.

*This work was done in conjunction with the Target Drug Institute (University of Oxford) in collaboration with the Kessler group. As this was prior to the COVID pandemic, I was able to be involved in the sample preparation (isolation, homogenisation and digestion) for the back to base sample cohort (57 samples) under the supervision of senior scientists within the TDI as the >400 COPE liver samples were being prepared.

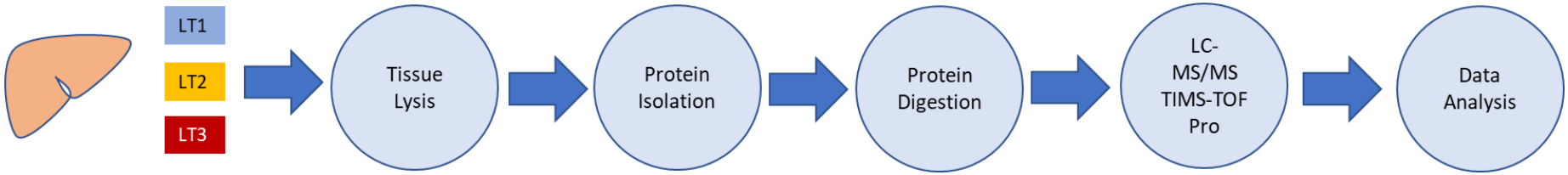


Figure 0.5: Overview of experimental workflow for proteomic analysis. Samples were collected at three timepoints (**LT1** – procurement or prior to normothermic machine perfusion (NMP), **LT2** at the end of preservation and **LT3** following reperfusion in the recipient. These samples were prepared, as proteins were extracted and digested into peptides ahead of label-free quantitation by liquid chromatography tandem mass spectrometry (LFQ LC-MS/MS) using the timsTOF Pro instrument (Bruker). The data output was then analysed using bioinformatic software (e.g Perseus®) (version 1.6.15.0),

1.14.5 Data Analysis

The raw data from the 'Back to base' and COPE study samples were analysed using MSFragger, to allow for protein identification and quantitation, which has formed the basis of this analysis (Figure 2). Principal Component Analysis (PCA) was used to assess, in an unbiased fashion whether changes in protein expression resulted in a discernible grouping (clustering) of samples by timepoint, based on components (and similarities) within the proteomic profile. Differential expression analysis was done using a combination of t-tests, 1-way and 2-way ANOVAs with post hoc tests (Tukey's HSD) and multiple testing corrections (False discovery rates using permutation-based FDR calculation) in Perseus® software (version 1.6.15.0). We considered proteins to be differentially expressed using a $p<0.01$ with FDR=5% as a threshold unless otherwise stated. For statistically significant differentially expressed proteins (DEPs) in our analyses, we performed supervised hierarchical clustering using Perseus® and we used the STRING® (version 11.5) online database to create protein-protein interaction (PPI) maps with k-means clustering and also to conduct Gene Ontology (GO) enrichment analysis. Comparisons of proteomic profiles were made between different timepoints, preservation approaches and clinical outcomes.

1.15 Transcriptomics

Liver tissue transcriptomic analysis was performed using the Nanostring ncounter® platform on frozen tissue samples from the 'Back to Base' study (n = 12).

1.15.1 RNA extraction and Quality Control

Extraction of RNA from frozen tissue samples was performed using the RNeasy kit (Quigen, Hilden, Germany). The kit was used following the manufacturer's instructions for the isolation of RNA. NanoDrop spectrophosphometer (NanoDrop ND 1000, Thermo Scientific, Wilmington, USA) was used for quantification and purity of RNA, assessed by determining the absorbance of RNA in RNase-free water at 230, 260, and 280 nm,. The RNA yield was calculated based on absorbance at 260 nm (A260) and the RNA integrity analysis was performed using the Algilent 2200 Tapestation was used for (Agilent Technologies, Santa Clara-CA–USA). This was performed in Oxford Molecular Diagnostic Centre (OMDC), within the Department of Oncology by their clinical scientists.

1.15.2 RNA array

The ncounter® Analysis system was used (Nanostring®, Seattle, USA) to analyse the expression levels of in the RNA extract using the 758 gene Human Organ Transplant Panel (HOT-P). The system works by a solution based RNA hybridization of probes which are subsequently fixed to a biotin coated cartridge which is digitally imaged and counted, quantifying the expression. The Human Organ Transplant panel includes a Panel Standard containing a pool of synthetic DNA oligonucleotides that correspond to the target sequence of each of the 770 unique probe targets in the panel. Includes 760 genes; 10 internal reference genes for data normalization

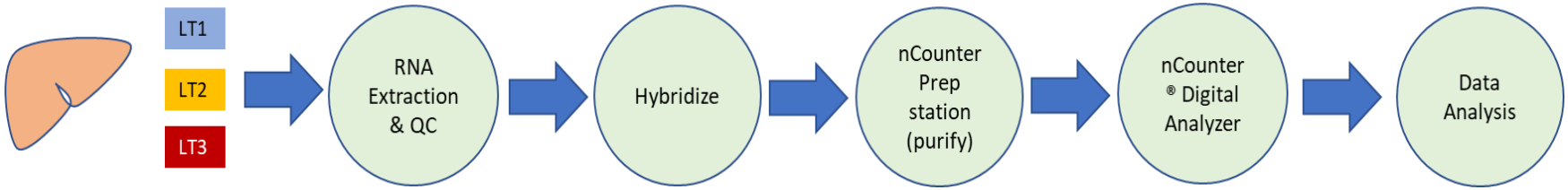


Figure 0.6: Overview of Transcriptomic analysis workflow. Samples were collected at three timepoints (LT1 – prior to normothermic machine perfusion (NMP), LT2 at the end of preservation and LT3 following reperfusion in the recipient). Using fresh frozen samples, RNA was isolated and extracted then undergoing quality control for yield, quantity and integrity. Total RNA from each sample was then hybridized to the nCounter® Human Organ Transplant Panel (NanoString Technologies, Seattle, WA, USA). Data analysis was performed using the bespoke nSolver® software (version 4.0.70) and advanced analysis module (version 2.0.115).

1.15.3 HOT-P gene list

Following the Banff 2019 meeting on molecular diagnostics a consensus report was published for the Human Organ Transplant (B-HOT) gene panel providing a data driven approach to molecular studies of the most well researched genes related to transplant immunology(176). Below are the tables with all the genes included in the panel, related to cell type, functional annotations and specific processes. (See appendix for full gene lists).

Table 0.1 **Immune Cell Profiling**: Genes sets associated with 14 different human immune cell types. Below summarizes each cell type represented by gene content in the panel, as qualified through biostatistical approaches and selected literature in the field of immunology(176).

Cell Type	Associated Human Genes
B cells	BLK, CD19, FAM30A, FCRL2, MS4A1, TNFRSF17, PNOC, SPIB, TCL1A
CD45	PTPRC
CD8 T cells	CD8A, CD8B
Cytotoxic Cells	CTSW, GNLY, GZMA, GZMB, GZMH, KLRB1, KLRD1, KLRK1, PRF1, NKG7
Dendritic Cells	CCL13, CD209, HSD11B1
Exhausted CD8	CD244, EOMES, LAG3, PTGER4
Macrophages	CD163, CD68, CD84, MS4A4A
Mast cells	MS4A2, TPSAB1, CPA3, HDC, TPSAB2
Neutrophils	CSF3R, S100A12, CEACAM3, FCAR, FCGR3A, FCGR3B, FPR1, SIGLEC5

NK CD56dim cells	IL21R, KIR2DL3, KIR3DL1, KIR3DL2
NK Cells	NCR1, XCL2, XCL1
T cells	CD3D, CD3E, CD3G, CD6, SH2D1A, TRAT1
Th1 Cells	TBX21
Treg	FOXP3

Table 0.2: Functional annotations for different pathways and processes were assigned to the genes in the Human Organ Transplant Panel.

Functional Annotations		
Adaptive Immune System	Inflammasomes	TGF-beta Signalling
Angiogenesis	Innate Immune System	Th1 Differentiation
Apoptosis & Cell Cycle Regulation	Lymphocyte Trafficking	Th17 Differentiation
Autophagy	MAPK	Th17 Mediated Biology
B-Cell Receptor Signalling	Metabolism	Th2 Differentiation
Cell-ECM Interaction	MHC Class I Antigen Presentation	Tissue Homeostasis
Chemokine Signalling	MHC Class II Antigen Presentation	TNF Family Signalling
Complement System	mTOR	Toll Like Receptor Signalling
Cytokine Signalling	NF-kappaB Signalling	Treg Differentiation
Cytosolic DNA Sensing	NLR Signalling	Type I Interferon Signalling
Cytotoxicity	Oxidative Stress	Type II Interferon Signalling
Epigenetics & Transcription		T-Cell Checkpoint Signalling
Hematopoiesis		T-Cell Receptor Signalling

1.15.4 Statistical analysis

Statistical analysis of Nanostring® nCounter RNA expression data was performed using a custom analysis pipeline based on the 'R' programming language, two-tailed t-test was used as recommended by manufacturer with significant genes to $p< 0.05$ with fold change >1 after Benjamini-Hochberg false discovery rate correction.

1.16 Histopathology

The scoring of pathological specimens was performed by a blinded Consultant Pathologist using H&E and PAS histological stained slides. Biopsies were scored according to a pre-agreed semi-quantitively scoring system agreed by the COPE Liver Pathology working group. Briefly, this score included steatosis, glycogen depletion, coagulative necrosis, congestion, red cells in space of Disse and portal oedema and haemorrhage.

1.17 Perfusate: Acquisition, handling & storage

Perfusate sampling was typically performed from the arterial sampling port on the metra, located on the shunt between the hepatic artery and IVC, unless otherwise stated, see individual chapter methods for variations (e.g. sampling from modified *metra* circuit). Perfusate samples were immediately analysed using point of care blood gas analysers, e.g., clinical ABL90 Flex or ABL800 Flex Analysers (Radiometer, Crawley, UK), which provided: pO2, pCO2, pH, haemoglobin (Hb), haematocrit (Hct), sodium (Na), potassium (K), calcium (Ca), lactate and glucose. For biochemical analysis of perfusate, samples were processed and stored at -80° C for future analysis. Processing of samples involved centrifugation at 3500rpm for 15 min at room temperature and then the supernatant was aspirated and pipetted in 500μL aliquots and initially stored on dry ice and then at -80o°C. For perfusate biochemistry analysis, samples were thawed, vortexed and analysed on a clinical biochemistry analyser via spectrophotometry (Abbott Architect c8000, Abbott diagnostics,

Illinois, USA) at the Department of Clinical Biochemistry, John Radcliffe Hospital, Oxford for the following: Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Gamma glutamyl transferase (GGT), Bilirubin, Alkaline Phosphatase (ALP), Lactate Dehydrogenase (LDH), Urea.

1.17.1 Bile sampling

Bile was sampled from the T-tube cannulating into the common bile duct. Bile biochemistry was analysed using the using point of care blood gas analysers, e.g., clinical ABL90 Flex or ABL800 Flex Analysers (Radiometer, Crawley, UK), which provided pH, haemoglobin (Hb), bicarbonate and glucose.

1.17.2 Nucleosome measurement

Levels of circulating H3.1 nucleosomes and H3R8cit nucleosomes were measured using Nu.Q™ ELISA assays (Belgian Volition SRL, Isnes, Belgium) according to the manufacturer's instruction. Briefly, plasma samples (20 µl in duplicate) were incubated for 2 h 30°C at room temperature in a 96-well microtiter plate coated with a monoclonal antibody raised again either a Histone H3.1 or a Histone H3R8cit epitope. After washing steps, the level of nucleosomes was quantified by adding 100 µl of a HRP-labelled anti-nucleosome detection antibody directed to a nucleosome conformational epitope (incubation 90 min at room temperature). The wells were washed and a peroxidase substrate: 3,3',5,5'-Tetramethylbenzidine (TMB) was added. After 20 min, the colorimetric reaction was stopped by adding 100 µ1 of Stop solution. The optical densities of the well were read at 450 nm using a microplate reader (Multiskan™, Thermo Fisher Scientific, Inc.).

Where the levels remained above the detectable range, samples were sent to a central laboratory at Volition HQ (Isnes, Belgium) for analysis by the manufacturers with multiple dilutions. These ELISAs were performed using the automated ELISA processing system DS2 (Dynex Technologies, Denkendorf, Germany).

1.18 NucleoCapture

1.18.1 Column

NucleoCapture (Santersus AG, Zurich Switzerland) is an adsorbent column containing porous, spherical agarose beads with covalently linked recombinant human N,N-bismethyonyl-histone H1.3 as ligand (via its N-terminus). The column binds cell free DNA in the plasma due to the high affinity of the analogue of linker histone H1protein to DNA. As plasma runs through the column this binding occurs, removing cell free DNA from the plasma. Linker histone H1 specifically tightly binds nucleosomes with a Kd between 0.022 and 3.3 nM and naked DNA with Kd value of 5 nM. It has been shown that the NucleoCapture selectively binds DNA of a broad range of lengths, with a positive correlation between the amount of the DNA adsorbed and the pre-treatment plasma cfDNA level, while keeping essential proteins such as albumin in the plasma. Figure 0.7)

1.18.2 Linker Histone H1.3

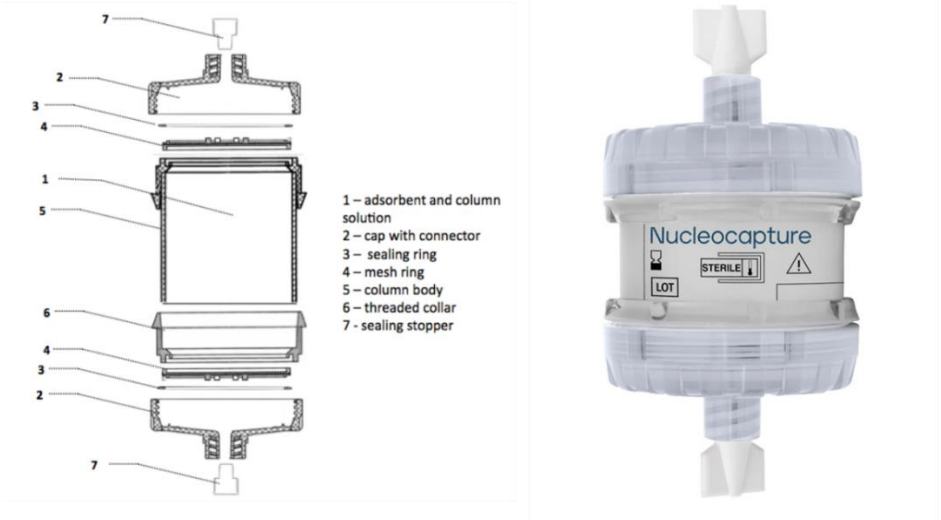


Figure 0.7: NucleoCapture Column. Biocompatible, highly porous polymer (Sepharose) beads conjugated with human recombinant histone H1.3 protein (Santersus AG, Zurich, Switzerland). Plasma separated from the circulating red blood cell based perfusate by the Terumo Spectra Optia system is passed through the column coming into contact with the H1.3 conjugated beads allowing cell free DNA, histones and nucleosomes to be bound powerfully and removed from the circulation. Left: schematic of composition of column. Right: photograph of column.

Ex situ reperfusion

"Life is, in general, cell activity"

"The body is a cell state in which every cell is a citizen"

— Rudolf Virchow

1.19 Background

Continuous or 'device to donor' liver NMP (cNMP) is associated with reduced graft injury and measurable reductions in IRI related clinical complications despite substantially prolonged total preservation times (140). In standard criteria donors, 'back to base' NMP (pSCS-NMP) has been shown to have similarly beneficial effects, however, in higher risk or ECDs, typically preserved with, the positive observations from cNMP are less apparent. In these organs, 'preservation reperfusion injury' (PRI) and its clinical consequences persist despite the application of NMP (172). The clinical manifestations of preservation injury following NMP, suggest that, as seen in severe ischaemia reperfusion injury following cold storage, there is an exaggerated inflammatory and immune-mediated injury occurring after transplantation, despite prior NMP (177). The composition and features of the "immune compartment" during NMP, its influence on graft injury during preservation and transplantation, as well as the overall effect it may have on post-transplant outcomes, is therefore, poorly understood (167).

1.19.1 Preservation reperfusion injury

Understanding the inflammatory and immune responses to "reperfusion" remains a central objective in the field of transplantation. The advent of machine perfusion has offered new reperfusion contexts (e.g normothermic regional perfusion (NRP), ex situ machine reperfusion

and in situ transplant reperfusion) as well as new paradigms: 'device to donor' (cNMP) and 'back to base' (pSCS-NMP), that necessitate a conceptual recapitulation of the immunobiology of organ reperfusion. A first step in this process is a reframing of what we have traditionally referred to as "ischaemia reperfusion injury" into the more specific yet inclusive term: "preservation reperfusion injury" promoted by Hann et al(177), thus capturing the events occurring during preservation (including application of machine perfusion technologies) in the reperfusion injury that follows transplantation.

Furthermore, investigating the crucial role this compartment, during ex situ preservation may play in determining the magnitude and consequences of preservation reperfusion injury (PRI) after transplantation, represents a major clinical challenge in liver transplantation, that is currently limiting the safe expansion of ECD liver use.

1.19.2 Immune mediators: Cells, Cytokines & Damage Associated Molecular Patterns

Immune cells, inflammatory cytokines and damage associated molecular patterns (DAMPs) are major constituents of the immune compartment and are the primary drivers of sterile inflammation (43,61,178). During preservation with machine perfusion, these elements can be assessed by analysing circulating perfusate. Pre-clinical machine perfusion studies in other organs (lung (179), heart (180), kidney (181)) have all demonstrated that the ex-situ removal of donor derived immune cells or "passenger leukocytes" can reduce the immunogenicity of grafts resulting in reduced immune cell infiltration following transplantation. Cytokines have also been proposed as therapeutic targets, particularly in ex vivo lung perfusion studies (182–184), and recent data from kidney perfusion shows that ex-situ cytokine adsorption is associated with anti-inflammatory alterations in the transcriptomic profiles of grafts preserved with NMP (185).

In recent years, DAMPS and more specifically, extracellular nucleic content: cell free DNA (cfDNA), extracellular histones, Neutrophil extracellular traps (NETs), Nucleosomes or

chromatin fragments have been mechanistically linked to the propagation and exacerbation of acute on chronic liver failure (186,187), hepatic ischaemia reperfusion injury (61,188), and transplant rejection (49). Nucleosomes are chromatic fragments comprised of an octamer of four paired histone proteins wrapped in 147 base pairs of DNA (see introduction), that are immunogenic and promote innate immune responses alongside other nucleic content such as free histone and extra cellular DNA (cfDNA) (65).

Nucleic DAMPs are intrinsically immunogenic via multiple mechanisms. NETs have implicated in the development of sinusoidal occlusive thrombi resulting in microcirculation failure during transplantation (189) and they are also directly hepatotoxic and proinflammatory (74,75). Other nucleic DAMPs drive sterile inflammation within the liver through Kupffer cell activation and catalysing innate immune responses that ultimately result in hepatic injury. These DAMPs play an important role in innate immunity under normal conditions, raising the alarm for injury as they are recognised by pattern recognition receptors (PRRs) (62,63,190). These receptors (PRRs) initiate downstream innate inflammatory responses when they encounter alarm molecules denoting damaged cells (DAMPs) or microbial pathogens – pathogen associated molecular patterns (PAMPS). This is an ancient and highly preserved innate immune response system deeply encoded and is critical for immunity. In the context of reperfusion injury, this plays a role in driving inflammation and therefore injury.

1.19.3 Chapter Rationale

It is unclear if these highly pro-inflammatory and immunogenic molecules are a) present in perfusate, b) the extent to which their levels change through perfusion and finally c) whether they constitute a possible immuno-therapeutic target. What is clear, is that to some extent "immune priming", is occurring during NMP of ECD livers, and this is associated with higher than expected

immune related complications post-transplant, such as severe IRI and acute cellular rejection: the latter has been reported at 62% in a recent series from Birmingham (191).

In this chapter I explore whether circulating perfusate can provide mechanistic insights into the immune compartment of liver grafts during ex situ perfusion, and determine the extent to which inflammation and immunologically driven 'ex situ reperfusion injury' (via DAMPS and NETs) is occurring during NMP.

1.20 Chapter Aims

1. To determine the characteristics and temporal kinetics of the key immunological elements of perfusate, immune cells and cytokines, during ex situ reperfusion.

2. To assess the extent to which these elements relate to donor liver characteristics and clinical outcomes

3. To assess the extent to which pro-inflammatory, immunogenic, nuclear DAMPs (nucleosomes, NETS, histones) are released into the circuit during pSCS-NMP

1.21 Methods

1.21.1 COPE Liver samples – continuous NMP (cNMP)

Twenty consecutive livers recruited from the NMP arm of the COPE Liver trial (ISRCTN 39731134) (140) (see Methods) were perfused on the NMP device (OrganOx® metra, Oxford, United Kingdom) according to the standard protocol (Methods). Once established on NMP, all 20 livers exhibited stable hepatic artery and portal venous flow rates throughout perfusion: however 2 livers failed to biochemical transplantability criteria and were discarded(140),(the interpretation of biochemical and perfusion data was left to the implanting teams and not stipulated in the study), leaving the remaining 18 that went onto be successfully transplanted and have been included in this study.

1.21.2 cNMP perfusate Sampling

Perfusate samples were obtained prior to initiation of NMP (during priming), at 30 mins, 60mins and 240mins. Samples for biochemistry, point of care testing blood gas analysis and cell storage were prepared, stored and analysed as previously described (see Methods).

1.21.3 cNMP perfusate immunological assessment

Flow cytometry analysis was used to determine the absolute cell count:((number of cells x 20)/ (number of beads x 100)) x bead concentration x 1000, as well as to characterise and quantify the immune cell repertoire against a human immune cell panel (see General methods). Inflammatory profiling of perfusate was performed using a human Luminex array, quantifying a total of 15 cytokines in perfusate samples, with 11 determined to be detectable (TNF-α, IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-9, IL-10, IL-17A, GM-CSF and MCP-1).

1.21.4 cNMP analysis of clinical characteristics and perfusate values

The relationships between the peak efflux of cells and donor liver characteristics including: donor type (DBD/DCD), age, BMI, degree of steatosis and liver weight were assessed. Clinical features of ischaemia reperfusion injury and preservation injury including peak serum AST, early allograft dysfunction (EAD) and Post Reperfusion Syndrome (PRS) were also explored in the context of leukocyte migration during perfusion.

1.21.5 : Damage Associated Molecular Patterns: pSCS-NMP or "Back to Base" Sample analysis

Livers from the pSCS-NMP livers (back to base) liver study (NCT03176433) (see methods) were procured and transported under SCS conditions to the recipient centre where the livers were perfused using the standard NMP protocol. 30/51 recruited livers were ultimately transplanted and included in the trial, perfusate and liver tissue samples were obtained at the described times (see below). All livers with available research samples at the three timepoints during perfusion (n = 14) were included in this study to assess the levels of Nucleosomes. Nucleosome and NET levels were measured using Nu.Q™ ELISA assays (Belgian Volition SRL, Isnes, Belgium).

1.21.6 pSCS-NMP Perfusate sampling

Perfusate samples were obtained prior to initiation of NMP (during priming), at 30 mins, 60mins and 240mins. Samples for biochemistry, point of care testing blood gas analysis and cell storage were prepared, stored and analysed as previously described levels of circulating H3.1 nucleosomes and H3R8cit nucleosomes were measured using Nu.Q™ ELISA assays (Belgian Volition SRL, Isnes, Belgium) according to the manufacturer's instruction.

1.21.7 Statistical analysis:

Descriptive statistics were reported as mean and standard deviation or median and interquartile range according to normality. Normality was determined by a combination of Shapiro-Wilks tests, Q-Q plots and visualisation (e.g histogram) assessment. One-way ANOVA (Kruskal-Wallis) repeated measures was used to assess changes in cells over time and post hoc analysis using the Bonferroni multiple comparisons test was applied to evaluate differences at each timepoint. In the case of non-parametric data failing to meet the normality assessments outlined above, a Freidman's test and Wilcoxon pairwise comparison test was performed. A p-value of 0.05 was considered statistically significant. Unpaired t-tests and linear regression models were used to compare continuous variables across independent samples. Pearson's r test was used to test for correlation analysis.

1.21.8 Tables

A Student t test was used to analyse parametric, continuous variables, and the Mann-Whitney U test was used for nonparametric continuous variables. Fisher's exact test was used for categorical variables and a p-value of 0.05 was considered statistically significant.

1.22 Results

1.22.1 Cells and cytokines during continuous NMP (COPE liver study livers)

Ex situ reperfusion induces an efflux of immune cells into the circulating perfusate

There was a substantial mobilisation of immune cells from the liver into the circulating perfusate following ex situ reperfusion (Figure 0.1). Starting from a baseline perfusate devoid of leukocytes (see methods for perfusate composition), there was an early peak in detected cells at the first measured timepoint (30 mins). This change from a mean level (range) of 5.1 $x10^3$ (1.35-77.45 $x10^3$) to 6 $x10^5$ (4.81 $x10^4$-2.72 $x10^6$) was statistically significant (p=0.0013). This was followed by a significant decrease in cells detected from the peak value at 30 mins to 60 mins, $4.10x10^5$ ($1.29x10^4$-$2.00x10^4$), p = 0.03. Between 60 mins and 240 mins there was no significant difference in cells detected (p = 0.64), but there was still a significant difference between baseline levels and the (final) 240min timepoint, (p = 0.0013).

When scaled up to the total perfusate volume (30min mean cell count/mL x circuit volume (1700mls)), this equated to $1.1x10^9$ leukocytes that had effluxed from the liver into the perfusate at the 30 min peak. The characteristics of the livers included in this analysis are outlined in (Table 0.1).

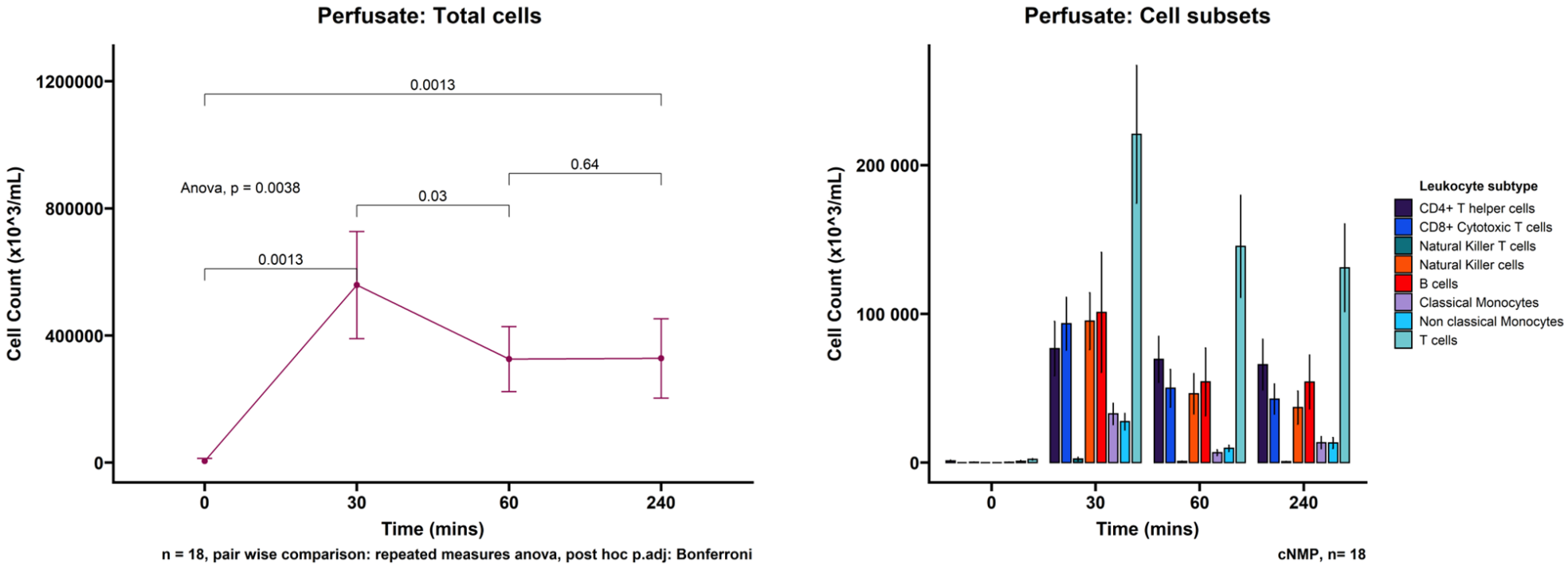


Figure 0.1: Immune cells (leukocytes) detected in perfusate through NMP. **Immune cells were quantified using flow cytometry**. There was a significant increase in detected cells from baseline to 30 mins and a significant decrease from 30 mins to 60mins but no difference from 60min to 240mins. Data is represented as mean ± SEM. Differences between means at the various timepoints were assessed using a repeated measures ANOVA test and then specific differences between time points with a pairwise comparison and Bonferroni's multiple comparisons test, a p-value of <0.05 was significant. (NMP = normothermic machine perfusion)

Table 0.1: Donor and Recipient Characteristics. Livers from COPE liver study, NMP arm.

	Donor/Recipient Characteristics	
Donor Type, n (%)	DBD	11 (55%)
	DCD	9 (45%)
Donor Age (Years)	Median (Range)	50.5 (21 – 79)
Recipient Age (Years)	Median (Range)	53 (24 – 68)
Recipient Sex, n (%)	Female	9 (45%)
	Male	11 (55%)
Donor BMI (m/kg2)	Median (Range)	25.74 (20.0 - 46.6)
Donor Cause of Death, n (%)	CVA	12 (60%)
	HBI	3 (15%)
	CNS malignancy	1 (5%)
	IECOPD	2 (10%)
	Trauma	2 (10%)
UKDRI (score)	Median (Range)	1.855 (0.98 - 6.35)
	Missing/NA	n = 2
ETDRI (score)	Median (Range)	1.81 (1.30 - 3.28)
	Missing/NA	n = 2
Degree of Steatosis*, n (%)	None	4 (20%)
	Mild	9 (45%)
	Moderate	5 (25%)
	Severe	2 (10%)
Liver weight (g)	Median (Range)	1786 (1180.0 - 1950.0)
	Missing/NA	n = 9
Transplanted, n (%)	Discarded	2 (10%)
	Transplanted	18 (90%)

DBD = donation after brainstem death, DCD = Donation after circulatory death, SD = standard deviation, BMI = body mass index, CVA = cerebrovascular accident, HBI = hypoxic brain injury, HCC = hepatocellular carcinoma, CNS= central nervous system, IECOPD = Infective Exacerbation of Chronic Obstructive Pulmonary Disease, UKDRI = United Kingdom Donor Risk Index, ETDRI = EuroTransplant Donor Risk Index, Steatosis (*Surgeons assessment).

1.22.2 Immune cell subtypes effluxed into the circuit

T-lymphocytes were the predominant immune cell subtype effluxing into circuit

T-cells (CD4+/CD8+ cells) were the dominant immune cell subtypes detected throughout perfusion, representing 39.8% of all cells at the 30 min (peak) timepoint and 47.6% by the final 240 mins time point. This population was equally divided between CD4+ helper and CD8+ cytotoxic T cells at 30 minutes of perfusion (17.17% CD4+ vs 22.65% CD8+) but by the final timepoint CD4+ T cell were the most abundant with numbers that remained stable throughout perfusion, yet the CD8+ decreased significantly by the last timepoint. NKT cells were detected in greatest number at 30 min which was significantly greater than baseline ($p = 0.008$) but fell back down by the 60 min time point to very low levels not statistically significantly different levels to baseline or the last timepoint.

NKCs and B-cells followed a similar overall pattern to that of the CD8+ cells of peaking early at 30 mins and then significantly falling from the peak at 30mins to the 60min time point where the detected cells remained at a similar level the subsequent time points (30mins vs 60 mins NKC $p=0.0086$, B-cell $p= 0.0460$, 60mins vs 240 mins NKC $p=0.9269$ B-cell $p = 1.000$).

This overall pattern was similar for the monocyte subtypes, with classical monocytes being detected in slightly higher numbers at the 30 min peak relative to nonclassical monocytes. Detected monocytes fell after 30mins and overall were detected in very low numbers.

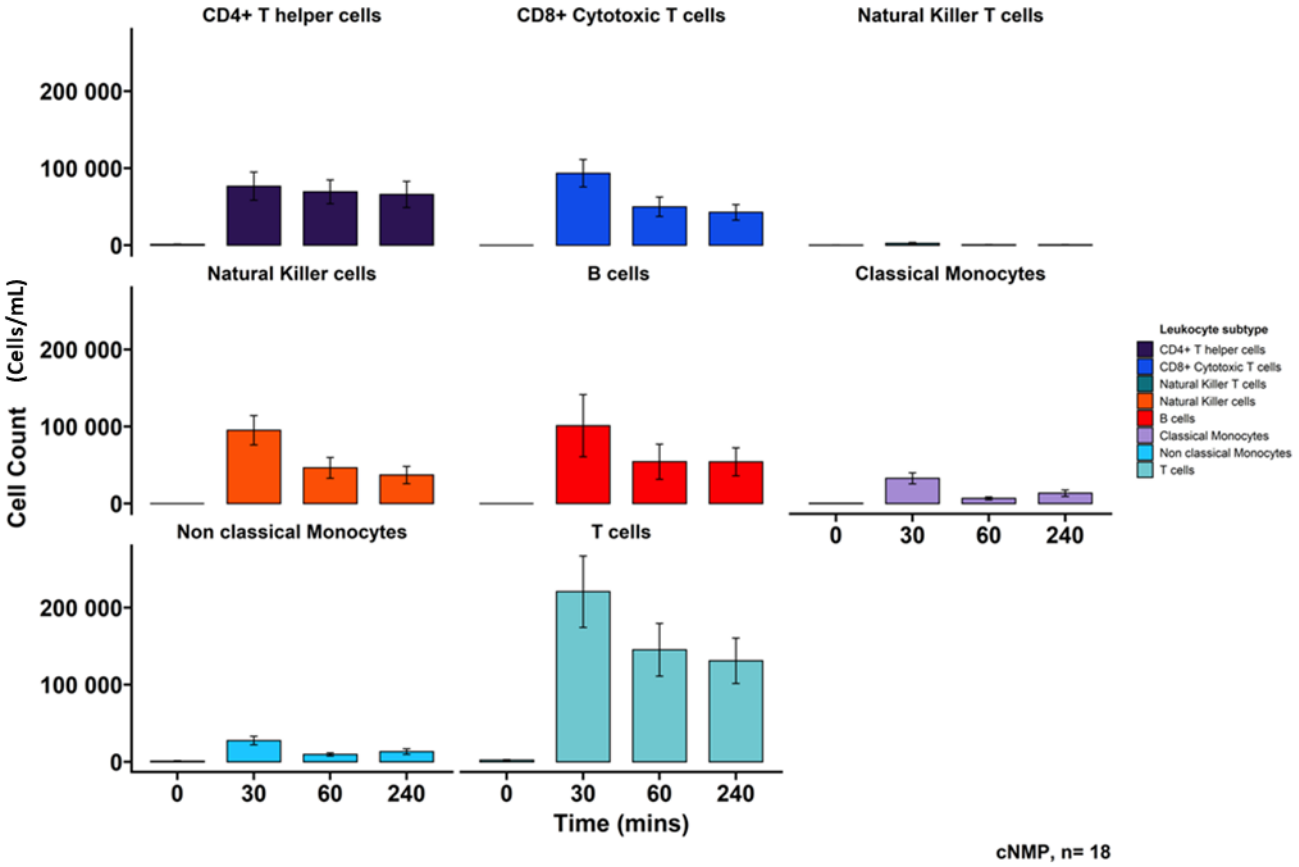


Figure 0.2: Immune cell (leukocyte) subtypes detected in perfusate through NMP. **Immune cells were quantified using flow cytometry**. (Top row) T-cell subsets ($CD4^+$ T helper, $CD8^+$ Cytotoxic T cells and NKT cells). (Middle row) NK cells, B cells and classical monocytes. (Bottom row) non classical monocytes and total T cells (all subsets combined). Data is represented as mean ± SEM. Differences between means at the various timepoints were assessed using a repeated measures ANOVA test and then specific differences between timepoints with a pairwise comparison and Bonferroni's multiple comparisons test, a p-value of <0.05 was significant. (NMP = normorthermic machine perfusion, NK = Natural Killer).

1.22.3 Relationship between immune cell efflux and donor characteristics

Immune cell efflux is not related to donor liver characteristics

The magnitude of the overall efflux of immune cells at reperfusion (peak cells, i.e. mean number of immune cells detected at 30min timepoint) was compared to donor liver characteristics to determine whether the extent of efflux into the perfusate was related to these characteristics. No differences in perfusate immune cell count were observed for donor type (DBD vs DCD, p=0.98) or composite measures of donor risk (e.g. UKDRI (p = 0.62) or ETDRI (p= 0.23)). Furthermore, donor BMI (p = 0.76), liver weight (p=0.92), were not related to efflux of cells (Figure 0.3). Donor livers assessed by surgeons to have moderate/severe steatosis were compared to livers assessed to have no/mild steatosis and found to have similar peak cells detectable in the perfusate (p-value = 0.74). However, the histological analyses of the biopsies from livers included in this study reported less than 5% steatosis in all cases.

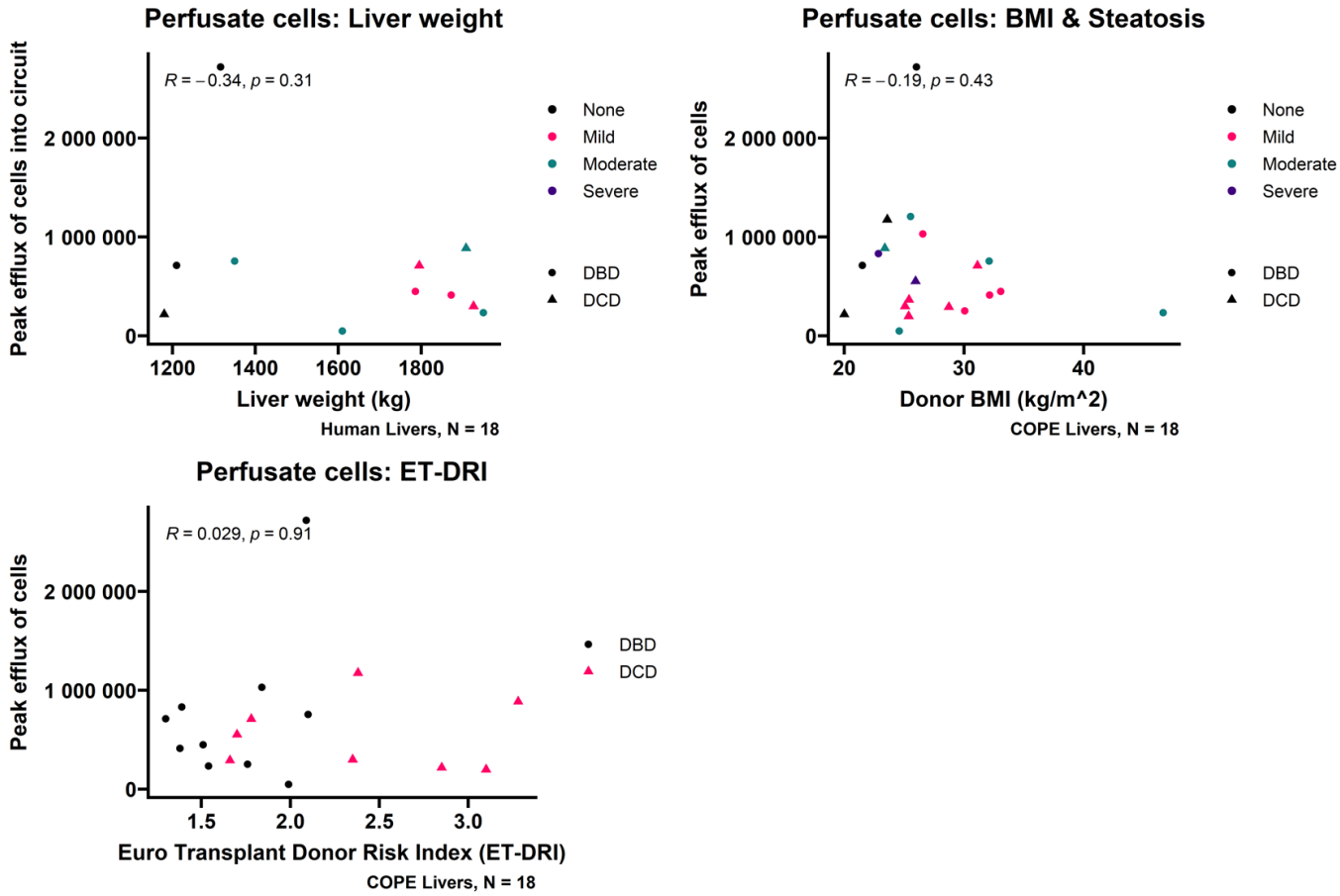
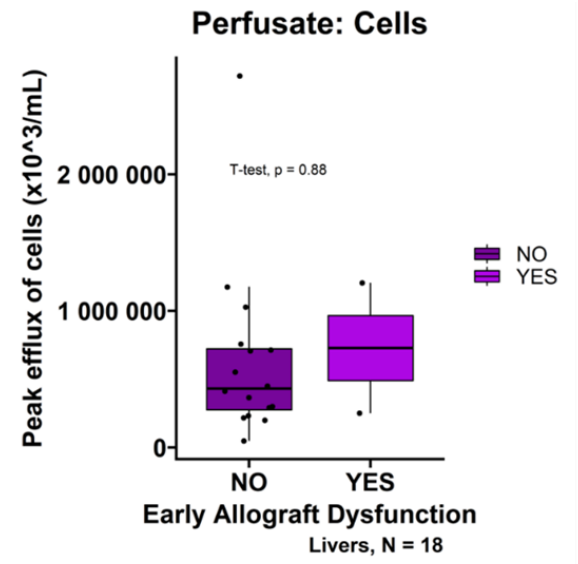


Figure 0.3: Correlation analysis of peak cells detected in perfusate with donor liver characteristics. A) ETDRI was not correlated with the magnitude of cell efflux into the circuit (p= 0.9). B) Liver weight (p= 0.31) and C) Donor BMI (p =0.43) were not correlated with efflux of cells. Immune cells were quantified using flow cytometry. Pearson's correlation analysis was performed, with a p value of <0.05 used to determine significance.

1.22.4 Immune cell efflux and clinical IRI

Immune cell efflux is not related to clinical measures of early graft injury and IRI

We saw no statistically significant relationship between the magnitude of the efflux and markers of early graft injury/severity of IRI (EAD and peak AST).



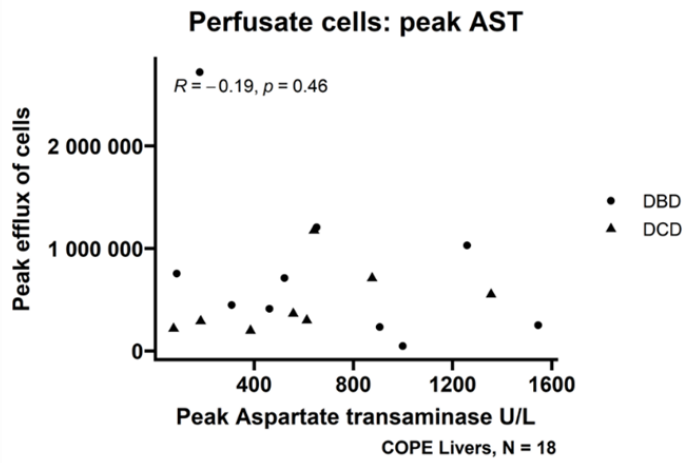


Figure 0.4 : Correlation analysis of peak cells detected in perfusate with early post operative outcomes. A) EAD was associated with an increase in peak cells (p =0.43) were not correlated with efflux of cells. B) Peak AST was not correlated with the magnitude of cell efflux into the circuit (p= 0.46). Immune cells were quantified using flow cytometry. Pearson's correlation analysis was performed, with a p value of <0.05 used to determine significance. NMP = normothermic machine perfusion, AST = Aspartate transaminase, EAD = early allograft dysfunction (Oltoff criteria).

1.22.5 Cytokines during NMP

Cytokines levels progressively rise through ex situ reperfusion

We detected 11 of the 15 cytokines in our human immune panel within the perfusate. We included livers in the analysis where individual cytokines were detectable at more than one timepoint in samples from the same liver. Overall, all detectable cytokines rose over time through perfusion. This was true of anti-inflammatory cytokines (IL-10) and well as the pro-inflammatory cytokines (e.g IL6, IFγ etc).

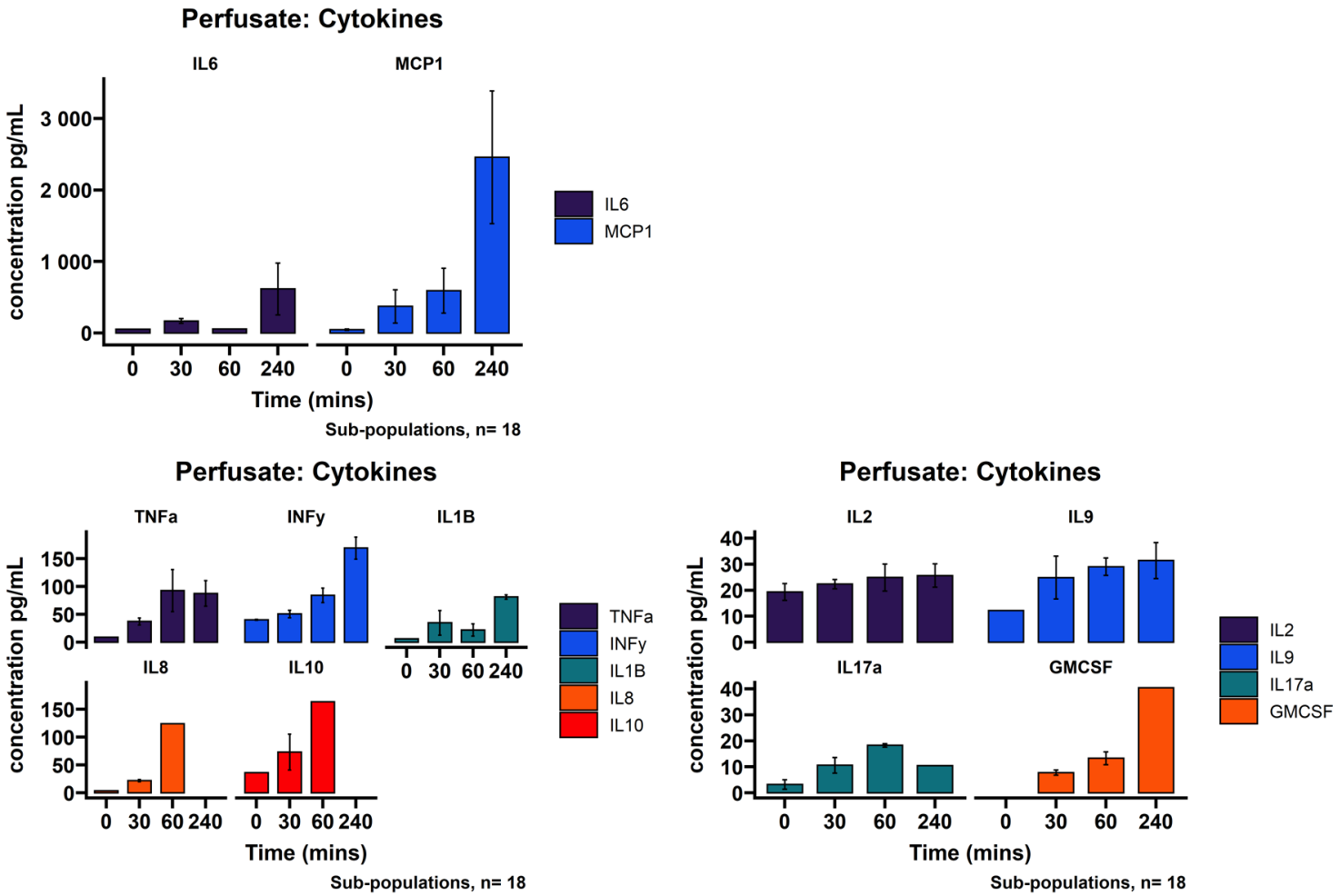


Figure 0.5 Cytokine analysis of perfusate during NMP. A) IL-6 and MCP1 were detected in the highest concentrations, with an increase in levels through perfusion. B) TNFa, INFγ, IL-1b, IL8, IL10 C) IL2, IL9, IL17a, GMSCF were all in the lowest range relative to other cytokines (0-40) but showed a progressive increase through NMP. All cytokines were measured using a human Luminex array 11 of the 15 were detected. NMP= normothermic machine perfusion.

1.22.6 Damage Associated Molecular Patterns, PRI and pSCS-NMP.

Perfusate biochemistry and post operative patient plasma biochemistry does not significantly differ between livers that have develop clinical manifestations of IRI defined as any of: PRS - post reperfusion syndrome, RRT - requirement for renal replacement therapy , EAD - early allograft dysfunction (Olthoff criteria (80)) or primary non function) and those that have immediate function following pSCS-NMP preservation. We found that within our cohort, livers that develop clinical IRI (defined as above) have similar perfusate biochemistry to livers that had immediate function following preservation with pSCS-NMP. Furthermore, post operatively, there were no significant biochemical differences in the plasma samples between livers with severe IRI versus those without

Table 0.2. The early recipient plasma AST is higher in the IRI group for the first two days but this quickly falls to the level of livers which do not have clinical manifestations of IRI.

Table 0.2: Comparison of donor liver preservation characteristics of livers that developed clinical ischaemia reperfusion injury with those that did not following pSCS-NMP.

Preservation Characteristics		No IRI	IRI*	p
Donor Type	DCD	10 (35.7)	1 (16.7)	0.671
	DBD	18 (64.3)	5 (83.3)	
ETDRI	Mean (SD)	2.0 (0.5)	1.7 (0.3)	0.123
CIT (mins)	Mean (SD)	365.8 (84.8)	343.0 (68.6)	0.548
NMP duration (mins)	Mean (SD)	478.2 (240.8)	610.5 (250.5)	0.242
Total Preservation (mins)	Mean (SD)	844.0 (276.6)	953.5 (287.0)	0.396

There were no significant differences in baseline characteristics. ETDRI – Eurotransplant donor risk index, CIT – cold ischaemic time, NMP – normothermic machine perfusion, IRI – ischaemia reperfusion injury (* defined as any of: PRS - post reperfusion syndrome, RRT - requirement for renal replacement therapy, EAD - early allograft dysfunction (Olthoff criteria) or primary non function. (Entire pSCS-NMP cohort from Back to Base study).

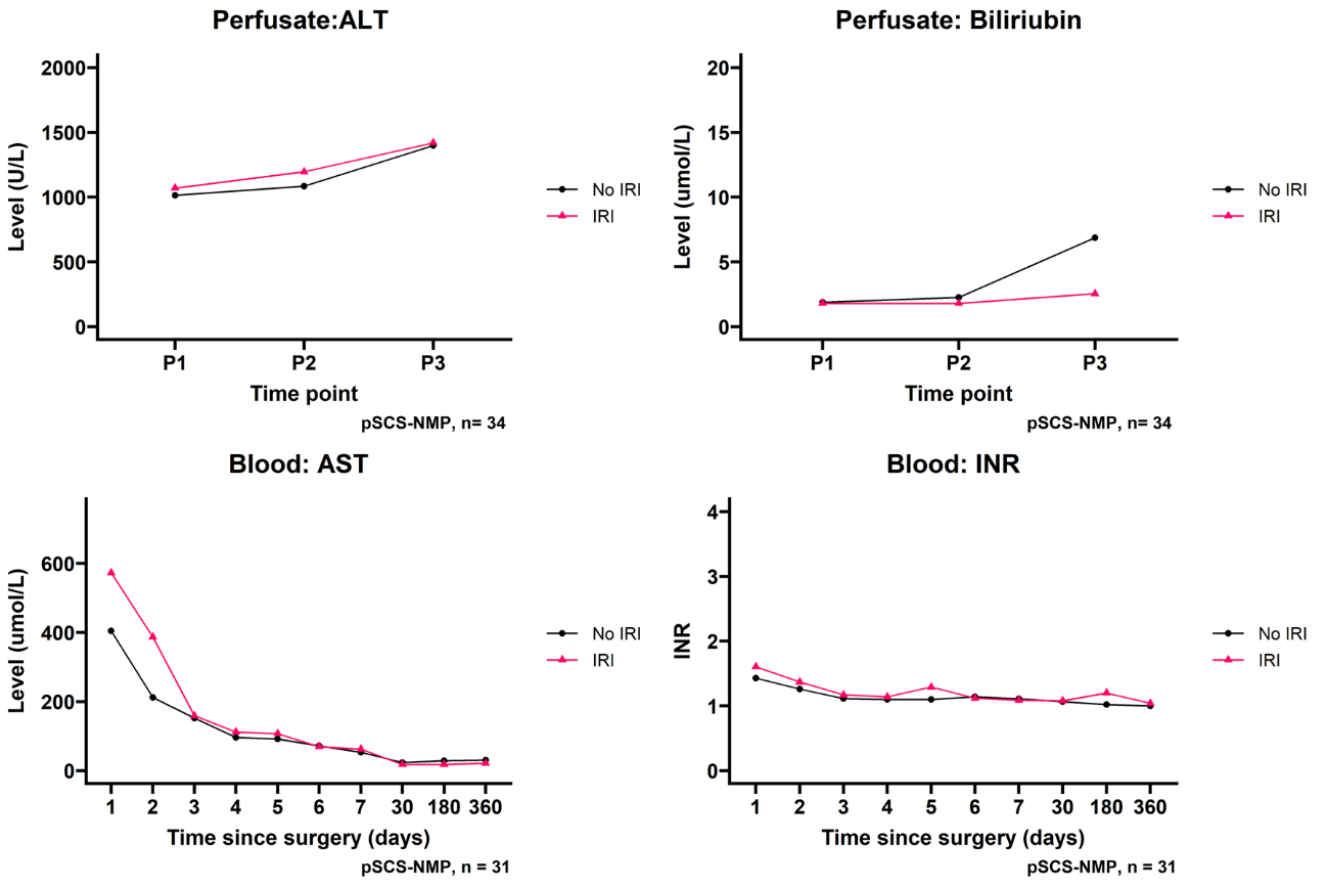


Figure 0.6: pSCS-NMP livers perfusate and blood biochemistry comparing livers that developed clinical manifestations of ischaemia reperfusion injury to those that did not. (Top row) Perfusate liver enzymes and bilirubin are similar in both groups. (Bottom row) Post operative recipient blood levels of liver enzymes and coagulation demonstrate an early difference in AST between the groups, but by day three post op, there are similar levels of AST, which continue to be similar throughout the study. INR levels are similar throughout. pSCS-NMP = prior static cold storage normothermic machine perfusion.

1.22.7 Nucleosomes and NETs are released into the perfusate during NMP

We measured circulating extracellular nucleosomes using the NuQ H3.1 assay (Belgian Volition SRL, Isnes, Belgium) and neutrophil extracellular traps (NETs) using the Nu.Q Hr8Cit assay (Belgian Volition SRL, Isnes, Belgium) in livers preserved with pSCS-NMP (Table 0.3) to determine the levels of these highly proinflammatory molecules in the perfusate. Circulating levels of pro-inflammatory nucleosomes were detected a high level during all phases of perfusion, with all readings were above the validated range of the commercially available ELISA assay. In highly inflammatory conditions such as COVID-19, where the levels of nucleosomes have been related to the severity of disease, the median level of nucleosomes in s COVID-19 = 689.2 ng/ml, compared to healthy controls = 81.2 ng/ml and for ctHr8Cit the mean levels in COVID-19 = 20.6 ng/ml, with healthy controls = 5.4 ng/ml, the levels associated with severe COVID-19 are illustrated by the red dashed line (192). In our study at the 15 min timepoint, levels of nucleosomes in the perfusate were already elevated at a median level (IQR) of 355.60 (753) ng/ml. This increased to a peak level at the 60 minute timepoint, reaching a median value of 6465.42 (7439.942) ng/ml. At the end of perfusion, we found that the levels of circulating nucleosomes remained high at 1427.955 (4435.838). The levels of circulating NETs (neutrophil extracellular traps) as measured by the NET specific assay Nu.Q Hr8Cit (Belgian Volition SRL, Isnes, Belgium), followed a very similar overall pattern, with an elevation and peak at the 60 min timepoint, but showed a more definitive reduction at the end of perfusion with levels dropping to levels similar to those at the early 15minute timepoint ($p = 0.16$) in 14 liver perfusions.

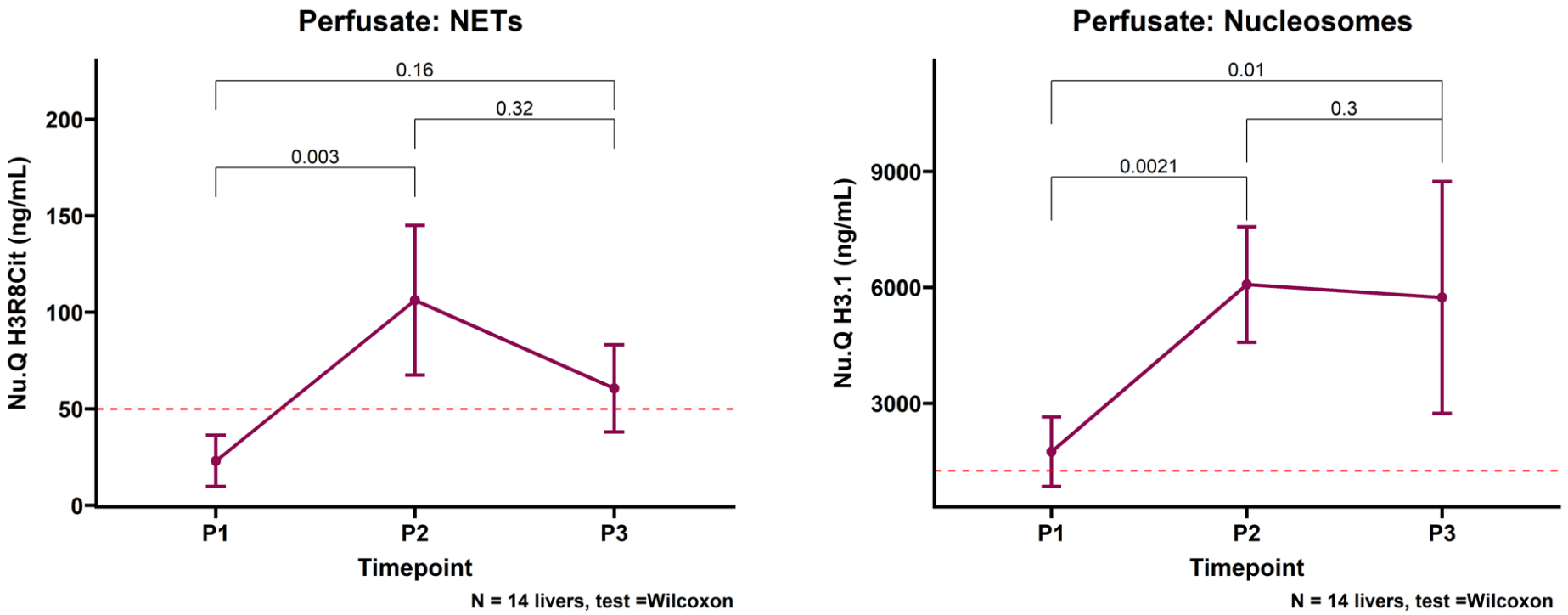


Figure 0.7: Circulating nucleosomes and neutrophil extracellular traps (NETs) in the perfusate of pSCS-NMP livers. A) Circulating Nucleosomes, measured using the NuQ H3.1 assay. The dashed red line represents the levels of nucleosomes detected in patients with severe systemic inflammation in ICU patients with COVID-19 infection. B) Circulating NETs measured using the Nu.QH3R8Cit (Citrullinated nucleosome) assay. The dashed red line represents the levels of nucleosomes detected in patients with severe systemic inflammation in ICU patients with COVID-19 infection(192). N = 14, Data is represented as mean ± SEM. Differences between means at the various timepoints were assessed using a repeated measures Friedmans test and then specific differences between timepoints with a pairwise (Wilcoxon) comparison and Bonferroni's post hoc multiple comparisons test, a p-value of <0.05 was significant. (NMP = nomorthermic machine perfusion. pSCS-NMP = prior static cold storage-NMP, ICU = intensive care unit, COVID-19 = SARS-CoV-2 respiratory infection) .

Table 0.3: Donor liver characteristics and early outcomes for livers (n = 14) included in Nucleosome analysis. Comparison of livers by donor type. There were no significant differences in baseline characteristics.

Donor Type		DCD	DBD	p
Donor Age	Median (IQR)	64.0 (50.5 to 70.8)	63.0 (52.0 to 69.0)	0.8
Donor Sex	F	1 (20.0)	6 (66.7)	0.2
	M	4 (80.0)	3 (33.3)	
Donor				
BMI(kg/m2)	Median (IQR)	26.2 (21.2 to 30.1)	30.6 (23.6 to 31.2)	0.4
ETDRI	Median (IQR)	2.5 (2.2 to 2.6)	1.8 (1.7 to 2.0)	0.0
Steatosis	None	2 (50.0)	2 (25.0)	0.4
	Mild	0 (0.0)	3 (37.5)	
	Moderate	1 (25.0)	3 (37.5)	
	Severe	1 (25.0)	0 (0.0)	
PRS	0	4 (100.0)	7 (87.5)	n/
	1	0 (0.0)	1 (12.5)	
EAD	No	4 (100.0)	7 (87.5)	n/
	Yes		1 (12.5)	
RRT	No	4 (100.0)	7 (87.5)	n/
	Yes		1 (12.5)	
Peak AST	Median (IQR)	332.0 (289.2, 702.8)	528.0 (252.0, 818.5)	0.8
NMP				
duration	Median (IQR)	343.5 (298.8, 394.8)	339.0 (288.8, 480.8)	0.8
CIT	Median (IQR)	377.0 (363.5 to 404.8)	326.0 (269.0 to 397.5)	0.3
Total				
preservation	Median (IQR)	768.0 (712.5 to 796.8)	713.5 (675.8 to 774.8)	0.0

1.23 Discussion

Ex situ reperfusion upon initiation of NMP induces an efflux of immune cells into the circulating perfusate, with cell numbers that peak early (30 mins), fall and then plateau through the first 4 hours of NMP, yet cytokine levels progressively increase through perfusion. We established that the immune cell efflux was a predominantly lymphocytic, with low levels of monocytes and granulocytes. Importantly we determined that this phenomenon occurred with all livers, regardless of donor characteristics and we saw no relationship between the magnitude of the immune cell efflux and conventional markers of early graft injury/severity of IRI (e.g peak AST). Nuclear damage associated molecular patterns (DAMPs), in the form of extra cellular nucleosomes and NETs are detected at high levels within perfusate of pSCS-NMP livers, with peak levels at the 60-minute timepoint. These findings represent novel insights into the immunological events that occur following ex situ reperfusion during NMP.

Immune cell efflux following 'ex situ reperfusion'

Traditionally, transplant immunology studies have investigated 'in situ reperfusion' (i.e within the recipient) focussing on graft infiltration with recipient leukocytes as a marker of reperfusion injury (168,193,194) or focused on the transfer of donor derived immune cells as "passenger cells" to the recipient during transplantation, primarily interested in their role in allorecognition (195–197). However, with the advent of machine perfusion, new areas of investigation are developing as new paradigms of 'reperfusion' have emerged, such as ex situ, machine based, organ reperfusion. Consequently, a better understanding the effects of this 'ex situ reperfusion' on immune process, such as the flux of donor derived cells into the perfusate during perfusion and their overall effect on subsequent events post-transplant, is increasingly important.

In this study, ex situ reperfusion during NMP resulted in an early and substantial efflux of donor-derived immune cells into the circuit in all livers. This result suggested the phenomenon is strongly linked to ischaemia reperfusion and not a feature linked to specific donor characteristics such DCD or presence/degree of steatosis. This highly reproducible pattern of perfusate leukocyte kinetics showed that following an early peak, there was a substantial reduction in detectable cells in the perfusate by the end of NMP, albeit not back down to the pre-NMP levels. This pattern was observed with almost all immune cell sub populations, with CD8+ T cells, NK cells and B-cells experiencing the most substantial reductions though perfusion. In contrast, numbers of CD4+ T cells did not reduce through the perfusion with levels remaining at a similar level to the peak.

Presumably the reduction in detectable cells represents a return of mobilised cells back into the graft, with different subtypes returning in different proportions. Alternatively, this reduction may represent an attrition of cells due to the circuit itself, with damage and loss of cells that find themselves in the circuit. This is less likely: as shown in my subsequent experimental porcine work (see Chapter 5), that the circuit alone (i.e. without a liver) doesn't significantly affect the leukocyte count over 6 hours of perfusion (i.e. well beyond the 240 min time point in this study). This implies that any changes in cell levels in the perfusate represent trafficking between the graft and perfusate. The phenotype of the cells that return into the liver and the extent to which these cells are activated/primed prior to returning the liver is unknown.

Passenger cells, that have historically been associated with the development of 'operational tolerance' in the context of microchimerism (102,195), are now thought to be of hematopoietic stem cell origin and thus potentially distinct from the leukocyte populations identified within this study (60,198).Haematopoietic stem cells are long-lived and have the potential to give rise to mature donor cells of several hematopoietic lineages, which allows them to maintain a degree of chimerism but has also implicated them in the development of graft versus host disease, which

can be fatal following liver transplantation in greater than 80% mortality (199). The proportion of donor stem cells within this initial efflux of cells into the perfusion circuit is unknown, however recent studies have suggested that the cell population (effluxed) represents a more responsive, mobile inflammatory compartment of the immune system which is more likely to potentiate immunogenicity as opposed to promote tolerance (74,200). The donor haematopoietic stem cells are considered distinct from the circulating and peripheral cells that constitute the effluxed cell and are graft residing cells (201). Therefore, it seems reasonable to assume that the removal of effluxed donor immune cells might be a valid immunomodulatory approach during ex situ preservation of the liver, as has been the case with kidney and lung, with limited negative consequences as the role of more long-lived passenger cell types is unclear.

This study is pertinent in the context of passenger cell depletion-based immunomodulation of donor organs during NMP, as described by Stone et al (179,181). The finding that cells return to the liver following an initial efflux informs ongoing work on cellular therapy delivery during NMP. Novel immunomodulatory approaches delivering cellular therapies (e.g. regulatory T-cells (Treg), tolerogenic dendritic cells (Tol-DC) and mesenchymal stem cells (MSCs)) require cells to be engrafted within the liver (126). The observation that cells re-enter the liver and the kinetics of this process might inform the optimum timing of cell therapy delivery during NMP.

Cytokines progressively increase in response to ex situ reperfusion

The liver's cytokine network is regulated by non-hepatocyte cells which make up a third of the cells in the liver, including liver sinusoidal epithelial cells (LSECs), hepatic stellate cells, Kupffer cells (macrophages), dendritic cells and other lymphocytes. These cells produce several cytokines (TNF-α, IL-5, IL-13, IL-2, CXCL8, IL-7, IL-1Ra) which are mediators of processes including IRI and have also been implicated in driving a switch from innate to adaptive immune responses in liver transplantation with detrimental effects on graft outcomes (202). The inflammatory

environment and circulating perfusate during NMP had not previously been investigated extensively in the liver.

We found that NMP was associated with a progressive rise in the concentration of all detected cytokines within the circulating perfusate. The significance of this finding and its implications on the graft microenvironment, however, remains unclear. It is well established that in the deceased donor setting, a systemic inflammatory response in the period prior to organ procurement is caused by a cytokine storm that creates an inflammatory environment for the graft (203). In cases of subsequent severe ischaemia reperfusion injury this inflammatory milieu persists, resulting in the graft remaining in a state of longer-term inflammation, subsequently mediated by adaptive immune responses (202). Our results showed a sustained pro-inflammatory cytokine environment following ex situ reperfusion with rising levels of almost all measured cytokines. The perfusion circuit is a closed system with a fully cannulated organ, therefore a continual rise in the cytokine levels may represent an accumulation of cytokines from the initial ex situ reperfusion injury that persists throughout NMP. Whether or not this is an active process being driven by NMP, as occurs in cardiopulmonary bypass (204) cannot be elucidated without the employment of an extraction strategy such as CytoSorb® as has been done in other organs (183,185) and serial measurement of levels over prolonged perfusions. What can be deduced is that despite the presence of these high cytokine concentrations, the livers included in this study functioned well during perfusion (using the clinical parameters for assessment of organs during NMP) and went on to be transplanted with positive outcomes (140). The role of cytokines during MP therefore, remains unclear. In ex-vivo lung perfusion (EVLP) and cardiopulmonary bypass procedures, the use of absorptive filters (CytoSorb®) to reduce pro-inflammatory cytokines had been proposed as potential option for reducing systemic inflammatory responses in these scenarios, but this has not been shown to improve clinical outcomes in recent clinical trials (184,205). In the context of liver NMP this an emerging approach and has only recently been integrated into a liver perfusion device (206): there

are no efficacy data yet. Furthermore, the liver is flushed with cold preservation fluid at the end of NMP, which may also be protective as the cytokine rich perfusate circulating during NMP is flushed out prior to implantation, potentially limiting the effects of cytokines to influencing the ex-situ reperfusion injury and therefore only indirectly impacting on preservation reperfusion injury.

Ex situ reperfusion is associate with high levels of circulating nuclear damage associated molecular patterns (DAMPS)

DAMPs include a heterogenous group of molecules that play a critical role in the pathophysiology of ischaemia reperfusion injury, and therefore cause inferior clinical outcomes in liver transplantation ranging from EAD and the requirement for RRT to higher T-cell mediated rejection rates (191,207). Nuclear DAMPS including (histones, HMGB1, nucleosomes and cell free DNA) are some of the best characterised DAMPS in liver injury (64,208,209). NETs (neutrophil extracellular traps) derived from the process of NETosis, represent an important subset of nuclear DAMPs that is known to have a significant effect on organ function, mediating sterile inflammatory organ injury, endothelial dysfunction and microthrombus formation (186,210,211). In the context of ex situ reperfusion injury, any role of DAMPs within the circuit and on the graft is not well understood, and so its significance regarding downstream clinical sequalae is unknown.

We found that the circulating nucleosomes and NETs increased in response to ex situ reperfusion, peaking at the 60 min timepoint and remained elevated through to the end of perfusion at extremely high levels. Indeed, the observed levels in our livers during NMP were much higher than those reported in any other setting, including in critically ill Covid-19 patients on ICU (192).

The effect of nuclear DAMPS including NETs during NMP of the liver is not well established, with little evidence regarding the kinetics and effect of circulating DAMPS on the graft during perfusion and following transplantation. It is possible that the removal of both DAMPS and pro-inflammatory cytokines may have a lasting effect on the immunogenicity of the graft: this represents an important area for future study.

Ex situ reperfusion injury/inflammation: a distinct entity

When considering the actions of immune cells, cytokines and DAMPs in the context of reperfusion on the device, the concept of ex situ reperfusion injury/inflammation emerges as a single overarching concept that describes the immune events occurring during NMP. Ex situ reperfusion initiates an efflux of cells into the circuit which catalyses an inflammatory response characterised by cytokine release and extremely high DAMP concentrations that peak at 60 mins and are thereafter sustained during perfusion. The data presented here supports the concept of ERI and establishes it as a potential target for interventions. Our unexpected finding that immune cells re-enter the liver after the initial efflux suggests that key cell populations are trafficking in and out of the graft: the extent to which this is linked to ERI is unclear, as is the potential therapeutic targeting of this phenomenon to mitigate immune activation. Further investigation of this phenomenon is required.

Although the livers included in this Chapter were obtained from one of the largest machine perfusion trials conducted to-date, the sample size remains small, limiting the conclusions that can be drawn. There are limits, however, to the interpretation, not least because there were very few graft failures: indeed, there were no livers that went on to develop PNF or graft loss. This has undoubtedly limited our ability to investigate how ERI relates to clinical outcome. In view of more recent clinical data that has emerged which has found that ECD livers preserved with pSCS-NMP have a propensity to develop preservation reperfusion injury despite NMP as well as an

unexpectedly high acute T-cell mediated rejection rate (172,173,212), greater attention to immune events (ERI) and the possible 'immune priming' occurring during NMP should be afforded. The retrospective nature of the analysis also limited the scope of immunological analysis to the basic characterisation and phenotyping that was done previously and I did not have access to the original sample material to perform any additional tests, such as functional and deeper immuno-phenotypical analysis of cells (for example suppressive function assays, transcriptomic analysis and regulatory cell characterisation).

My observations have provided novel insights into the immune environment during perfusion, characterising the kinetics of passenger cells within the perfusate, quantifying anti- and pro-inflammatory cytokine concentrations and measuring nuclear DAMPs during NMP. These findings suggest that the following approaches that utilise NMP to facilitate interventions targeting these elements of perfusate should be considered: (i) the removal of cells that are deleterious if passed into the recipient (applied early after reperfusion during the initial efflux); (ii) delivery of cell-based therapies (while cells re-entering the liver); (iii) removal of pro-inflammatory cytokines (absorption i.e CytoSorb®); (iv) DAMP burden reduction (addressed later in the thesis).

This study/Chapter has generated several questions and highlighted important issues that require further investigation in order to fully appreciate the immune events occurring during perfusion and develop possible interventions. Since the clinical studies from which the data for this work was generated, the field has progressed and the clinical landscape shifted significantly. DCD liver transplantation has increased sharply, facilitated, in part, by machine perfusion, (but also by novel technologies such as NRP (30,131)). Clinical NMP has been applied predominantly in this higher risk cohort of livers and mostly for 'viability assessment' and 'reconditioning' of ECD livers more generally. However, in these ECD livers, preservation reperfusion injury-related outcomes (especially the immune-mediated outcomes) are incompletely mitigated by NMP, suggesting that the observations we have reported, characterising ex situ reperfusion injury/inflammation, may

play a critical role in determining the magnitude of residual preservation injury in DCDs and ECDs.

In the next chapter, I investigate the impact of ERI on livers during preservation and transplantation by conducting multi-omics (proteomic and transcriptomic) analysis of tissue samples at various times through preservation, to gain understanding of the impact ERI related immune-molecular events on the graft itself.

Immuno-molecular profiling of NMP livers

"In the protein molecule, nature has devised an instrument in which an underlying simplicity is used to express great subtlety and versatility; it is impossible to see molecular biology in proper perspective until this peculiar combination of virtues has been clearly grasped."

– Sir Francis Crick

Chapter introduction

The circulating perfusate during NMP is comprised of highly inflammatory molecules and cells that have been characterised in the preceding chapter. The impact of this perfusate milieu on the graft itself, over many hours of NMP is not well understood on a molecular level. It is clear that preservation in a "near-physiological", normothermic environment, continuously from retrieval to implant with a "device to donor" i.e continuous NMP (cNMP) approach, has benefits over exclusive SCS. Extensive pre-clinical data demonstrates molecular evidence of graft recovery, ATP repletion and reduced preservation injury (128,150,160,163,170,213). However, much of the available data on cNMP from both clinical and pre-clinical studies have been derived from relatively healthy donor livers with minimal exposure to cold ischaemia prior to NMP, and this has largely shaped the understanding of how NMP affects grafts on a molecular level. Furthermore, when NMP is initiated after substantial periods of SCS (pSCS-NMP), the extent to which cold ischaemia during organ transport abrogates the molecular benefits of subsequent NMP is yet to be fully explored.

However, as the technological developments in NMP have rapidly transformed the field of liver transplantation; financial and logistical considerations have resulted in "back to base" or pSCS-NMP emerging as the dominant clinical application of machine perfusion technology: this includes

a large proportion of ECD livers being preserved in this way for "viability assessment" as well as cases where NMP is used to facilitate complex recipient procedures (172,174,212,214) Although non-randomised and retrospective comparative studies have shown reassuringly comparable clinical data to cNMP in standard criteria donor livers (170,171) mechanistic understanding of the impact of pSCS remains limited and the molecular and immunological status of grafts undergoing this modality of preservation is poorly understood.

1.23.1 Background: Molecular profiling and 'omics' analysis

To gain a deeper understanding of the molecular changes occurring within the graft, we adopted a combined proteomic and transcriptomic (multi-omics) approach, also referred to as "transplantomics" (215), analysing donor liver tissue samples. These molecular techniques help us to understand the proteome and transcriptome within a given sample under certain conditions by identifying the proteins and transcripts present and quantifying them in relation to a comparative sample, often a control or gold-standard treatment, providing differential expression data (216–218). The identified differentially expressed proteins (DEPs) and genes (DEGs) are then analysed using bioinformatic software to determine which cellular component, molecular function and biological processes are represented by these differentially expressed proteins and transcripts (genes) and determine which biological pathways they are involved in. This downstream analysis is referred to as Gene Ontology (GO) enrichment analysis.

Proteomic analysis has been extensively utilised in drug discovery and biomarker research; however, expertise has been concentrated in centres with resources to run expensive instruments and process large amounts of bioinformatic data (219). The cost of analysis coupled with the novelty of machine perfusion technology has resulted in a lack of multi-omics studies on NMP livers (217). Similarly, few studies have investigated the transcriptomic profile of livers during NMP

(220,221), and to my knowledge, no human clinical studies have investigated the transcriptomic profile of livers preserved with pSCS-NMP that went on to be transplanted (217).

In this chapter, I aimed to gain a deeper understanding of changes in the molecular and proteomic profile of donor livers preserved using a 'back to base' approach, principally to assess the impact of a substantial period of SCS prior to NMP on donor livers (pSCS-NMP), but also to appreciate longitudinal changes occurring within the graft through this type of preservation. Furthermore, I sought to explore the proteomic profile of donor livers that went on to have severe preservation reperfusion injury (PRI), as manifest by clinical markers/syndromes associated with the graft reperfusion injury in the recipient (i.e PRS, EAD and elevated pAST). Importantly, this was an exploratory observational multi-omics study designed to discover pathways and processes that might reveal previously unknown aspects of molecular events occurring during pSCS-NMP and was not an exercise in biomarker discovery or 'viability assessment'.

1.24 Chapter aims

The overall objective of this Chapter is to characterise the molecular and immune profile of donor livers preserved with pSCS-NMP.

Specifically, I aimed to:

1. Determine the longitudinal changes (changes over time) in the proteomic and transcriptomic profiles of grafts preserved with pSCS-NMP.

2. Assess and characterise the differences in proteomic and transcriptomic profile of ex situ perfusion (on the device) vs transplant/in situ reperfusion (in the recipient).

3. Analyse the impact of prolonged SCS prior to NMP on the proteome of donor livers at the end of preservation and following transplant reperfusion.

4. Compare the proteomic profiles of livers that developed severe clinical preservation reperfusion injury (PRI) with livers that had no clinical evidence of PRI

1.25 Materials & Methods

1.25.1 Clinical studies

Samples were taken from our previous clinical studies of NMP in liver transplantation, including both modalities of use of the technology. These studies and groups are summarised in Table 0.1. These studies were highly standardised, with protocolised sample preparation and storage which was identical in both (see Methods) the timing of sampling is outlined in.

Donor and recipient clinical characteristics have previously been shown to have excellent comparability between the two studies from which we obtained our samples (171), and this remained the case for the specific samples included in our analysis, summarised in Table 0.2.

Table 0.1: Human Liver machine perfusion studies. Clinical studies of normothermic machine perfusion, including study characteristics and nature of samples included in analysis. COPE = Consortium for organ preservation in Europe, NMP = normothermic machine perfusion, SCS = static cold storage, cNMP = continuous NMP, p SCS-NMP = prior SCS-NMP, UW = University of Wisconsin, FFPE = Formalin fixed paraffin embedded, UK = United Kingdom

	COPE Liver Trial (ISRCTN 39731134)	'Back to Base' pSCS-NMP study (NCT03176433)
Brief Study Description	Livers were procured and placed on OrganOx metra® NMP device at the donor centre (cNMP) or transported in standard SCS conditions in UW® solution to the recipient centre in the COPE study	Livers were procured and transported to recipient centres in standard SCS fashion (for between 3-8hrs) and NMP-L only initiated at the recipient centre for a minimum of 4 hours
Study arms	NMP (minimal cold ischaemia) SCS	Single arm study: SCS+ NMP
Sites	7 Transplant Centres UK & Europe	3 UK Transplant Centres
Samples	Tissue: Snap frozen (stored at -80˚C) Formalin Fixed Paraffin Embedded (FFPE)	Tissue: Snap frozen (stored at -80˚C) Formalin Fixed Paraffin Embedded (FFPE)

	LT1 - Procurement LT2 - End of preservation LT3 - Reperfusion (in situ)	LT1 -End of SCS LT2 - End of NMP LT3 – Reperfusion (in situ)
	Perfusate: stored at -80˚C	Perfusate: stored at -80˚C
	P1 – 15 mins P2 – 60 mins P3 – End of NMP	0 min (baseline sample prior to NMP), 30 min, 60 min, 240 min

Table 0.2: Clinical characteristics: Livers included in Proteomic analysis. **Comparison of the clinical characteristics of the groups from which samples were obtained within the clinical studies.**

Sample timepoints, donor characteristics, preservation details and ischaemia reperfusion injury-related outcomes are included in the table. cNMP = continuous normothermic machine perfusion, SCS = static cold storage, pSCS-NMP = "back to base" or prior static cold storage normothermic machine perfusion, Liver Tissue (LT) Timepoints: LT1 = time of procurement or prior to normothermic machine perfusion, LT2 at the end of preservation and LT3 following reperfusion in the recipient. DBD = donation after brainstem death, DCD = donation after circulatory death, Renal replacement therapy (RRT) or new Acute Kidney Injury (AKI), MEAF = Model for early allograft function (MEAF score 0-10), Peak AST = maximum aspartate aminotransferase in the first 7 days post-transplant), EAD = early allograft dysfunction (Olthoff et al., 2010). Preservation Reperfusion Injury = any one of RRT/AKI, EAD (Oltholff criteria) or PRS, Post Reperfusion Syndrome (PRS). NA – missing data/not available.

		cNMP	***SCS***	***pSCS-NMP***	***p***
Samples					
Number per Timepoint	LT1	97 (34.3)	48 (33.3)	**22 (37.3)**	0.983
	LT2	96 (33.9)	49 (34.0)	**20 (33.9)**	
	LT3	90 (31.8)	47 (32.6)	**17 (28.8)**	
Donor Characteristics					
Donor Type	DBD	194 (68.6)	126 (87.5)	**38 (64.4)**	
	DCD	89 (31.4)	18 (12.5)	**21 (35.6)**	
Donor Age (years)	Mean (SD)	53.8 (16.2)	59.1 (13.6)	**57.6 (16.5)**	
Biological Sex	Male	168 (59.4)	90 (62.5)	**31 (52.5)**	0.422
	Female	115 (40.6)	54 (37.5)	**28 (47.5)**	
Body Mass Index (kg/m^2)	Mean (SD)	27.9 (6.4)	27.1 (4.9)	**27.7 (7.3)**	0.47
Steatosis (surgeon assessment)	None	82 (29.1)	45 (31.2)	**18 (31.6)**	
	Mild	111 (39.4)	75 (52.1)	**14 (24.6)**	
	Moderate	56 (19.9)	21 (14.6)	**21 (36.8)**	
	Severe	33 (11.7)	3 (2.1)	**4 (7.0)**	
Preservation					
CIT (mins)	Mean (SD)	95.3 (23.3)	367.5 (146.1)	**366.2 (70.3)**	<0.001
NMP time (mins)	Mean (SD)	567.0 (257.2)	0.0 (0.0)	**458.6 (235.3)**	<0.001
Total Preservation time (mins)	Mean (SD)	635.7 (289.6)	637.8 (247.0)	**2328.6 (7888.9)**	<0.001
Liver Discarded	No	271 (95.8)	141 (97.9)	**54 (91.5)**	0.113

	Yes	12 (4.2)	3 (2.1)	**5 (8.5)**	
Ischaemia Reperfusion Injury (IRI) related clinical outcomes					
PRS	No	247 (90.1)	120 (85.1)	**49 (90.7)**	0.271
	Yes	27 (9.9)	21 (14.9)	**5 (9.3)**	
EAD (Olthoff criteria)	No	236 (87.1)	107 (75.9)	46 (85.2)	0.014
	Yes	35 (12.9)	34 (24.1)	8 (14.8)	
RRT/AKI	No	210 (77.5)	111 (78.7)	**44 (81.5)**	0.802
	Yes	61 (22.5)	30 (21.3)	**10 (18.5)**	
EAD (MEAF score)	Mean (SD)	4.0 (2.2)	4.9 (2.2)	**(NA)**	<0.001
Peak AST	Mean (SD)	870.0 (1143.1)	1443.9 (1839.2)	**589.5 (1797.8)**	<0.001
PRI	No	207 (76.4)	109 (77.3)	**38 (70.4)**	0.58
	Yes	64 (23.6)	32 (22.7)	**16 (29.6)**	

1.25.2 Proteomics & statistical analysis

The experimental workflow for molecular analysis is summarised below and in Figure 0.5, (a full description is in the (Methods section). Briefly, proteins were extracted, digested and analysed by quantitative label-free liquid chromatography tandem mass spectrometry (LFQ LC-MS/MS) using the timsTOF Pro instrument (Bruker). The raw data from the 'Back to base' and COPE study samples were analysed using MSFragger, to allow for protein identification and quantitation, which has formed the basis of this analysis (Figure 0.5). Principal Component Analysis (PCA) was used to assess, in an unbiased fashion, whether changes in protein expression resulted in a discernible grouping (clustering) of samples by timepoint, based on components (and similarities) within the proteomic profile. Differential expression analysis was done using a combination of t-tests, 1-way and 2-way ANOVAs with post hoc tests (Tukey's HSD) and multiple testing corrections (False discovery rates using permutation-based FDR calculation) in Perseus® software (version 1.6.15.0), we considered proteins to be differentially expressed using a $p<0.01$ with FDR=5% as a threshold unless otherwise stated. For statistically significant differentially expressed proteins (DEPs) in our analyses, we performed supervised hierarchical clustering using Perseus® and we used the STRING® (version 11.5) online database to create protein-protein interaction (PPI) maps with k-means clustering and also to conduct Gene Ontology (GO) enrichment analysis. Comparisons of proteomic profiles were made between different timepoints, preservation approaches and clinical outcomes.

1.25.3 Transcriptomics analysis

Immunological analysis of samples was performed using the Banff Human Organ Transplant (B-HOT) transcriptomic panel, which is a Nanostring® gene expression profiling tool(Mengel et al., 2020). This customised panel is constructed to characterise messenger RNA expression of 758

genes related to transplant molecular and immunological processes (see Methods) for full description). An overview of the process is outlined in (Figure 0.6). Only samples from the 'Back to base' study were included in this analysis (n=12) and the clinical details are outlined in Table 0.3,.

Clinical characteristics of transcriptomic patients* (Summary statistics)

Table 0.3: Transcriptomic analysis - Donor characteristics:

Liver	Age (years)	BMI (kg/m2)	Sex	Donor Type	Cause of Death	ETDRI	DLI
1	68	16.7	F	DBD	CVA	1.96	1.4
2	58	32.4	M	DCD	HBI	2.23	2.09
3	52	46.4	F	DBD	Meningitis	1.76	0.92
4	26	24.6	M	DCD	HBI	1.61	1.41
5	51	23.4	M	DBD	CVA	1.89	1.17
6	59	29.2	M	DCD	CVA	2.52	2.36
Mean	**52**	**28.8**			**CVA**	**1.99**	**1.55**

BMI = body mass index, CVA = cerebrovascular accident, HBI = hypoxic brain injury, ET-DRI = EuroTransplant donor risk index, DLI = donor liver index (UK)

Table 0.4: Transcriptomic analysis - Organ Preservation details.Perfusate samples were obtained at three time points during perfusion, 15 mins, 1 hour and the end of preservation. CIT = cold ischaemic time, NMP = normothermic machine perfusion, ALT = Alanine transaminase, AST = aspartate aminotransferase, LDH = lactate dehydrogenase. * steatosis was assessed by the retrieving surgeon.

Liver	**CIT (mins)**	**NMP duration (mins)**	**Total preservation (mins)**	**Steatosis ***	**Max ALT**	**Max AST**	**Max LDH**
1	402	298	700	None	388.6	725.1	769.6
2	373	377	750	Severe	2837.3	4116.2	8088.4
3	396	331	727	Moderate	1126.7	1798.8	4181.3
4	450	511	515	None	NA	NA	NA
5	311	563	568	Mild	433.3	739.1	1983.9
6	432	NA	NA	Mild	4385	NA	NA
Mean	**386.4**	**416**	**652**		**1834.18**	**1844.8**	**3755.8**

Table 0.5: Transcriptomic analysis - Recipient characteristics. BMI = body mass index, PSC = Primary Sclerosing Cholangitis, HCC = hepatocellular carcinoma, NASH = Non-alcoholic steatohepatitis, MELD = model for end-stage liver disease, UKELD =, United Kingdom model for end-stage liver disease.

Liver	Sex	BMI (kg/m2)	Cause of Liver Failure	MELD score	UKELD score
1	F	24.2	PSC	10	34
2	M	23.4	HCC Cirrhosis	17	41
3	M	43.4	Hep C Cirrhosis	17	41
4	M	31.8	NASH	24	49
5	M	30.1	Alcohol	14	37
Mean		**30.58**		**16.4**	**40.4**

Table 0.6: Transcriptomic analysis - Clinical outcomes of livers. EAD = early allograft dysfunction (Olthoff et al., 2010). PNF = Primary non-function (no function post-transplant requiring urgent re-listing, RRT = Renal Replacement Therapy, ITU = intensive therapy unit.

Liver	EAD	PNF	ITU stay	RRT	30-day patient survival	30-day graft survival
1	No	No	2	No	Alive	Functioning
2	No	No	7	No	Alive	Functioning
3	No	No	6	No	Alive	Functioning
4	Yes	No	20	Yes	Alive	Graft loss
5	Yes	No	14	Yes	Alive	Graft loss
Mean			**9.8**			

1.26 Results

1.26.1 Data & Bioinformatic work flow

In total, 57 samples from the 'Back to base' study underwent LFQ LC-MS/MS analysis alongside the 437 COPE liver study samples, as summarised in Table 0.1. Once the data was uploaded into Perseus®, it was filtered for human only proteins, transformed (Log2) and normalised (median) finding adequate protein abundances in all pSCS-NMP samples and initially identifying a total of 7714 proteins. We then filtered out some of the identified proteins, maintaining proteins identified in 70% of samples in at least one timepoint, with at least one unique peptide and we imputed missing values from a normal distribution within Perseus®. In our data set, we finally had 1073 proteins identified from which we ran our further analysis. The data was split into three study groups, pSCS-NMP (the focus of this study), cNMP and SCS, (both of which were from the COPE liver study), which were used for comparison and not in themselves a focus of my investigation.

1.27 Longitudinal proteomic analysis of pSCS-NMP liver preservation

We first assessed the degree of variation in proteome profile in our samples using principal component analysis (PCA) and we found clear grouping or clustering of the different sample timepoints, (Figure 0.1). We went on to perform differential expression analysis on samples taken at different timepoints through preservation. A total of 17 proteins were identified as differentially expressed between timepoints, we used a one-way ANOVA with Tukey's HSD post hoc test, FDR 0.05) (Figure 0.2). Using these identified proteins, we performed supervised hierarchical clustering analysis (Figure 0.2a) revealing clusters of proteins associated with changes during pSCS-NMP preservation revealing three major clusters of proteins, these are highlighted in the orange boxes. These proteins related to lipid metabolism, transport and fatty acid oxidation as well as haemoglobin (Hb)-based oxygen transport (Figure 0.2b&c). Hb-related proteins were upregulated immediately during LT2, which was anticipated owing to the blood-based perfusate on the

perfusion circuit with down regulation of lipid metabolism. During the LT3 phase, the fatty acid oxidation and lipid transport proteins are significantly upregulated alongside the oxygen binding and transport proteins.

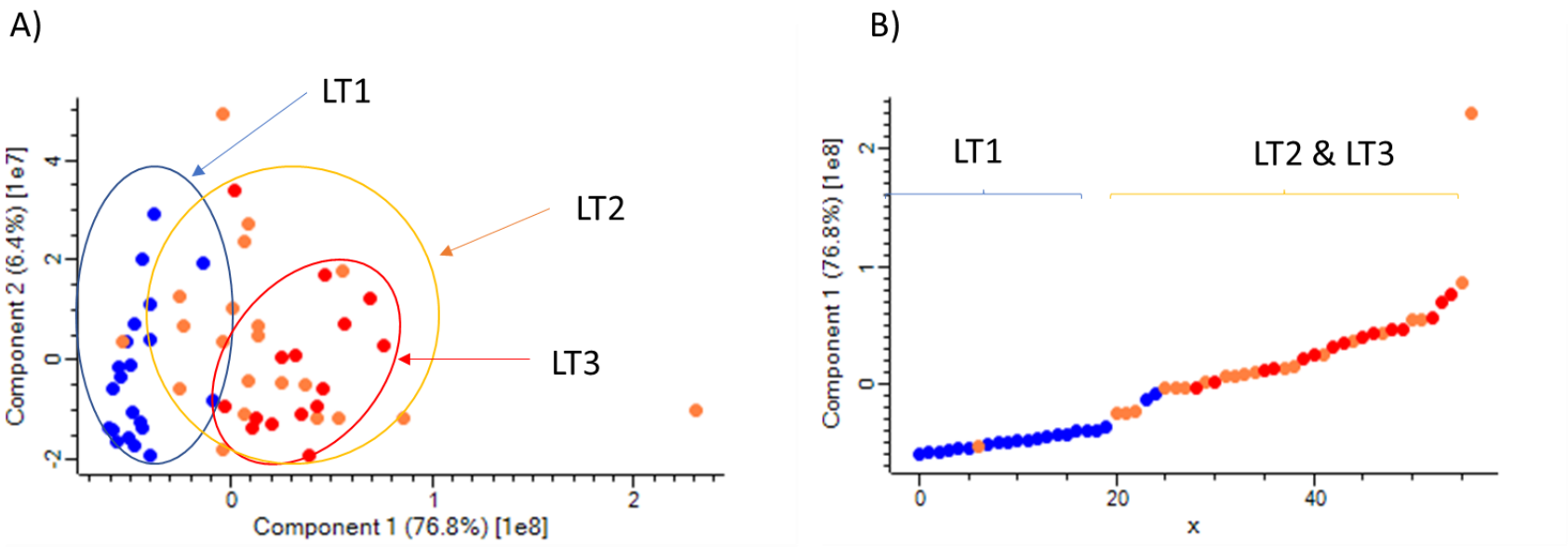
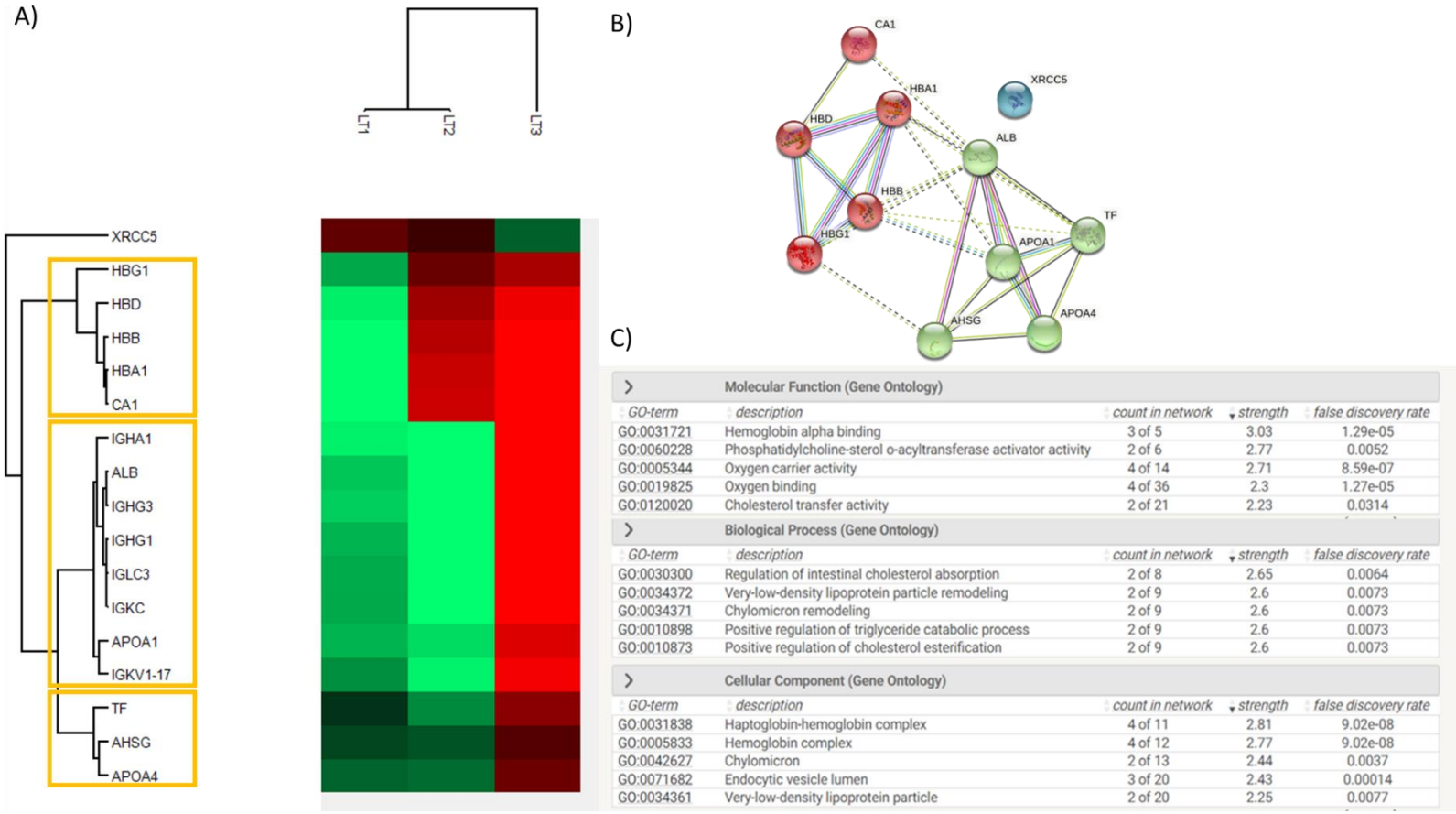


Figure 0.1: Proteomic Analysis - Principal Component Analysis (PCA). Label-free quantitation (LFQ) proteomic data of liver tissue samples taken at different time points of preservation was analysed by PCA. A) Three distinct clusters associated with the different time points were found, clearly demonstrating that LT1 samples (taken at the end of SCS (blue)) group together, while the LT2 samples (taken at the end of NMP (orange)) group separately. Furthermore, LT3 samples (taken following in situ reperfusion (red)) grouped separate to LT1 and seemingly as a subset within the LT2 samples. B) The clustering of samples according to timepoint is most apparent when the data is arranged according to component 1 with SCS (blue) clustering very clearly away from the other timepoints. NMP = normothermic machine perfusion, SCS = static cold storage.



Molecular Function (Gene Ontology)

GO-term	description	count in network	strength	false discovery rate
GO:0031721	Hemoglobin alpha binding	3 of 5	3.03	1.29e-05
GO:0060228	Phosphatidylcholine-sterol o-acyltransferase activator activity	2 of 6	2.77	0.0052
GO:0005344	Oxygen carrier activity	4 of 14	2.71	8.59e-07
GO:0019825	Oxygen binding	4 of 36	2.3	1.27e-05
GO:0120020	Cholesterol transfer activity	2 of 21	2.23	0.0314

Biological Process (Gene Ontology)

GO-term	description	count in network	strength	false discovery rate
GO:0030300	Regulation of intestinal cholesterol absorption	2 of 8	2.65	0.0064
GO:0034372	Very-low-density lipoprotein particle remodeling	2 of 9	2.6	0.0073
GO:0034371	Chylomicron remodeling	2 of 9	2.6	0.0073
GO:0010898	Positive regulation of triglyceride catabolic process	2 of 9	2.6	0.0073
GO:0010873	Positive regulation of cholesterol esterification	2 of 9	2.6	0.0073

Cellular Component (Gene Ontology)

GO-term	description	count in network	strength	false discovery rate
GO:0031838	Haptoglobin-hemoglobin complex	4 of 11	2.81	9.02e-08
GO:0005833	Hemoglobin complex	4 of 12	2.77	9.02e-08
GO:0042627	Chylomicron	2 of 13	2.44	0.0037
GO:0071682	Endocytic vesicle lumen	3 of 20	2.43	0.00014
GO:0034361	Very-low-density lipoprotein particle	2 of 20	2.25	0.0077

Figure 0.2: Proteomic analysis: Changes in protein expression through preservation with sequential static cold storage (SCS), normothermic machine perfusion (NMP) and in situ reperfusion. A) Supervised hierarchical clustering heatmap of differentially expressed proteins (DEPs) (n=17) during different phases of liver preservation (LT1 = end of SCS, LT2 = end of NMP and LT3 = following reperfusion). There is a progressive upregulation in the expression of these proteins through preservation, initially with a subset upregulated following NMP and then the remaining DEPs upregulated during reperfusion. The three clusters are highlighted by orange boxes. B) String® protein-protein interaction map with k-mean clustering of the n=17 DEPs demonstrating the network of Haemoglobin-related, oxygen carrying and transport related proteins (red) as well as an interconnected cluster of lipid metabolism-related proteins (green). C) The Gene Ontology (GO) enrichment analysis performed in String® displaying p-values corrected for multiple testing within each category using the Benjamini–Hochberg model. NMP = normorthermic machine perfusion, DEP = differentially expressed proteins, SCS = static cold storage.

1.27.1 Impact of a period of SCS prior to initiation of NMP on the molecular profile of livers preserved with machine perfusion.

Profile at the end of preservation (LT2)

We found that any application of NMP, (i.e a pSCS-NMP or cNMP), significantly alters the proteome of livers when compared to SCS alone when analysing the proteomic profiles of biopsy samples obtained from the grafts at the end of preservation (LT2 timepoint) (**Figure 0.3)**. Expectedly, the overall expression profiles within the NMP groups (pSCS-NMP and cNMP) were far more similar to each other than they were to the SCS group. However, there were also some significant differences in the protein expression profiles between cNMP and pSCS-NMP (Figure 0.4, highlighted by the orange rectangle).

DEPs that are *up*regulated during normothermic preservation common to all NMP modalities (Figure 0.3, a-c: top orange box, top table and PPI mapping) were related to oxygen transport and haemoglobin, with increased ribosomal activity and protein synthesis related proteins also present. Proteins involved in fatty acid degradation, biosynthesis of fatty acids and fatty acid metabolic processes were universally downregulated in all NMP livers at the end of preservation relative to SCS (Figure 0.3, a-c: bottom orange box and bottom table).

When we consider differences *between* the NMP cohorts in relation to SCS, enrichment analysis of DEPs that were specifically *down*regulated in pSCS-NMP conditions and not in cNMP were related to mitochondrial activity, mitochondrial Complex 1 activity and the electron transport chain. Yet protease subunits and protein degradation related proteins are comparatively *up*regulated under pSCS-NMP conditions (Figure 0.4 a-c).

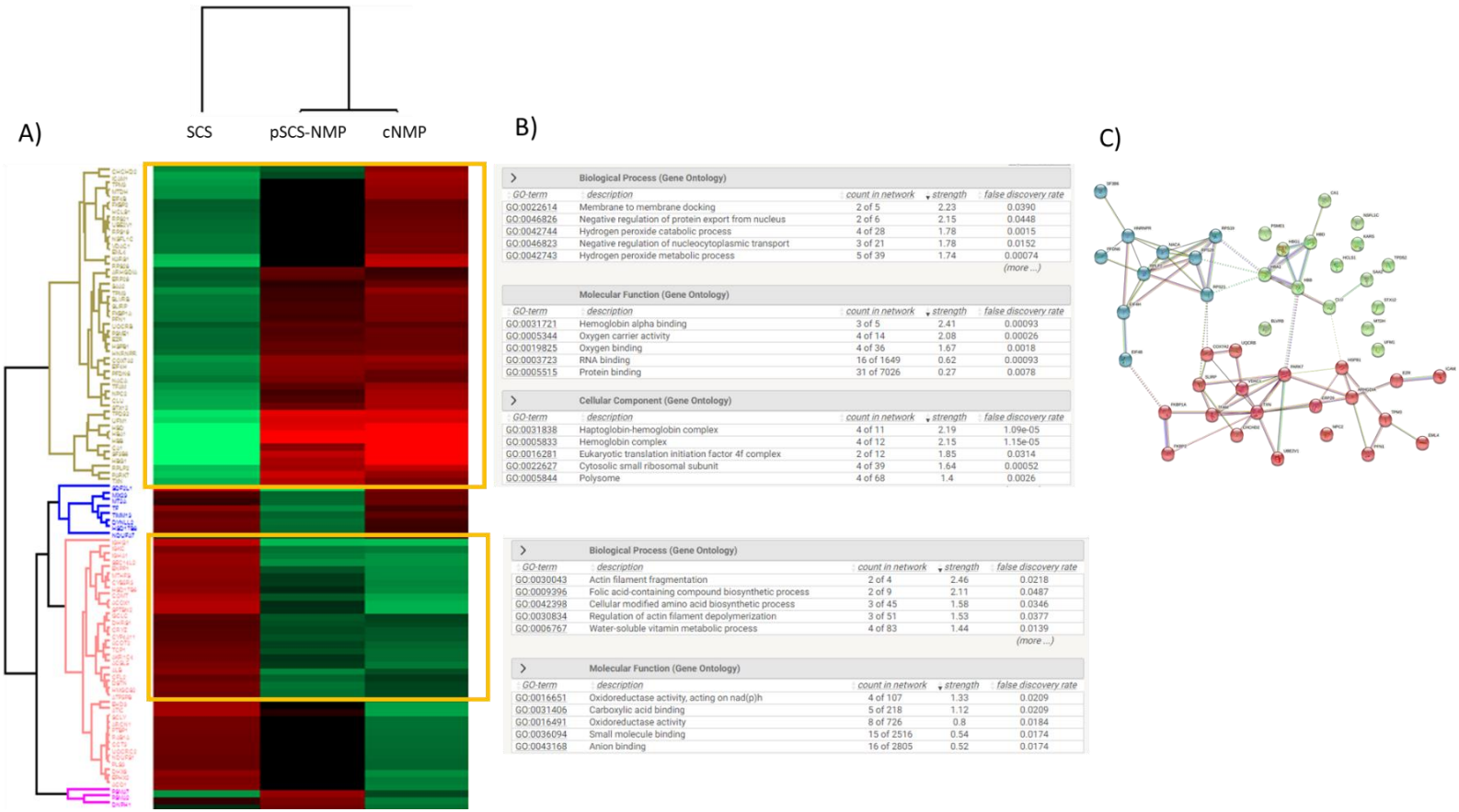
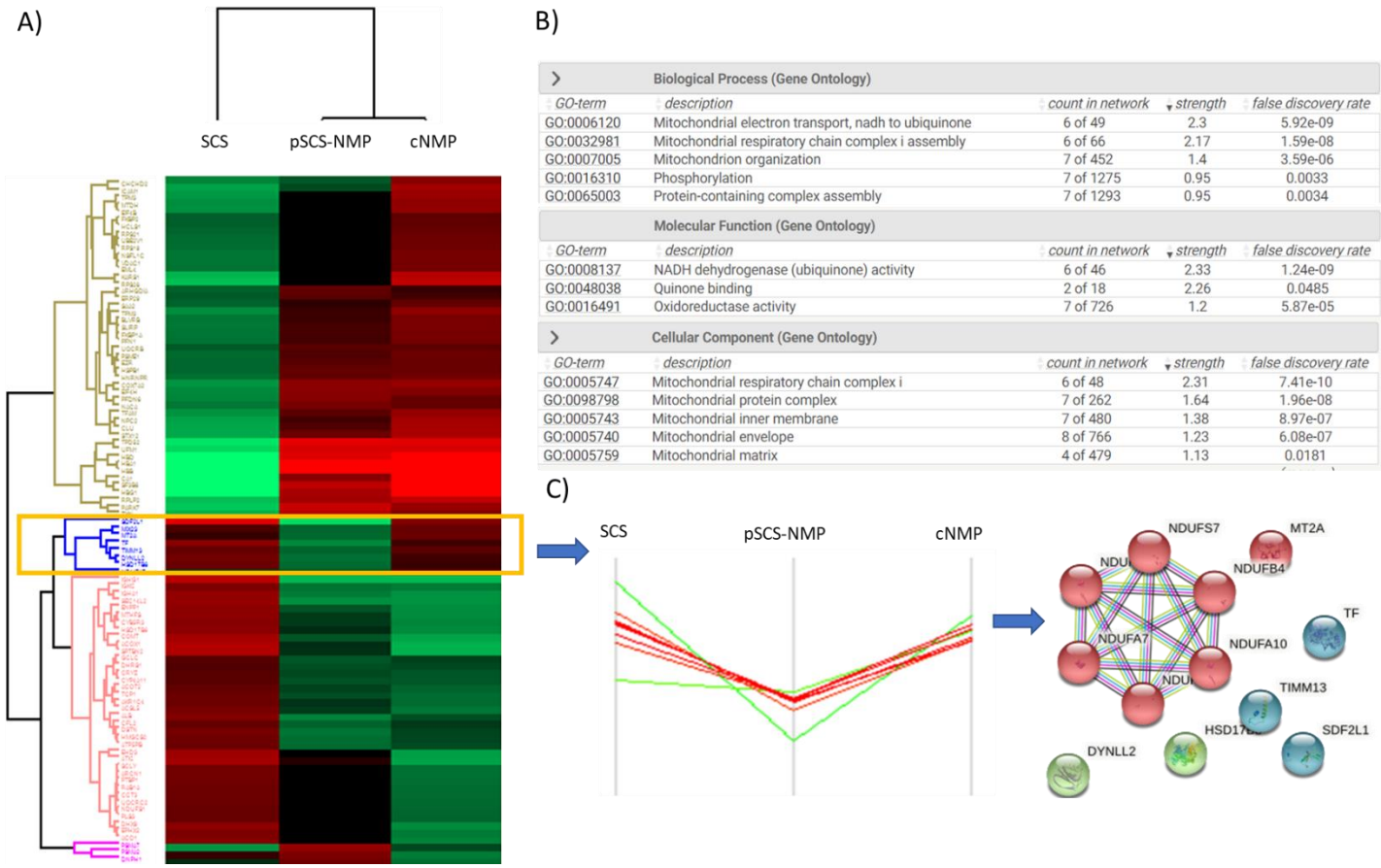


Figure 0.3: Proteomic analysis: Profiles at the end of preservation (LT2): impact of different preservation approaches. A) Hierarchical clustering analysis of the significantly differentially expressed proteins (DEPs) n=92, between the different preservation approaches. B) **The Gene Ontology (GO) enrichment analysis** performed in String® displaying the count in network, strength (enrichment effect size) and FDR shown as the p-value corrected for multiple testing within each category using the Benjamini–Hochberg model for both the upregulated (Top tables) and downregulated (bottom tables) proteins from the highlighted clusters (orange boxes) **C) Protein-protein interactions (PPI) map** (mapped via k-mean clusters (String®)) of significantly upregulated DEPs in the pSCS-NMP & cNMP cohort at the LT2 timepoint. Upregulated proteins related to oxygen transport and haemoglobin (green), ribosomal proteins and proteins synthesis (red & blue). The universally down regulated proteins during NMP did not form significant clusters. cNMP = continuous normothermic machine perfusion, SCS = static cold storage, pSCS-NMP = "back to base" or prior static cold storage normothermic machine perfusion.



B)

Biological Process (Gene Ontology)				
GO-term	description	count in network	strength	false discovery rate
GO:0006120	Mitochondrial electron transport, nadh to ubiquinone	6 of 49	2.3	5.92e-09
GO:0032981	Mitochondrial respiratory chain complex i assembly	6 of 66	2.17	1.59e-08
GO:0007005	Mitochondrion organization	7 of 452	1.4	3.59e-06
GO:0016310	Phosphorylation	7 of 1275	0.95	0.0033
GO:0065003	Protein-containing complex assembly	7 of 1293	0.95	0.0034
Molecular Function (Gene Ontology)				
GO-term	description	count in network	strength	false discovery rate
GO:0008137	NADH dehydrogenase (ubiquinone) activity	6 of 46	2.33	1.24e-09
GO:0048038	Quinone binding	2 of 18	2.26	0.0485
GO:0016491	Oxidoreductase activity	7 of 726	1.2	5.87e-05
Cellular Component (Gene Ontology)				
GO-term	description	count in network	strength	false discovery rate
GO:0005747	Mitochondrial respiratory chain complex i	6 of 48	2.31	7.41e-10
GO:0098798	Mitochondrial protein complex	7 of 262	1.64	1.96e-08
GO:0005743	Mitochondrial inner membrane	7 of 480	1.38	8.97e-07
GO:0005740	Mitochondrial envelope	8 of 766	1.23	6.08e-07
GO:0005759	Mitochondrial matrix	4 of 479	1.13	0.0181

Figure 0.4: Proteomic analysis: Profiles at the end of preservation (LT2): Differences between pSCS-NMP and cNMP. A) Hierarchical clustering analysis of the significantly differentially expressed proteins (DEPs) showing a cluster of DEPs (n= 8) were significantly different between pSCS-NMP and cNMP at LT2. B) The Gene Ontology (GO) enrichment analysis performed in String® displaying the count in network, strength (enrichment effect size) and FDR shown as the p-value corrected for multiple testing within each category using the Benjamini–Hochberg model for proteins downregulated in pSCS-NMP but upregulated in cNMP.) Protein-protein interactions (PPI) map (mapped via k-mean clusters (String®)) of the DEPs. cNMP = continuous normothermic machine perfusion, SCS = static cold storage, pSCS-NMP = "back to base" or prior static cold storage normothermic machine perfusion.

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Profile after transplantation: LT3 Post reperfusion

Analysis of the post [transplant] reperfusion (LT3) timepoint revealed even more DEPs (n=139 in total) were present between the different groups (Figure 0.5). Similar to observations at the end of preservation (LT2), following reperfusion in the recipient, the majority of significantly DEPs were upregulated in both groups where normothermic preservation was employed when compared to the SCS only cohort of livers (Figure 0.5 (orange box), b & c). However, as observed in the LT2 samples, there was a cluster of proteins that were down regulated in the pSCS-NMP while being up regulated in the SCS and cNMP cohorts (Figure 0.6 top orange rectangle). These 21 DEPs are not strongly interconnected when analysed using String® PPI mapping, but in the GO analysis, they present significant enrichment in biological pathways related to fatty acid derivative metabolic processes, fatty acid biosynthesis and oxidoreductase activity. Conversely, some DEPs were upregulated in pSCS-NMP at the LT3 timepoint yet down regulated in the continuous NMP which related to cellular proteostasis and chylomicron remodelling. (Figure 0.6 bottom orange box)

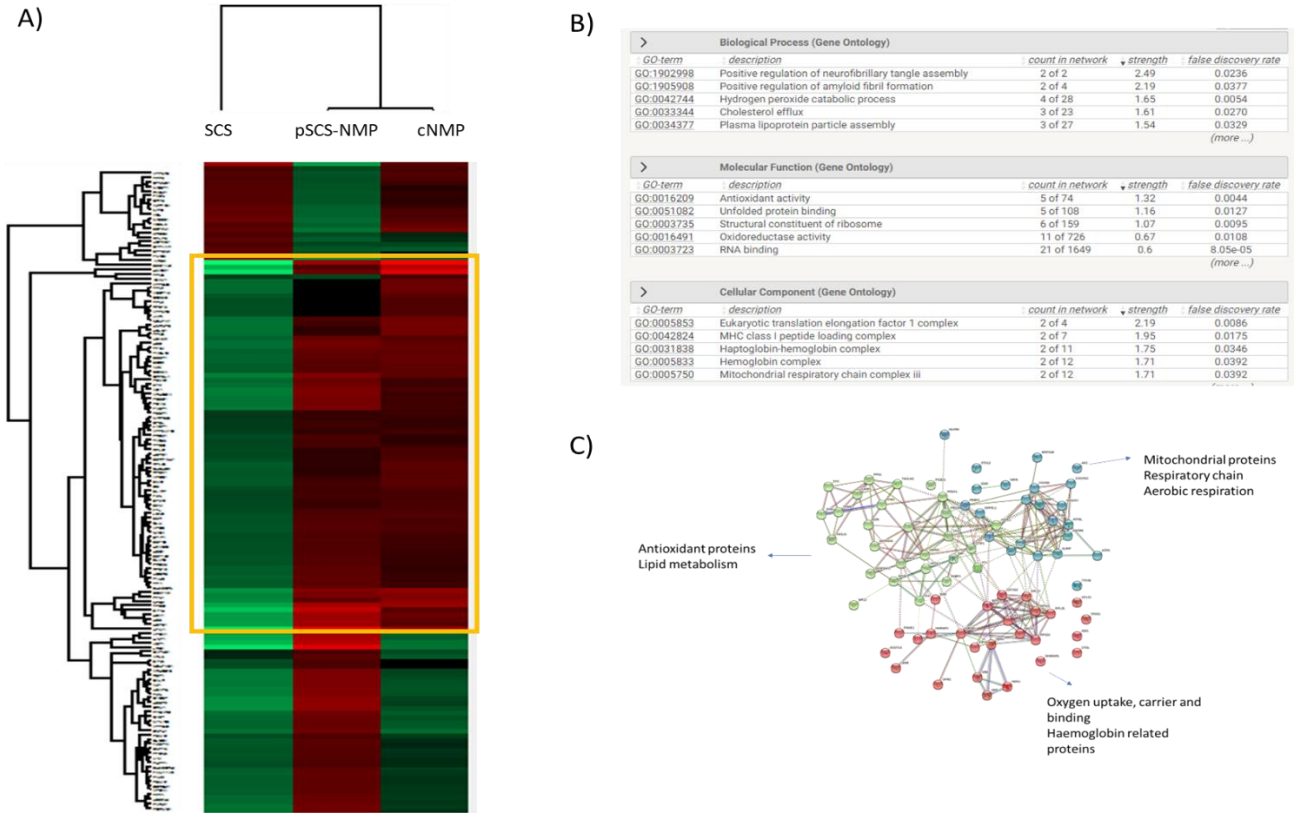
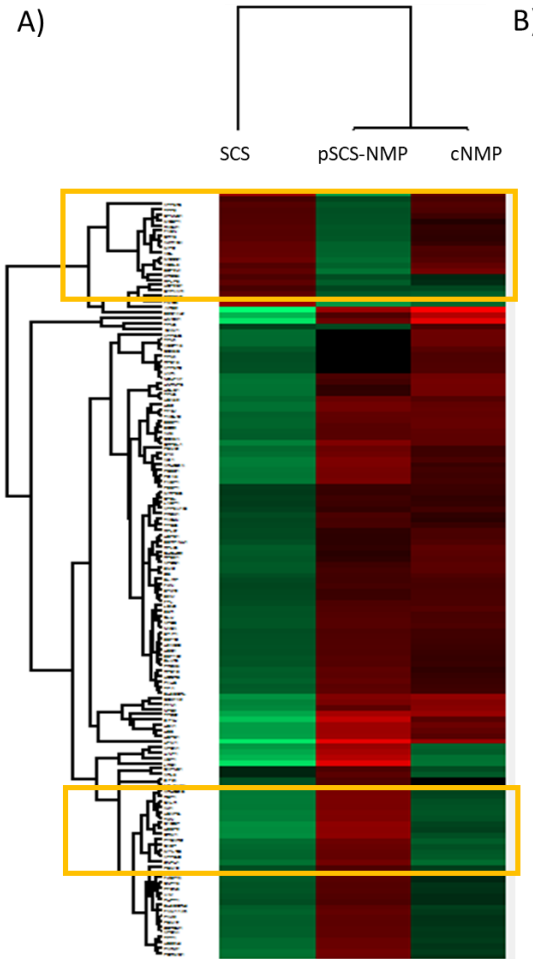


Figure 0.5: Proteomic analysis: Profiles following reperfusion (LT3): impact of preservation approach. A) Hierarchical clustering analysis of the significantly differentially expressed proteins (DEPs) (N=139) between the different preservation approaches. Highlighted are the proteins upregulated at the reperfusion stage (LT3) in both pSCS-NMP and cNMP livers (i.e all normothermically preserved livers). **B) The Gene Ontology (GO) enrichment analysis performed in String®** displaying the count in network, strength (enrichment effect size) and FDR shown as the p-value corrected for multiple testing within each category using the Benjamini–Hochberg model for proteins upregulated in NMP **C) String® protein-protein interaction (PPI) map of highlighted DEPs**. k-mean clustering revealed distinct clusters of proteins that are upregulated: antioxidant proteins and lipid transport and degradation proteins (green), oxygen uptake, binding and haemoglobin related proteins (red) and mitochondrial proteins and the respiratory chain (blue). cNMP = continuous normothermic machine perfusion, SCS = static cold storage, pSCS-NMP = "back to base" or prior static cold storage normothermic machine perfusion.



B)

Biological Process (Gene Ontology)

GO-term	description	count in network	strength	false discovery rate
GO:1901568	Fatty acid derivative metabolic process	4 of 162	1.36	0.0311
GO:0032787	Monocarboxylic acid metabolic process	6 of 515	1.04	0.0244
GO:0055114	Oxidation-reduction process	8 of 939	0.9	0.0144
GO:0043436	Oxoacid metabolic process	8 of 944	0.9	0.0144
GO:0019752	Carboxylic acid metabolic process	7 of 853	0.88	0.0307

(more ...)

Molecular Function (Gene Ontology)

GO-term	description	count in network	strength	false discovery rate
GO:0016709	Oxidoreductase activity, acting on paired donors, with incor...	3 of 47	1.77	0.0217
GO:0050661	NADP binding	3 of 54	1.71	0.0242
GO:0016491	Oxidoreductase activity	7 of 726	0.95	0.0205
GO:0043168	Anion binding	12 of 2805	0.6	0.0205
GO:0003824	Catalytic activity	15 of 5486	0.41	0.0299

C)

Biological Process (Gene Ontology)

GO-term	description	count in network	strength	false discovery rate
GO:1990592	Protein k69-linked ufmylation	2 of 5	2.23	0.0361
GO:1901164	Negative regulation of trophoblast cell migration	2 of 6	2.15	0.0392
GO:0046826	Negative regulation of protein export from nucleus	2 of 6	2.15	0.0392
GO:0030300	Regulation of intestinal cholesterol absorption	2 of 8	2.03	0.0478
GO:1901163	Regulation of trophoblast cell migration	3 of 13	1.99	0.0361

(more ...)

Molecular Function (Gene Ontology)

GO-term	description	count in network	strength	false discovery rate
GO:0120020	Cholesterol transfer activity	3 of 21	1.78	0.0382
GO:0005515	Protein binding	32 of 7026	0.29	0.0120

Cellular Component (Gene Ontology)

GO-term	description	count in network	strength	false discovery rate
GO:0016272	Prefoldin complex	2 of 7	2.08	0.0148
GO:0019773	Proteasome core complex, alpha-subunit complex	2 of 8	2.03	0.0177
GO:0071682	Endocytic vesicle lumen	4 of 20	1.93	5.13e-05
GO:0031838	Haptoglobin-hemoglobin complex	2 of 11	1.89	0.0260
GO:0005833	Hemoglobin complex	2 of 12	1.85	0.0289

Reactome Pathways

pathway	description	count in network	strength	false discovery rate
HSA-9613829	Chaperone Mediated Autophagy	3 of 22	1.76	0.0145
HSA-9615710	Late endosomal microautophagy	3 of 34	1.57	0.0256
HSA-2173782	Binding and Uptake of Ligands by Scavenger Receptors	3 of 41	1.49	0.0361
HSA-5687128	MAPK6/MAPK4 signaling	4 of 87	1.29	0.0256
HSA-109581	Apoptosis	5 of 173	1.09	0.0256

(more ...)

Figure 0.6: Proteomic analysis: Profiles following reperfusion (LT3): impact of preservation approach. A) Hierarchical clustering analysis of the significantly differentially expressed proteins (DEPs) (N=139) between the different preservation approaches. Highlighted are the proteins downregulated at the reperfusion stage (LT3) in pSCS-NMP yet upregulated in cNMP livers (Top orange box) and vice versa (bottom orange box)). **B) The Gene Ontology (GO) enrichment analysis performed in String®** displaying the count in network, strength (enrichment effect size) and FDR shown as the p-value corrected for multiple testing within each category using the Benjamini–Hochberg model for the different clusters (top orange box and bottom orange box). cNMP = continuous normothermic machine perfusion, SCS = static cold storage, pSCS-NMP = "back to base" or prior static cold storage normothermic machine perfusion.

1.27.2 Impact of prolonged SCS prior to NMP

To further assess the effect of SCS prior to NMP, the length of SCS was divided into long (> 6 hours), intermediate (between 3h and 6h) and short CIT (<3 hours) prior to initiation of NMP and the groups were subsequently compared. In this analysis, a 1-way ANOVA with Post hoc Tukey's HSD with a significance threshold of p= 0.01 was applied and an FDR = 0.05. We performed this analysis on the LT2 and LT3 timepoints separately.

LT2 analysis: Profile at the end of preservation

We found 25 DEPs related to the length of CIT prior to NMP with the majority of DEPs found between short and long CIT with very few DEPs related to intermediate CIT and either short or long. Proteins related to the mitochondrial respiratory chain, oxidative phosphorylation and leukocyte trans endothelial migration were downregulated with prolonged CIT in the enrichment analysis. Proteins involved in regulation of transcription, proteasome regulation and other proteolysis related proteins were up regulated with longer CIT (Figure 0.7).

LT3 analysis: profile following transplantation - post reperfusion

An analysis of the LT3 timepoint revealed similar findings to those of LT2, but had slightly more DEPs (66 in total) with the majority related to differences between the long and short SCS periods (as opposed to between intermediate and either short or long). The DEPs were analysed using String® GO enrichment analysis and this showed similar results to the LT2, with an upregulation of protein degradation related proteins and down regulation of mitochondrial complex 1 associated protein (Figure 0.8).

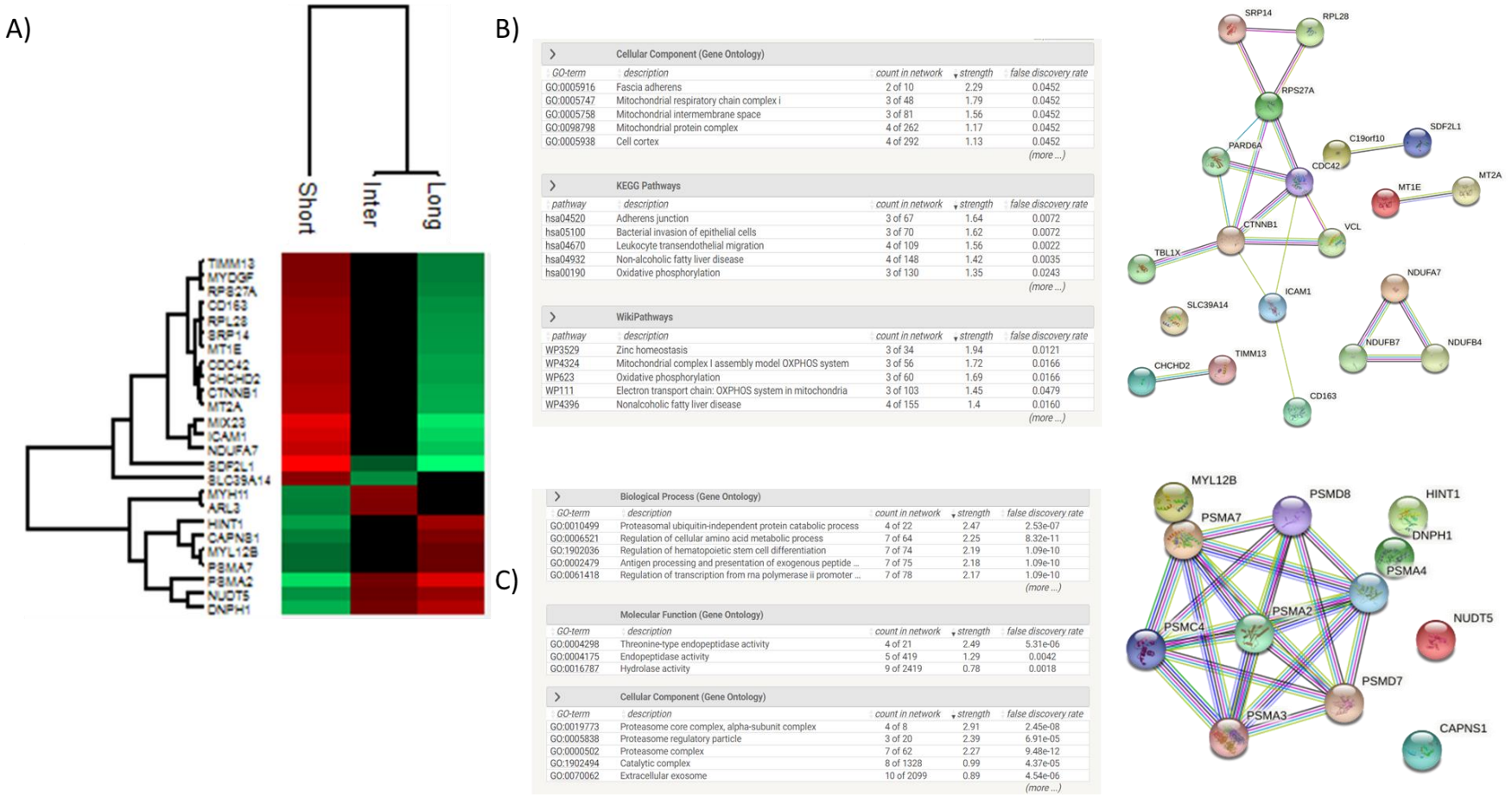


Figure 0.7: Proteomic analysis: Impact of length of static cold storage (SCS) prior to initiation of normothermic machine perfusion (NMP) on the profile of livers at the LT2 (end of preservation) timepoint. A) Hierarchical clustering analysis of the significantly differentially expressed proteins (DEPs) between the different lengths of SCS prior to NMP. The Gene Ontology (GO) enrichment analysis performed in String® displaying the count in network, strength (enrichment effect size) and FDR shown as the p-value corrected for multiple testing within each category using the Benjamini–Hochberg model for the different clusters and String® protein-protein interaction (PPI) maps B) (top row) Downregulated proteins after prolonged CIT prior to NMP GO enrichment and PPI mapping. C) Upregulated DEPs after prolonged CIT prior to NMP, GO enrichment and PPI mapping.

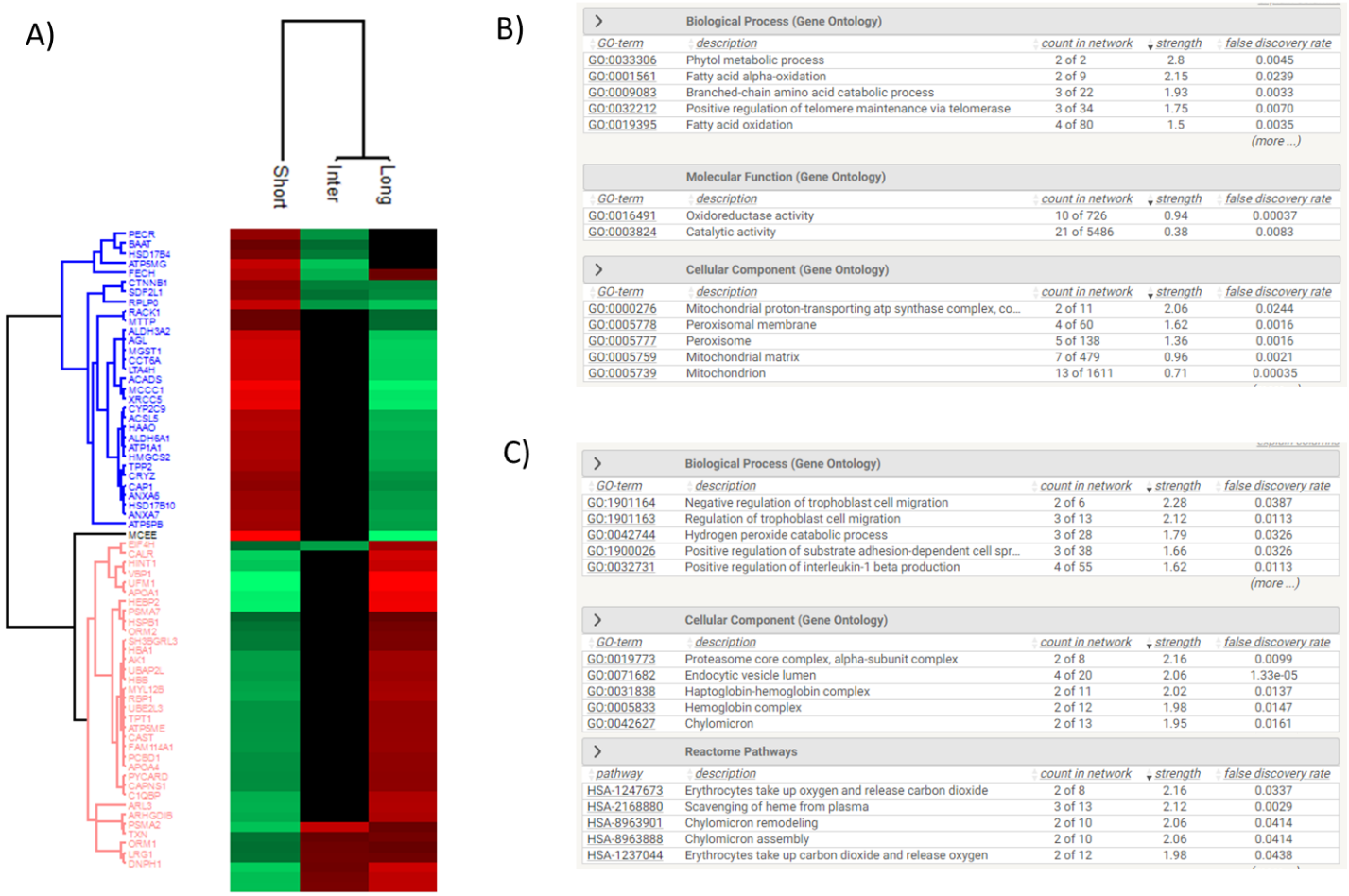


Figure 0.8: Proteomic analysis: Impact of length of static cold storage (SCS) prior to initiation of normothermic machine perfusion (NMP) on the profile of livers at the LT3 (Reperfusion timepoint). A) Hierarchical clustering analysis of the significantly differentially expressed proteins (DEPs) between the different lengths of SCS prior to NMP. The Gene Ontology (GO) enrichment analysis performed in String® displaying the count in network, strength (enrichment effect size) and FDR shown as the p-value corrected for multiple testing within each category using the Benjamini–Hochberg model for the different clusters: B) downregulated proteins following reperfusion after prolonged CIT C) upregulated proteins following reperfusion after prolonged CIT. CIT = cold ischaemic time (prior to NMP), SCS = static cold storage, long = > 6 hours, inter = 3-6 hours, short = <3 hors NMP = continuous normothermic machine perfusion.

1.27.3 Proteomic analysis of clinical PRI in pSCS-NMP livers

Finally, we compared the proteomic profile of donor livers at LT2 and LT3 between livers that went on to develop clinical PRI (one of PRS, AKI/RRT or EAD) versus those that did not. We found that at the end of preservation (LT2) there were very few, only 2 out of the 1073 detected proteins, that were significantly differentially expressed. Yet, after transplant reperfusion (LT3), we observed some, albeit modest differences, with 25 proteins significantly more abundant in PRI livers compared to liver that had no PRI (FDR0.05, p-value 0.01)(Figure 0.9). These DEPs were predominantly related to protein translation initiation, associated with 'ribosomal' cellular components and 'nuclear-transcribed mRNA catabolic processes' on STRING® GO and enrichment analysis (Figure 0.10).

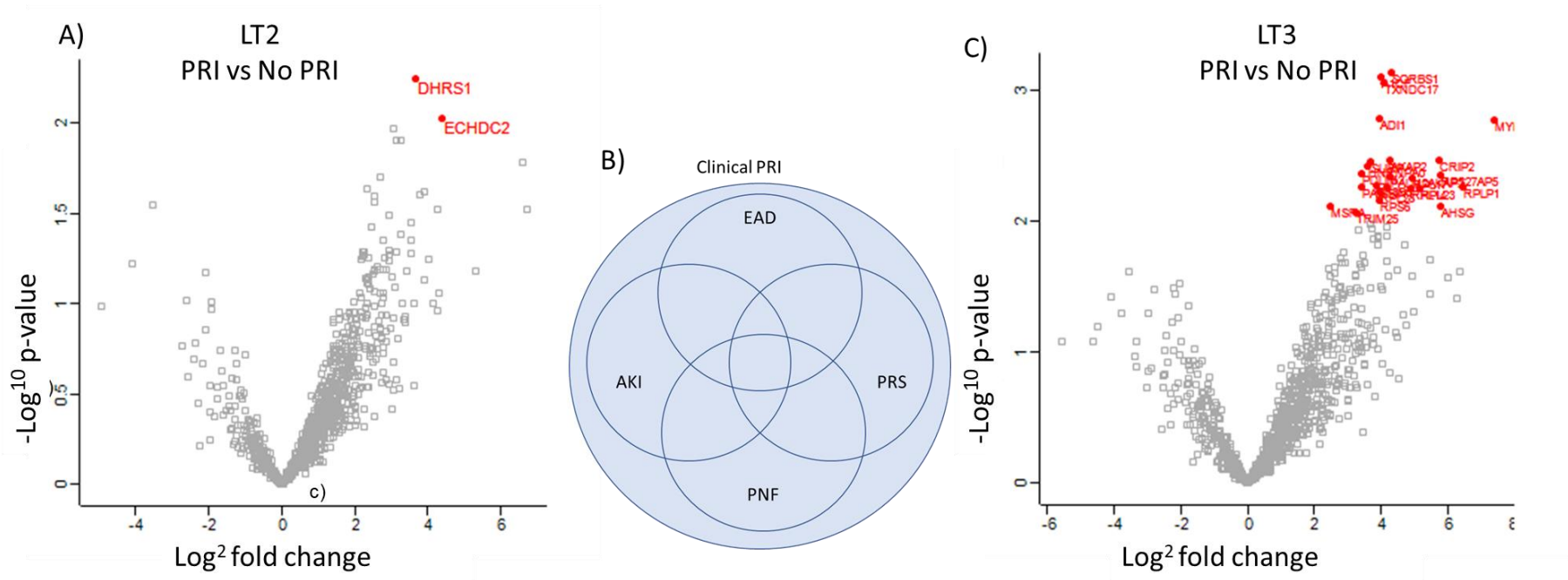
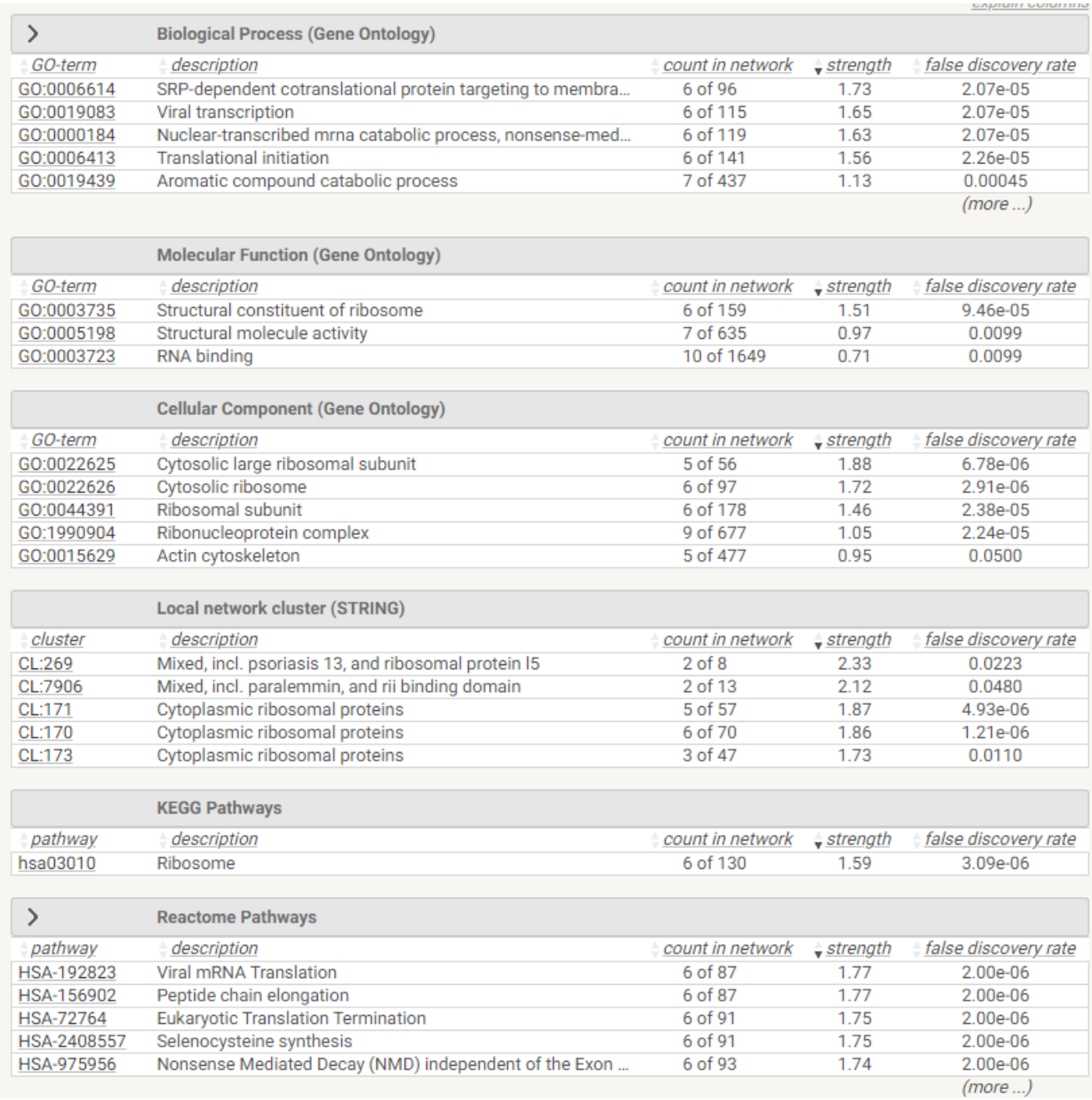


Figure 0.9: Proteomic analysis: Volcano plots displaying proteomic profile differences between livers with or without Preservation Reperfusion Injury (PRI) at different timepoints. A) Differences between PRI and no PRI at LT2: showing only 2 significantly differentially expressed proteins (DEPs) Analysis was by a paired students t-test, with a p-value threshold of 0.01. B) Differences between PRI and no PRI at LT3: showing 25 DEPs upregulated at this timepoint. These proteins are predominantly related to protein translation and ribosomal proteins. C) Venn diagram of the overlapping clinical syndromes associated with reperfusion injury that encompass clinical PRI. PRI = Preservation Reperfusion Injury (any one of RRT/AKI, EAD (Oltholff criteria) or PRS, PRS = Post Reperfusion Syndrome, RRT = Renal replacement therapy or AKI = new Acute Kidney Injury. Timepoints: LT2 at the end of preservation and LT3 following reperfusion in the recipient.



Biological Process (Gene Ontology)

GO-term	description	count in network	strength	false discovery rate
GO:0006614	SRP-dependent cotranslational protein targeting to membra...	6 of 96	1.73	2.07e-05
GO:0019083	Viral transcription	6 of 115	1.65	2.07e-05
GO:0000184	Nuclear-transcribed mrna catabolic process, nonsense-med...	6 of 119	1.63	2.07e-05
GO:0006413	Translational initiation	6 of 141	1.56	2.26e-05
GO:0019439	Aromatic compound catabolic process	7 of 437	1.13	0.00045

(more ...)

Molecular Function (Gene Ontology)

GO-term	description	count in network	strength	false discovery rate
GO:0003735	Structural constituent of ribosome	6 of 159	1.51	9.46e-05
GO:0005198	Structural molecule activity	7 of 635	0.97	0.0099
GO:0003723	RNA binding	10 of 1649	0.71	0.0099

Cellular Component (Gene Ontology)

GO-term	description	count in network	strength	false discovery rate
GO:0022625	Cytosolic large ribosomal subunit	5 of 56	1.88	6.78e-06
GO:0022626	Cytosolic ribosome	6 of 97	1.72	2.91e-06
GO:0044391	Ribosomal subunit	6 of 178	1.46	2.38e-05
GO:1990904	Ribonucleoprotein complex	9 of 677	1.05	2.24e-05
GO:0015629	Actin cytoskeleton	5 of 477	0.95	0.0500

Local network cluster (STRING)

cluster	description	count in network	strength	false discovery rate
CL:269	Mixed, incl. psoriasis 13, and ribosomal protein l5	2 of 8	2.33	0.0223
CL:7906	Mixed, incl. paralemmin, and rii binding domain	2 of 13	2.12	0.0480
CL:171	Cytoplasmic ribosomal proteins	5 of 57	1.87	4.93e-06
CL:170	Cytoplasmic ribosomal proteins	6 of 70	1.86	1.21e-06
CL:173	Cytoplasmic ribosomal proteins	3 of 47	1.73	0.0110

KEGG Pathways

pathway	description	count in network	strength	false discovery rate
hsa03010	Ribosome	6 of 130	1.59	3.09e-06

Reactome Pathways

pathway	description	count in network	strength	false discovery rate
HSA-192823	Viral mRNA Translation	6 of 87	1.77	2.00e-06
HSA-156902	Peptide chain elongation	6 of 87	1.77	2.00e-06
HSA-72764	Eukaryotic Translation Termination	6 of 91	1.75	2.00e-06
HSA-2408557	Selenocysteine synthesis	6 of 91	1.75	2.00e-06
HSA-975956	Nonsense Mediated Decay (NMD) independent of the Exon ...	6 of 93	1.74	2.00e-06

(more ...)

Figure 0.10: Proteomic analysis - Preservation reperfusion injury: The Gene Ontology (GO) enrichment analysis performed in String® displaying the count in network, strength (enrichment effect size) and FDR shown as the p-value corrected for multiple testing within each category using the Benjamini–Hochberg model for the DEPs at reperfusion (LT3) in livers that went on to develop clinical PRI . . PRI = Preservation Reperfusion Injury (any one of RRT/AKI, EAD (Olthoff criteria) or PRS, PRS = Post Reperfusion Syndrome, RRT = Renal replacement therapy or AKI = new Acute Kidney Injury. Timepoint: LT3 following reperfusion in the recipient.

1.27.4 Transcriptomic analysis of ex situ vs in situ reperfusion

We conducted gene expression profiling on (n = 12) samples from 6 livers preserved exclusively with pSCS-NMP, but with different perfusion and clinical outcomes (Table 0.3, Table 0.4, Table 0.5 and Table 0.6).

The quality and quantity of extracted mRNA was adequate in all cases nd analysis was performed using the bespoke nSolver™ Analysis software (v4.0) from Nanostring®. We initially compared the LT2 timepoint to LT1, thereby assessing the effect of ex situ reperfusion and NMP on the liver's gene expression profile. We also compared the LT3 to the LT1 timepoint to assess the overall impact of the transplantation process including the in situ reperfusion effects on livers. We found that through preservation (pSCS-NMP) and subsequent transplant reperfusion, there was a progressive increase in the number of differentially expressed genes (DEGs), with the LT3 vs LT1 profile (i.e. the profile representing reperfusion in the recipient after prior machine preservation) (Figure 0.12) having more significantly DEGs than in the end of preservation comparison of LT2 vs LT1 (Figure 0.11 & Figure 0.11).

Rather than simply looking at individual gene changes, we investigated groups of genes or 'gene sets' associated with important immunological processes. This was performed in two ways. Firstly, we assessed 'global significance', evaluating the magnitude of differential expression regardless of the direction (e.g. up or down) of the differential expression. We found an increased number of gene sets with a greater magnitude of differential expression in the LT3 vs LT1 comparison than we found in the LT2vLT1 and the genes sets that were most differentially expressed at LT2 were a subset of the genes that were most differentially expressed at LT3 (Figure 0.13, red box) suggesting that that changes occurring at the end of NMP (LT2) are sustained through to transplant reperfusion (LT3), where even more changes seem to occur.

Secondly, we analysed the gene sets from the perspective of directed global significance scores, where the emphasis was on the direction of expression (i.e. increased or decreased) of immunologically important gene sets at various timepoints. Overall, during the NMP phase of pSCS-NMP many of the gene sets were downregulated, compared to the post pSCS-NMP in-situ reperfusion phase, where a greater proportion of gene sets, were upregulated, albeit there were many gene sets which were similarly up or down regulated in both phases (**Figure 0.14**).

We found that a subgroup of gene sets that were upregulated in both LT2 and LT3, when compared to a baseline of LT1 (**Figure 0.15**(A)). The strongest effects related to 'Toll like Receptor (TLR) Signalling' and 'Cytosolic DNA sensing' genes but also proinflammatory 'TNF signalling', 'Apoptosis' and 'Cell cycle regulation. Similarly, in **Figure 0.15** (B), we see universally down regulated gene sets related to MHC class II Antigen presentation, B cell receptor signalling and the complement system. However, **Figure 0.15**(C) demonstrates the gene sets that moved in opposing directions under the two conditions (timepoints). NMP is associated with a down regulation of genes sets such as those related to 'Adaptive immunity', 'Cytokine Signalling' and 'Autophagy', which are then upregulated following in situ reperfusion. Likewise, NMP is associated with an upregulation of genes related to 'Oxidative stress', 'Chemokine signalling' and 'NF kappaB signalling', which are all subsequently downregulated following reperfusion in the recipient **Figure 0.15**(C).

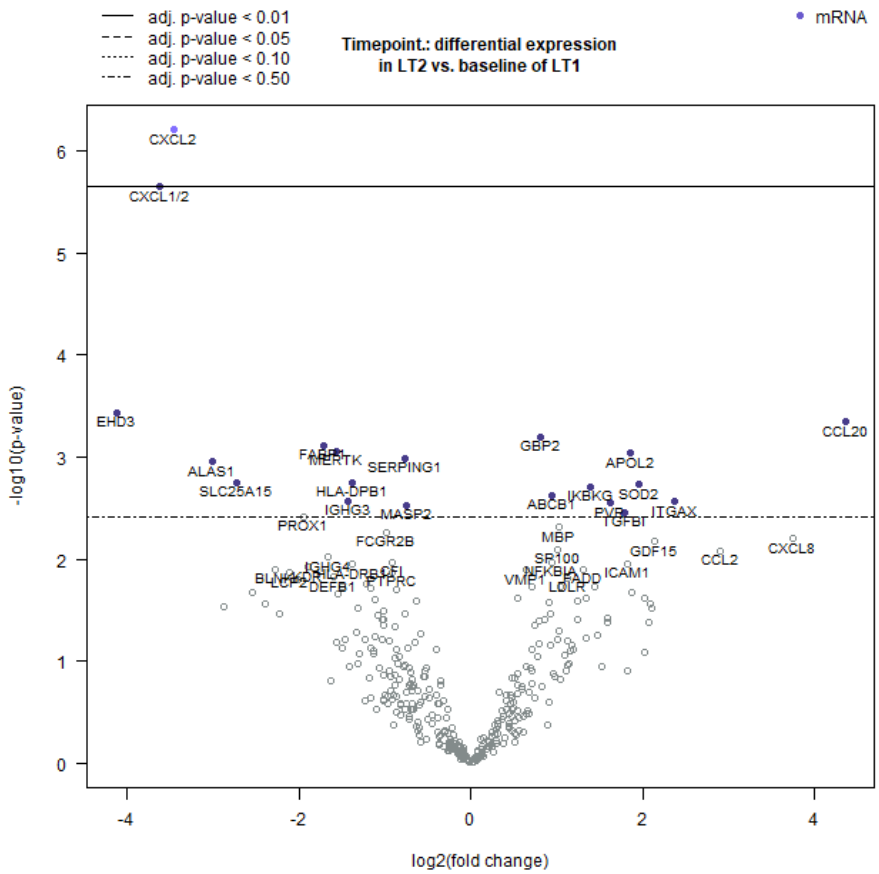


Figure 0.11 Transcriptomic Analysis - Volcano plot LT2 v LT1. Volcano plot displaying -log10(p-value) and log2 fold change for each identified gene in the two different timepoint comparisons (LT2 vs LT1). Highly statistically significant genes fall at the top of the plot above the horizontal lines. Negative log2 fold change values indicate downregulation and positive values indicate upregulation. Horizontal lines indicate various p-value thresholds. The 40 most statistically significantly differentially expressed genes are labelled. Only 2 genes were significantly differentially expressed following NMP when the p-value of 0.01 was adopted. Timepoints: LT1 – procurement or prior to normothermic machine perfusion, LT2 at the end of preservation and LT3 following reperfusion in the recipient

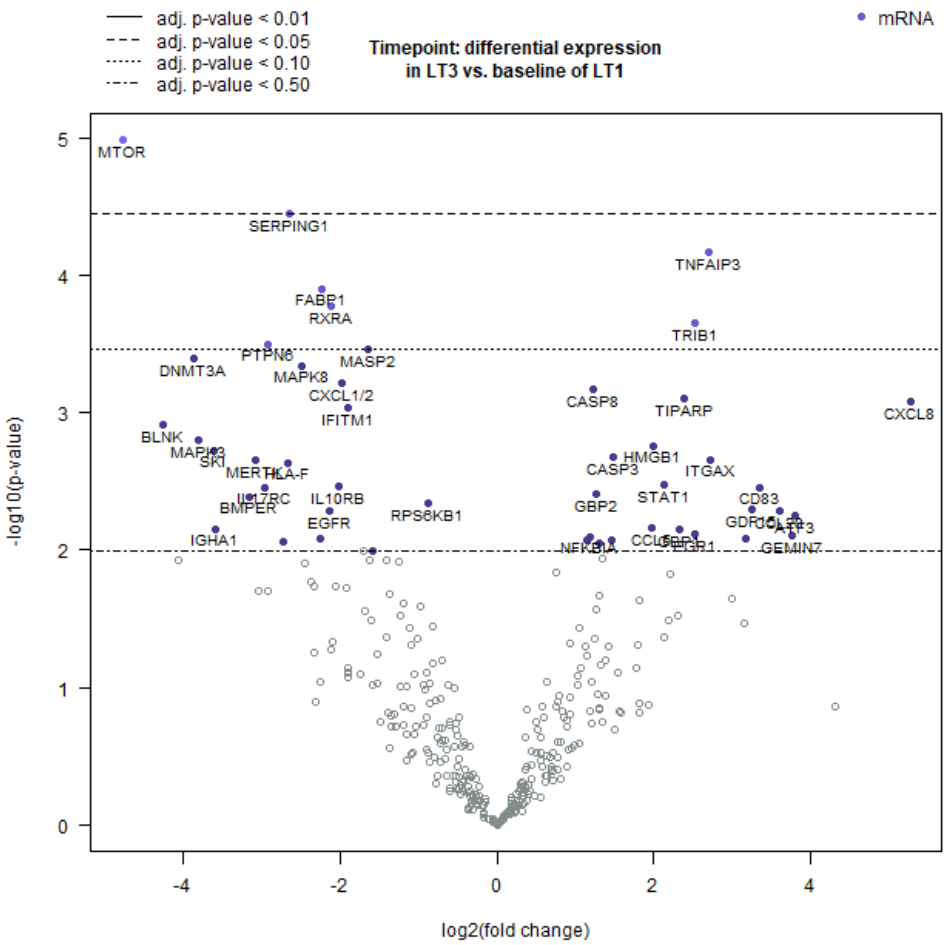


Figure 0.12. Transcriptomic Analysis - Volcano plot LT3 v LT1. Volcano plot displaying -log10(p-value) and log2 fold change for each identified gene in the two different timepoint comparisons (LT3 vs LT1). Highly statistically significant genes fall at the top of the plot above the horizontal lines. Negative log2 fold change values indicate downregulation and positive values indicate upregulation. Horizontal lines indicate various p-value thresholds. The 40 most statistically significantly differentially expressed genes are labelled. we find a greater number of significantly DEGs at higher significance thresholds than in the LT2 vs LT1 analysis. Timepoints: LT1 – procurement or prior to normothermic machine perfusion, LT2 at the end of preservation and LT3 following reperfusion in the recipient

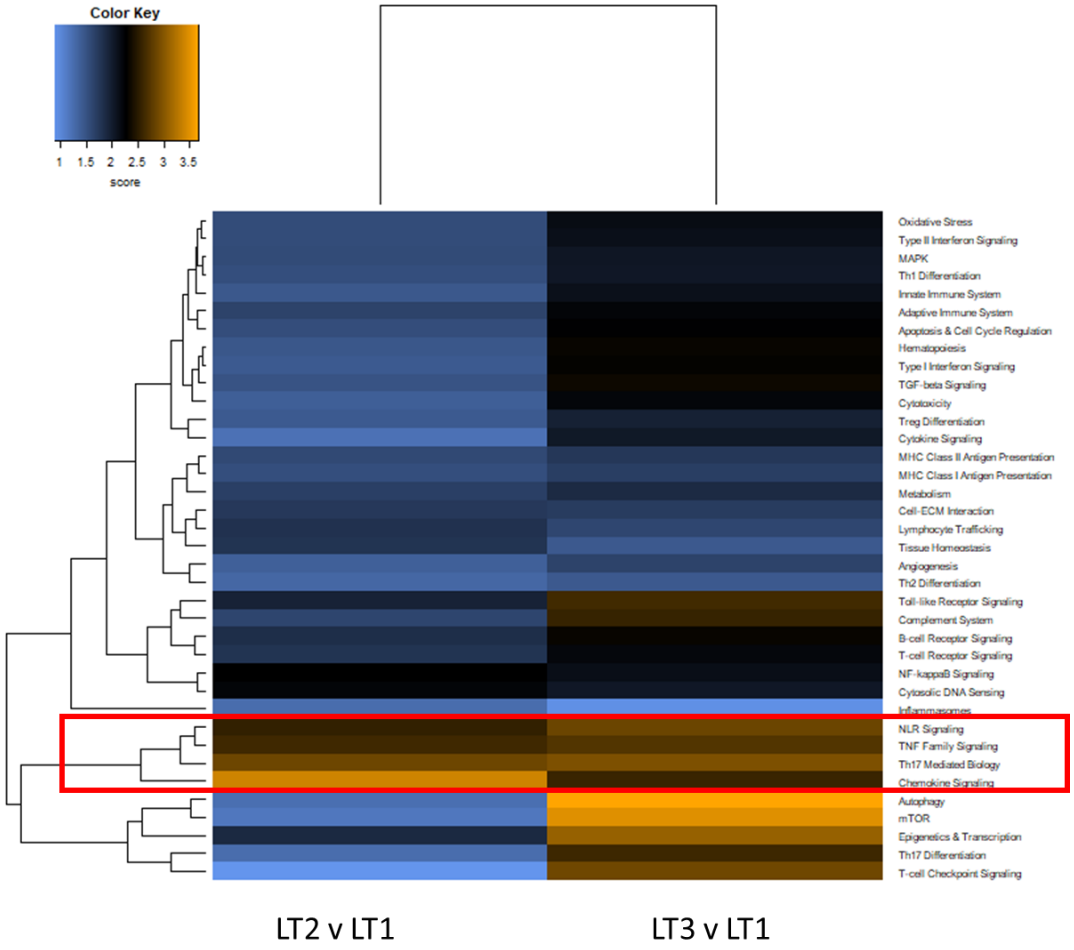


Figure 0.13. Transcriptomic analysis - Global significance Heatmap. Heatmap displaying the differential expression of a gene set's genes with timepoint, ignoring whether each gene within the set is up- or down-regulated. Orange denotes gene sets whose genes exhibit extensive differential expression with timepoint, blue denotes gene sets with less differential expression. We found that a greater number of gene sets were more extensively differentially expressed in LT3vLT1 compared to LT2vLT1. The gene sets differentially expressed in the LT2vLT3 were a subset of those differentially expressed in the LT3vLT1, suggesting that some of the changes occurring during ex situ reperfusion are sustained through to transplantation (red box). Timepoints: LT1 – procurement or prior to normothermic machine perfusion, LT2 at the end of preservation and LT3 following reperfusion in the recipient

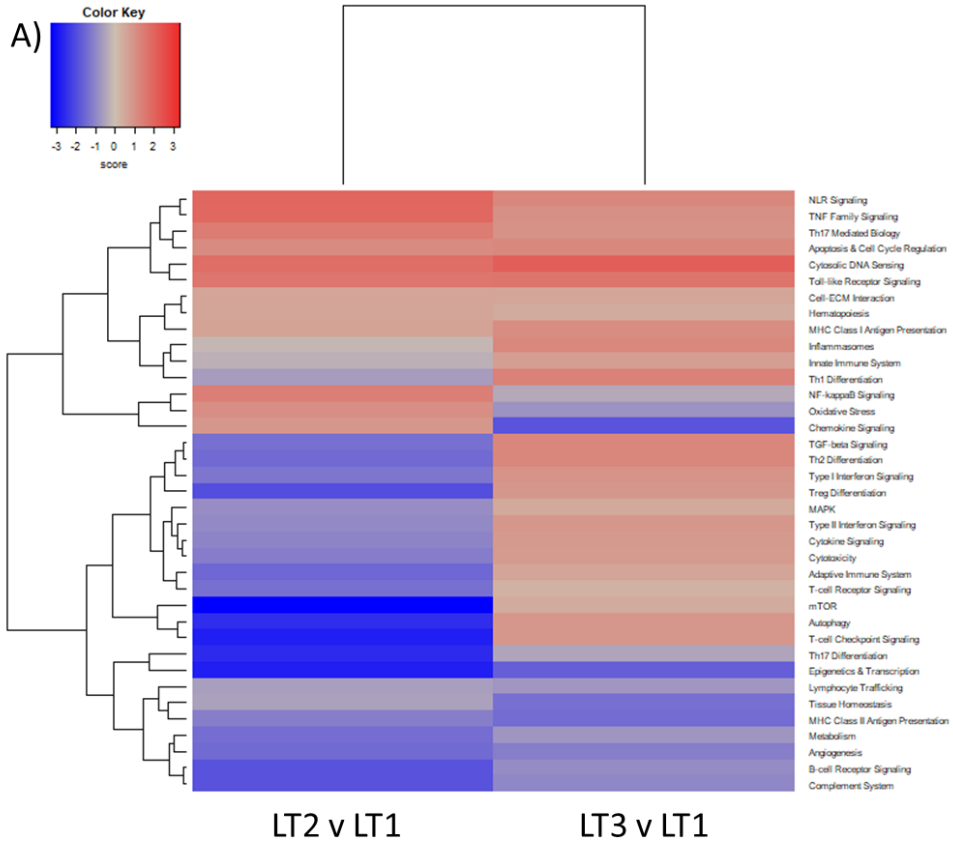


Figure 0.14 Transcriptomic analysis – Directed Global Significance Heatmap (a). Heatmap displaying the extent to which a gene set's genes are up- or down-regulated at LT2 and LT3 from a baseline of LT1. Red denotes gene sets whose genes exhibit extensive over-expression with the change from LT1 to LT2/LT3, blue denotes gene sets with extensive under-expression. We found that although many of the gene sets were up/down regulated under both LT3 and LT3, that a large proportion of gene sets were expressed in different directions. Timepoints: LT1 = prior to NMP (end of SCS), LT2 = end of preservation (NMP) and LT3 following reperfusion in the recipient. NMP = normothermic machine perfusion, SCS = static cold storage.

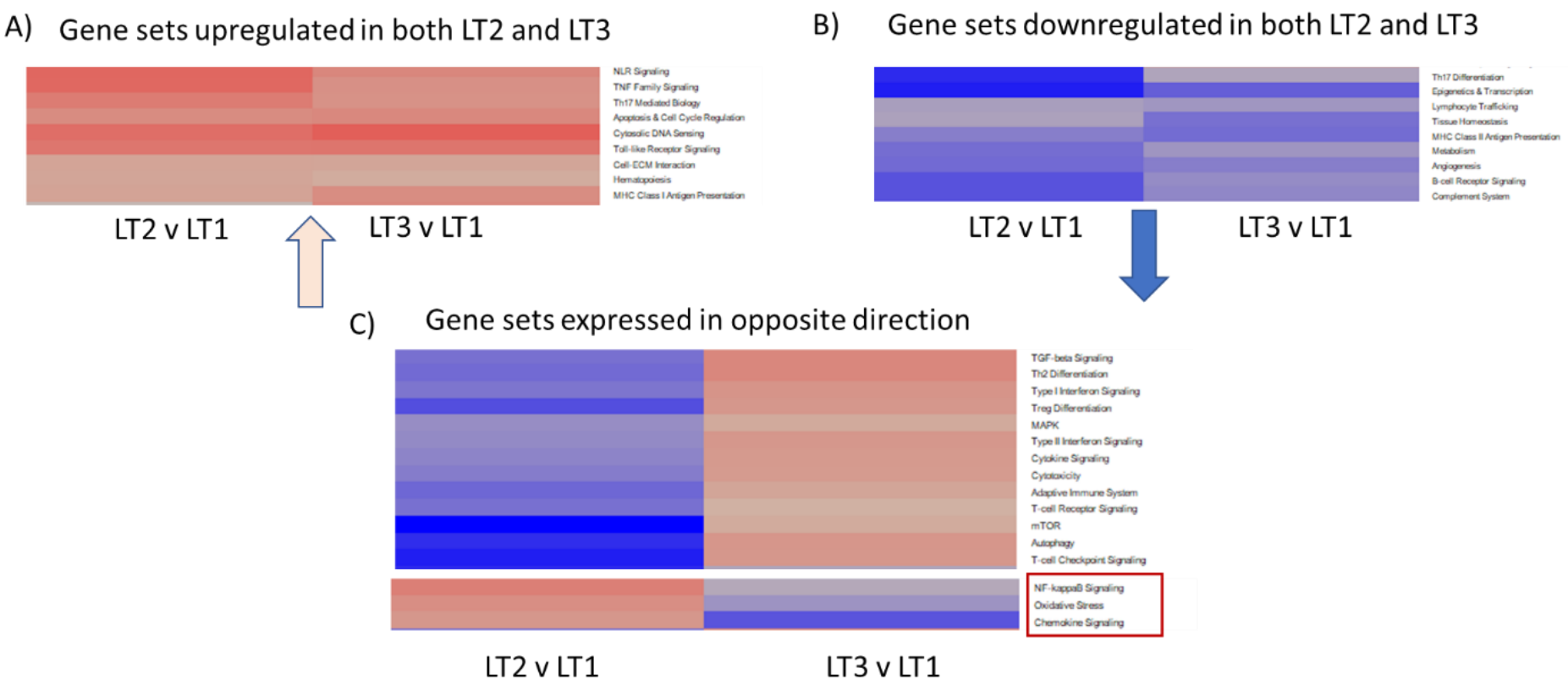


Figure 0.15: Transcriptomic analysis – Directed Global Significance Heatmap (b) Adapted heatmaps of directed global significance scores showing the extent to which a gene set's genes are up- or down-regulated at the timepoints LT2 and LT3 from a baseline of LT1. Red denotes gene sets whose genes exhibit extensive over-expression with the change from LT1 to LT2/LT3, blue denotes gene sets with extensive under-expression. A) Demonstrates upregulated gene sets in both LT2 and LT3 (vs LT1), (B) shows those that were down regulated and (C) shows those gene sets that moved in opposite directions under the different conditions. Highlighted in the red box were gene sets that having been upregulated during NMP were down regulated following reperfusion. Timepoints: LT1 = prior to NMP (end of SCS), LT2 = end of preservation (NMP) and LT3 following reperfusion in the recipient. NMP = normothermic machine perfusion, SCS = static cold storage.

1.28 Discussion

We found that proteomic and transcriptomic profiles of donor livers preserved with pSCS-NMP, changed substantially and sequentially through preservation and reperfusion, as the conditions changed. We demonstrated that although the LT2 (end of NMP preservation) protein expression profile had similarities to LT3 (following in situ reperfusion), these profiles were also distinct and had important differences. We found that in keeping with the clinical data, pSCS-NMP (back to base) preservation was very similar to continuous NMP (device to donor NMP) even on a molecular level. The exception to this was in cases of particularly prolonged SCS prior to NMP (greater than six hours), where substantial molecular differences related to mitochondrial function and protein degradation were evident when compared to shorter SCS times. When we attempted to dichotomise the livers into those that went on to develop clinically important early complications attributable to reperfusion injury (i.e. clinical PRI) and those that did not, we found no convincing evidence of a significant or detectable difference on a molecular (proteomic) level during preservation (**Figure 0.9**). However, we were able to demonstrate that a greater number of significantly DEPs were present between the two groups (PRI vs No PRI) following transplant reperfusion, suggesting that at this early-stage post-transplant, changes associated with PRI may be detectable at a molecular level. Taken together, this represents the first detailed molecular analysis of pSCS-NMP liver preservation, can provide important mechanistic insights into the impact of ex situ reperfusion on the molecular profile of grafts, the subsequent effect on reperfusion in the recipient and the impact of length of SCS prior to NMP.

Machine perfusion technologies remain novel approaches to organ preservation and have only recently become regularly utilised clinical modalities. As such, the availability of samples and tissue has been very limited, resulting in a paucity of 'omics' studies related to MP. In a recent (2021) systematic review of proteomics research in liver transplantation by Lopez et al (217), none of the studies included investigated machine-perfused organs and only 12 studies reported on human

samples, of which just two were analysing liver tissue (222,223). However, following this review, two notable studies have reported on the proteomic analysis of samples collected in the context of liver NMP. The first of these, Laing et al 2020 (224), was a discarded human liver NMP study assessing the capacity to deliver multipotent adult progenitor cells (MAPC®) during preservation. Six livers deemed unsuitable for transplantation were initiated on NMP and labelled MAPC® were delivered via the portal vein or hepatic artery and subsequently localised using immunofluorescence as measure of "engraftment". The perfusate of these livers underwent proteomic analysis, primarily to determine if MAPC® related proteins were present following delivery of therapy, therefore supporting the idea that these "engrafted cells" were indeed modulating the graft. The authors found a number of proteins uniquely expressed in the treatment group (for example IL6, ICAM1, TIMP-1, STIP1) and attributed them to their cells. Aside from the small number of livers included and a large number of proteins identified, the perfusate compartment was the focus, which may or may not provide any insight into what is going on within the graft. Furthermore, the heterogeneity of livers was marked and reflected by the fact that out of 1300 unique proteins identified, only 48 were present in all samples. Consequently, the study, although employing proteomic techniques, did not aim to nor actually provide any insight into the graft proteomic environment during NMP.

In the second notable study, Ohman and colleagues (225), the authors performed perhaps the most insightful multi-omics study on NMP livers to date, where discarded human livers (n = 12) were perfused and the tissue sampled. Predetermined functional criteria were used to determine "good function" and "poor function" and a comparison of molecular signatures was made between these two groups. Here, the authors were able to demonstrate that innate adaptive immune activation and subsequently, repair mechanisms and autophagy were prominent processes in livers that functioned well. In those with "poor function", there was a marked delay in the appearance of cellular repair related proteins and autophagy related proteins. This strongly suggests

that certain molecular processes are associated with differential performance during NMP using the currently available functional criteria.

Transcriptomic analysis of NMP livers, on the other hand, has been performed in the context of continuous NMP (163). Jassem et al (2018) conducted a comparative study of continuous NMP livers that were transplanted (n=12) and a matched cohort of SCS livers (n = 27) from the same time period, looking at the impact of NMP on IRI. This study involved immune profiling with immunohistochemistry and flow cytometry but also a microarray RNA analysis of transcripts related to IRI. Crucially, they used liver tissue for their analysis therefore truly representing the donor organ with their samples. They were able to demonstrate increased anti-inflammatory gene expression and reduced oxidative stress in comparison to SCS and also observed an enrichment of regulatory T cells within the grafts following NMP. This important initial mechanistic work was followed up by the COPE liver clinical study that demonstrated that these insights, in the context of continuous NMP could underpin the observed reduction in graft injury and IRI related complications (140). However, with the rapid adoption of back to base NMP (pSCS-NMP) as the default approach to clinical NMP more molecular understanding is required to appreciate how comparable these approaches are in terms of mechanism and also what new considerations are required, such as the appropriate length of SCS prior to NMP, none of which, to our knowledge, has been explored in the literature.

Thus, our study represents a large cohort of unique samples, previously unreported in the literature and representing a clinically pertinent group. Characterising the protein expression profile of livers preserved with pSCS-NMP that went on to be transplanted, provides valuable insight into how NMP impacts grafts in the clinical context and serves as an important reference for future work in different cohorts of NMP livers.

The findings of our PCA of pSCS-NMP samples revealed a clear grouping of samples according to the timepoint in preservation (**Figure 0.1)**. This was expected and reassuring yet a critically important finding for validating our methodology as it demonstrated that at the level of the proteome, we could expect to find detectable changes under different preservation conditions within the timeframe of clinical preservation. Therefore, protein expression, as well as transcriptomic gene expression data were valid approaches to molecular profiling of donor livers, even within the relatively short timeframes of clinical NMP. We also expected to find that sample profiles after NMP (LT2) and transplant/in situ reperfusion (LT3) would be very different to samples taken at the end of SCS (LT1), but more similar to each other as they are both normothermic and therefore more physiologically and metabolically similar. This was borne out in our proteomic data, as we found that LT2 and LT3 were relatively similar (**Figure 0.2**). The implications of this are significant, as this underpins the conceptual framework that NMP provides a "near physiological" environment for organs during preservation and may be able to support livers for longer periods of time.

Our transcriptomic data suggested a stepwise, progressive alteration in the liver profile through preservation, with the most marked difference being between LT1 and LT3 (**Figure 0.13**). The most prominent findings within our downstream enrichment and GO analysis were a consistent picture of upregulation of mitochondrial proteins involved in 'Oxidative phosphorylation' and 'Fatty acid oxidation', both key co-determinants of cellular energetics. We also found a consistent picture of down-regulation of proteins involved in protein degradation. This is in keeping with the limited literature on transcriptomic changes associated with NMP (220,221,225), Sanders and colleagues, performed an analysis of lean and steatotic discarded livers during NMP, finding no obvious differences between groups, but a consistent activation of innate immunity, with IL-10, IL-6 and Toll like receptors differentially expressed (221).

The impact of SCS on the molecular profile of livers subsequently preserved with NMP and then transplanted has not been investigated in the literature. Clinical studies have demonstrated comparable outcomes between pSCS-NMP and continuous NMP when using standard criteria donor (SCD) organs (170,171). However, we hypothesised that, although no clear clinical difference was detected in the aforementioned studies, we may be able to detect subclinical differences in the livers observed only on a molecular level, which may be valuable and possibly clinically significant in the context of higher risk extended criteria donor livers. In this analysis, we found that pSCS-NMP was in fact very similar to continuous NMP, even on a molecular level, corroborating the clinical data. However, we were able to show that prolonged periods of SCS prior to NMP (>6 hours), were associated with downregulation of proteins related to mitochondrial electron transport and upregulation of proteins related to degradation These findings were present in both the LT2 and LT3 timepoints when comparing the different lengths of SCS prior to NMP (**Figure 0.8** & **Figure 0.7**). This suggests that long periods of CIT prior to NMP can impair mitochondrial function on the device and following reperfusion. These findings are important when we consider that in studies of higher risk liver enrolling ECD grafts(172–174,212), which are particularly sensitive to cold ischaemia, the rates of clinical preservation reperfusion injury (i.e. EAD, requirement of RRT, PRS) are significantly higher than those of continuous NMP and in keeping with the rates seen in matched cohorts preserved with SCS alone. This strongly suggests that ECD livers, which are already sensitive to static hypothermic ischaemic preservation, that undergo pSCS-NMP with prolonged SCS may have a greater degree of mitochondrial dysfunction which, at least in part, may explain the significant PRI rate these grafts experience despite NMP.

A key finding in this work has been the characterisation of ex situ reperfusion as a similar yet distinct entity from transplant/in situ reperfusion, which can also be influenced by factors such as the length of SCS prior to NMP. Having demonstrated important differences in the LT2 and LT3

samples it was clear that the phenomenon of ex situ perfusion/re-perfusion has a distinct molecular profile from in situ reperfusion. The conditions during NMP are of course different to those of in situ reperfusion. In the former, the liver is reperfused in the absence of platelets, complement and leukocytes on an isolated circuit, whereas the latter is a whole blood, allogenic reperfusion in a truly physiological environment. The protein expression profile of LT2 in pSCS-NMP compared to LT3 suggested a down regulation of some mitochondrial related proteins and upregulation of protein degradation related proteins, suggesting that some degree of injury (mitochondrial and cellular) does occur during NMP and is exacerbated by length of CIT. These subtle but demonstrable molecular differences are important insights into the mechanisms of NMP, albeit clinically insignificant in this cohort of standard criteria livers. These observations are crucial when we consider the data presented in earlier chapters describing the inflammatory circulating perfusate during NMP, providing some insight into the possible downstream effects on the graft ex-situ reperfusion inflammation/injury.

Our findings in the Chapter suggest that PRI could not be detected, at the level of the proteome, during preservation as we found very few significantly DEPs related to PRI at the LT2 timepoint. The inherent biological heterogeneity of the clinical syndromes that constitute PRI may have influenced our results, as the number of subjects with PRI was limited and 'event rate' was relatively low, making any correlation analysis challenging. PRI involves multiple clinical entities largely related to but not solely dependent on IRI. Proteomic changes take hours to be translated and, in some cases, the processes may evolve over days, hence, we may not see all the relevant changes within the timeframe of NMP. At LT3 however, we were able to see more DEPs and a separation in the groups (Figure 0.12). This may not be clinically helpful, but does suggest that even during the early phases of in situ reperfusion, PRI is beginning to be detectable and the molecular process that ultimately manifest in the clinical syndrome are evidently being initiated.

There were a number of limitations to this work. The pSCS-NMP livers included in this study were relatively few (in comparison to the SCS only and cNMP cohorts from the COPE study). This limited the ability to address important subgroups such as DCD and DBD livers separately, which may be quite biologically distinct entities. Furthermore, the length of normothermic preservation may potentially have affected the profile of grafts, with varying degrees of gene expression, transcription and protein translation occurring over varying preservation times, therefore impacting the sample profiles on this basis and potentially confounding our results. However, we found no significant impact of donor type or length of NMP on the proteomic profile of livers during our analysis, therefore we were confident that grouping deceased donor livers together and not dividing the donor livers into groups according to total/NMP preservation times was the appropriate approach. The validity of this work is supported by the fact that this represents a clinically relevant cohort of livers, using the technology in the way it is most commonly used and with demonstrably good outcomes, thus it provides important mechanistic insights to a clinically relevant application. The ability to compare the data with large cohorts of SCS and cNMP livers is powerful in isolating the effect of prior SCS before NMP, which was the principal aim of this chapter. However, having a larger cohort of discarded livers, including PNFs and livers with poor outcomes, would have strengthened this study and permitted us to start to define molecular characteristics associated with quality, something not in the scope of this work.

This unbiased observational 'multi-omics' work conducted on what is a large sample size, breaks from more traditional proteomics work that has been primarily focused on biomarker discovery, limited to very small cohorts. By avoiding a targeted proteomics approach, as seen in the majority of published studies, we were able to minimised bias. Moreover, previous studies have often focused on easily accessible tissue e.g. serum, urine, plasma or perfusate presuming that this would provide a surrogate for what is occurring in the graft or organ of interest, whereas we have directly accessed graft tissue.

In summary, we have characterised the molecular and immunological profile of livers preserved with pSCS-NMP, demonstrating that, although there are similarities to continuous NMP, there are evident proteomic differences which are more exaggerated with longer periods of SCS prior to NMP. Furthermore, we have more precisely characterised the molecular profile of ex situ (re)perfusion and demonstrated its differences from transplant/in situ reperfusion following transplantation. We did not seek to, nor did we identify any protein expression patterns within these cohorts of standard criteria livers that are associated with organ quality or resilience to PRI, but instead would postulate that our findings represent an initial library of profiles associated with good clinical outcomes, which may serve a comparative cohort for other experimental work in the future.

Porcine model development

"A model is a lie that helps you see the truth."

— Siddhartha Mukherjee, The Emperor of All Maladies

1.29 Background:

In the clinical liver NMP studies presented earlier, the characteristic features of ex situ reperfusion injury/inflammation (ERI) and its impact on the molecular and immunological profile of grafts during NMP and transplantation were described. Although valuable insights were gained, there were several drawbacks to the use of clinical samples, including being limited to standard criteria donor livers, study protocol constraints on sampling regimens and restricted access to bio-banked tissue. These factors led us to develop an appropriate animal model to obtain a mechanistic understanding of ERI, especially in the context of DCD and extended criteria livers that were not well represented in the human liver clinical studies (140,171). Due to the extensive experience with porcine models within the group, efforts were focused on this model.

Porcine models have been important pre-clinical models in translational transplant research, used extensively in the field and, in particular, they have been crucial in the development of ex situ perfusion technology (150,226,227). Pigs are well matched to humans in terms of their abdominal visceral and vascular anatomy and have with similarly sized organs (228), while also having a high degree of genomic similarities with humans permitting molecular biology and 'omics' research. The immune system and consequent inflammation-related protein expression patterns are therefore consistent between species and this further increases the value of this specific model (229) Moreover, with the clear benefits of the porcine model, there has been a dramatic increase in the development of many porcine species-specific laboratory resources with specific antibodies,

assays and kits that have facilitated in-depth molecular analysis and allowed the exploration of multiple new avenues of enquiry.

Despite the evident scientific advantages of large animal models, important ethical issues remain, and the acceptability of animal experimentation remains highly controversial and consequently heavily regulated. To meet these stringent ethical standards, researchers and institutions face prohibitively high costs, related to animal husbandry and the logistical considerations around meeting these standards in large animal experiments (230). I was no exception, requiring me to adopt an innovative approach to overcome these challenges.

Therefore, I set out to design a reliable and efficient procurement process for porcine livers from the slaughterhouse to facilitate pre-clinical liver perfusion research. Utilizing the slaughterhouse as a source of organs was not only cost effective, but ethically advantageous and in line with the principle of 'Replacement' within the 3Rs (Reduction, Replacement & Refinement), which form the ethical framework for scientific animal research (230). Additionally, this approach would provide organs retrieved using a technique that closely resembles the UK DCD clinical pathway. Moreover, this DCD construct was closely linked to the critical area of marginal or extended criteria donor research that is the focus for many NMP research groups.

Although slaughterhouse porcine organ procurement methods have been described for various organs used for ex-vivo perfusion experiments (liver (231), pancreas (232), kidney (233) and lungs (234)) the published experience is heterogenous and success rates highly variable.

This chapter reports on a procurement system that can standardise the approach to this procedure and provide a dramatic increase in the availability of DCD organs suitable for ex-vivo organ perfusion and transplantation experiments without the requirement for extensive large animal facilities. This can be achieved at an affordable cost to researchers, reducing the number of animals used for research.

1.29.1 Aims

The overall objective of this chapter was to develop a cost-effective, ethically acceptable, valid large animal model of ECD liver NMP with a surrogate transplant reperfusion readout to be able to assess preservation injury. Specifically, this involved: (a) developing a scientifically-acceptable and reproducible method of liver procurement from the slaughterhouse; (b) developing the core ex situ machine perfusion model and finally (c) developing and modifying a simulated (allogenic) transplant model using ex situ NMP (235,236).

1.30 Methods

1.30.1 Slaughterhouse Organ Procurement Protocol

A collaboration was developed with Long Compton Abattoir (Warwickshire, UK) which permitted access to their facility to obtain porcine organs for the purposes of research. This collaboration pre-dated the development of the specific retrieval technique outlined in this chapter, as previous researchers had established a procurement system (predominantly for porcine kidneys). Briefly, their approach involved procurement from the animal directly, while it was hanging head-down, immediately after stunning, exsanguination and the hot water bath. This approach, increased the risk of injury, particularly to the liver, from traction (associated with gravity) and also increased the chances of retrieval injury during the dissection phase given the non-intuitive anatomical arrangement: it also took a long time to perform, which was disruptive to the flow within the abattoir (when the liver was being procured), which undermined the premise of an efficient system with minimal impact on the slaughterhouse workflow. The new procurement method (described below) was designed to minimise injuries, reduce warm ischaemic time and increase efficiency within the slaughterhouse. Working closed with the slaughtermen and Dr Flavia Neri to change the organ procurement process, a technique was devised that was reproducible and reliable.

1.30.2 Set-up and preparation

At the facility we were provided with a space in an adjacent space/room next to the main slaughter room. We set up our equipment on a large metallic table upon which we used to complete the multi-organ procurement procedure reliably and efficiently (Figure 0.1). The table was draped and an intravenous fluid delivery stand and giving-set (Baxter Medical, Deerfield, USA) set up in preparation for ex situ cold flush-out of organs. A large bowl of ice and gauze was required for liver back-table perfusion. Two litres of NaCL 0.9% (normal saline) and two litres of organ

preservation solution (UW® Solution) was heparinized with 1000 IU/L of unfractionated heparin sodium (Wockhardt UK Ltd) and kept at 4^{0}C (39.2^{0}F).

1.30.3 Animals

Sus domesticus pigs (50-70kg) were electrically stunned and while unconscious, exsanguinated by stab incision to the heart and great vessels, in line with the standard abattoir process and in compliance the Welfare of Animals at the Time of Killing (England) Regulations 2015 (WATOK) and EU regulation 1099/2009. Whole blood was collected into a container with 20,000IU of unfractionated heparin sodium (Wockhardt UK Ltd), or alternatively directly into blood collection bags and anticoagulated with citrate-phosphate-dextrose solution, for storage and preparation at the lab. The blood collection process was standardised and performed in the same way regardless of whether the pig was a blood donor only or going on to have its liver procured. Once the incision is made for exsanguination, the blood is collected in a 'clean catch' fashion into a large funnel connected via an IV giving set spike to citrated blood collection bags or heparinesd (emptied) 1L NaCL bags. The animals were briefly cleaned in a hot water bath at 60˚C (140^{0}F) for a pre-set time of 3 mins. The time from exsanguination to commencement of retrieval operation of the animal took between 5-7 mins per animal and was comparable to the 'no-touch' period following cardiac arrest before commencing the retrieval operation in clinical human DCD retrieval procedures in the UK. (237)

1.30.4 En bloc thoracoabdominal organ excision

Following the hot bath, the animals were then suspended from their posterior limbs, according to the local slaughterhouse procedure. A longitudinal midline incision was made to expose the abdominal and thoracic cavities and a circumferential incision made around the anus and rectum allowing it to be pulled down in continuity with the gastro-intestinal tract. An en bloc removal of the abdominal and thoracic viscera was conducted to minimize damage to the organs and facilitate

an ex-situ separation of the required organs. The visceral block was explanted by detaching it, starting caudally, from the longitudinal vertebral ligament the retroperitoneal space cutting these attachments flush against the vertebral column. The visceral bloc included the small and large bowel, duodenum, pancreas, stomach, oesophagus, kidneys, spleen, heart, lungs, aorta and trachea are removed alongside the liver and diaphragm en bloc (Figure 0.2).

1.30.5 Liver procurement

The multi-organ block was transferred to the back-table where the liver was quickly inspected for damage or disqualifying tears and the liver parenchyma covered with slushed ice as the process of organ cannulation and perfusion commences. The distal (infra-renal) aorta could be quickly and easily identified and a 21Fr Argyl straight aortic cannula ™ (Cardinal Health, Dublin Ohio, USA) inserted for anatomical guidance (Figure 0.3). The organs are swiftly arranged in standard anatomical position but with the liver reflected caudally exposing the hepatoduodenal ligament, under-surface of the liver and gall bladder, where more slushed ice was placed.

Infra-hepatic vena cava: The right kidney is identified and retracted laterally exposing the infra-hepatic inferior vena cava (IVC) as it enters the liver, it should be noted that in pigs, the hepatic segment of the inferior vena cava is intra-hepatic. From here the IVC is dissected inferiorly to the origin of the left renal vein, and is then divided, ensuring an adequate cuff of IVC beyond the most inferior part of the liver. The liver tissue extends on the posterior aspect of the vessel and is susceptible to damage if this is divided before the renal vein origin (**Figure 0.4**).

Dual perfusion option: Although not routinely performed in this way, we were able to successfully dual perfuse via both the hepatic artery and portal vein. However, it's often difficult to determine what amount of fluid manages to reach the liver without being lost via pancreatic and splenic arteries. To perform dual perfusion, a modified giving set is prepared to allow simultaneous direct cannulation of both renal arteries by connecting a rapid giving set to a Y-

connector using sterile silicone tubing. The limbs of the Y-connector are extended using 20-30cm lengths of silicone tubing, to which a small arterial cannula is secured to one limb and the standard portal cannula (mentioned below) secured to the other. Plastic tubing clamps are incorporated to allow independent control of the two limbs

Portal cannulation: The portal vein (PV) is approached well away from the hepatic hilum where it lies deep to the pancreatic parenchyma at the junction of the superior mesenteric vein (SMV) and splenic vein (SV). The portal vein can be easily visualized once the pancreatic parenchyma has been spread exposing it within the pancreas. Once identified it is immediately incised, cannulated and flushed with cold heparinized preservation fluid via a soft 18 Fr T-tube (Summit Medical, Cheltenham, UK) or a portal cannula attached to the giving set (**Figure 0.5**a-c).

Hepatic artery: The porcine arterial inflow is variable and often branches very early, thus, preserving an aortic patch of the coeliac is advisable at this stage of the procurement process. This is helped by using the previously placed aortic cannula (figure 3) as a guide. Superiorly, the thoracic aorta can be divided just above the diaphragm and inferiorly, the aorta is divided above the kidneys, leaving a cylinder of aorta around the cannula. The aortic tube can then be split on the posterior surface creating a superior mesenteric artery (SMA) and coeliac artery patch. Once the aortic patch has been fashioned, the artery is flushed with preservation fluid via a wire-reinforced cannula.

Bile duct and liver attachments: Once the arterial inflow, the PV and infra-hepatic IVC were divided, the remaining hepatoduodenal attachments were divided flush with the duodenum. The common bile duct (CBD) was identified and divided as it enters the duodenum and was left long, to facilitate cannulation and bile collection during any experimental perfusions. This dissection was extended along the lesser curve of the stomach to the oesophagus, dividing the diaphragmatic crus and the remaining diaphragmatic attachments were also carefully divided isolating the liver. The remaining vascular outflow, the supra-hepatic inferior vena cava was divided with a minimum

length of 3cm and circumferential cuff of the diaphragm. The separation of the liver was now complete, and it was transferred into the ice bowl for ongoing flushing.

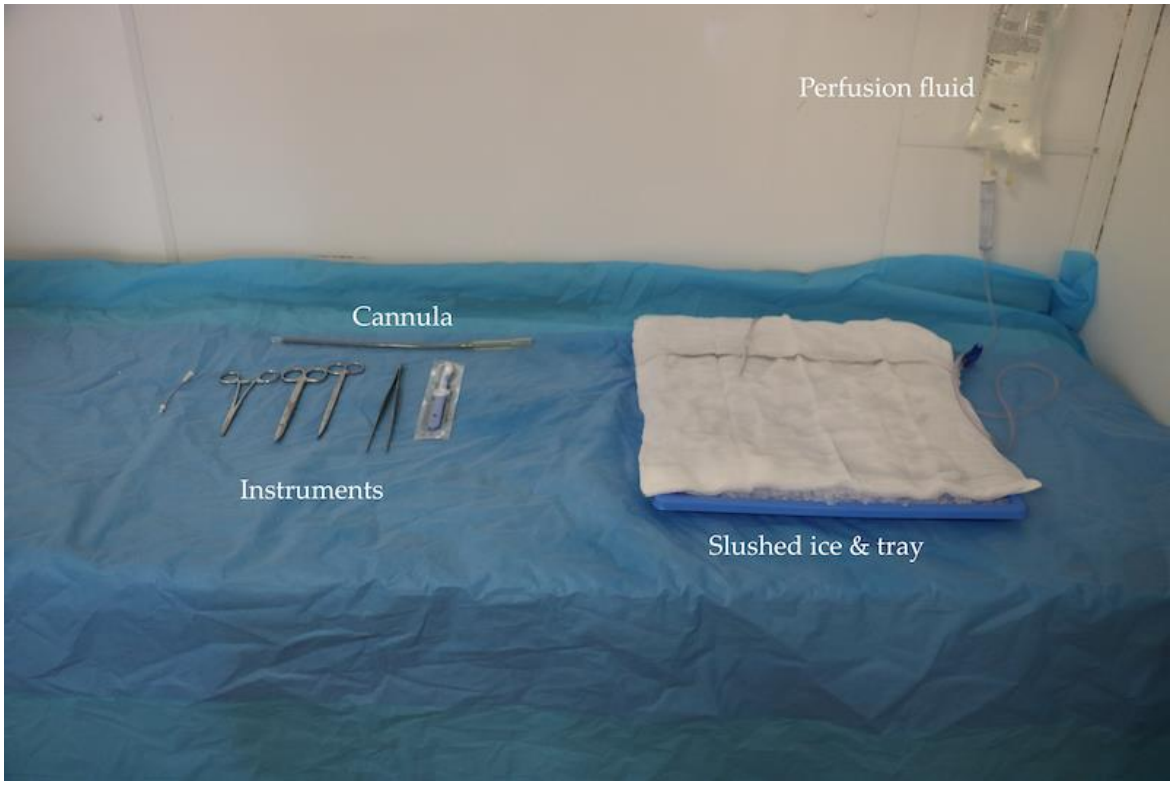


Figure 0.1: On-site back table preparation. Back table with prepared instruments, selection of cannulas, a tray containing slushed ice and overlying cold damp gauze and heparinized preservation fluid at elevation and ready for perfusion.

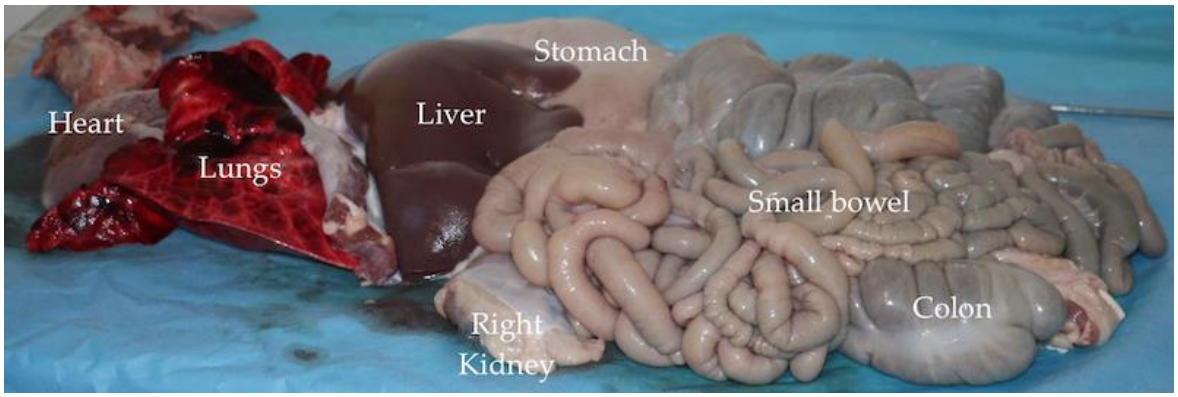


Figure 0.2: Thoraco-abdominal viscera. Labelled en bloc thoraco-abdominal organs on the back-table to facilitate ex-situ cold perfusion and separation.

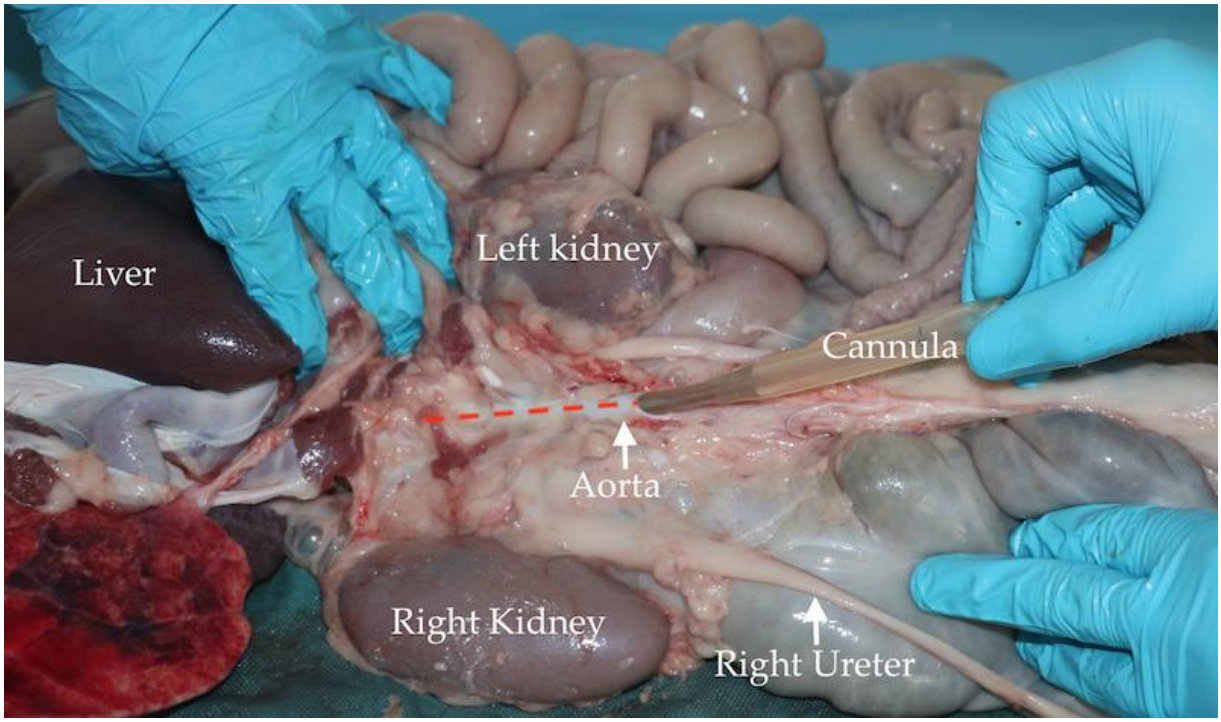


Figure 0.3 . **Aortic cannulation**. Insertion of a straight aortic cannula into the infra-renal aorta to aid anatomical orientation. Dashed line indicates the line of division of the aorta prior to individual cannulation of the renal ostia using the modified perfusion set up described in section "Slaughterhouse organ procurement protocol".

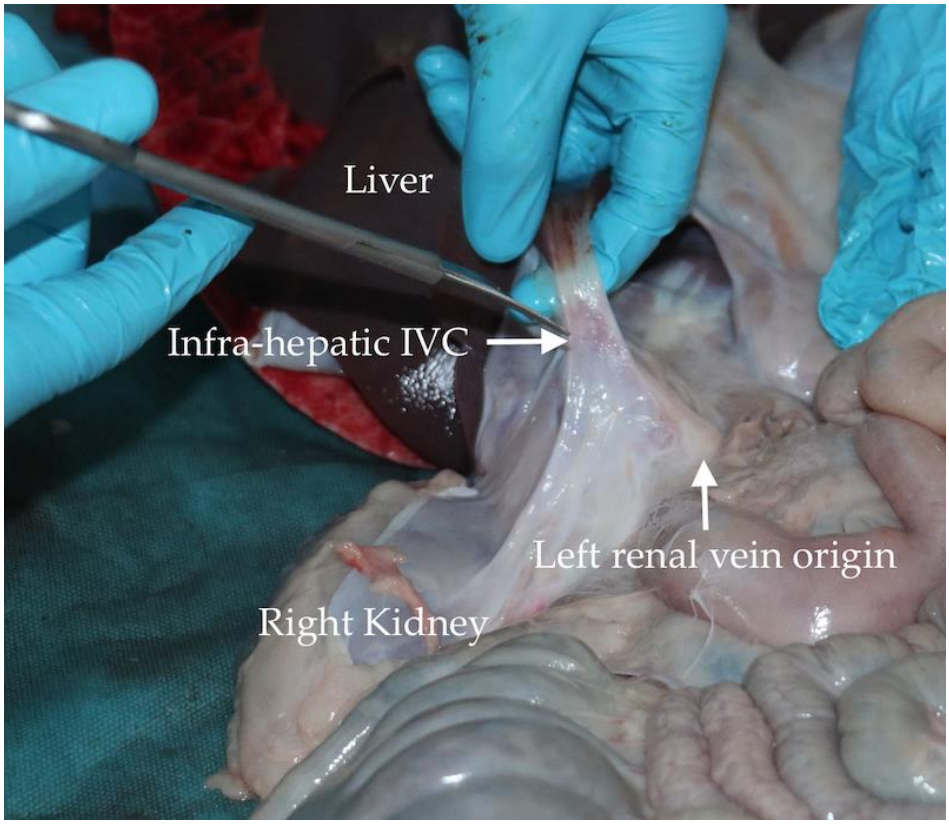


Figure 0.4 . Liver Isolation. Dividing the infra-hepatic inferior vena cava (IVC) at the level just above the origin of the left renal vein. Note that the IVC in pigs is intra-hepatic unlike in humans.

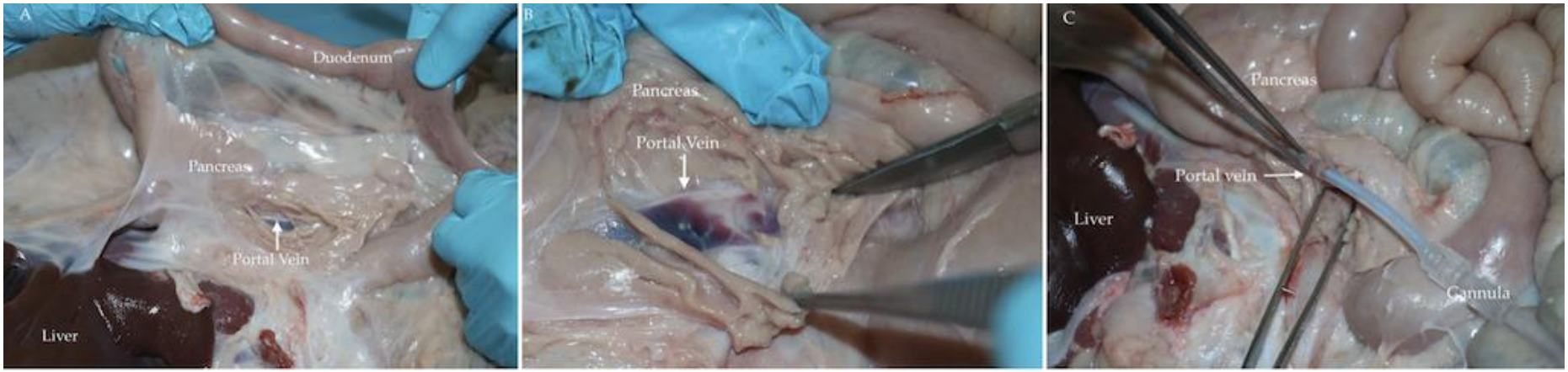


Figure 0.5: Portal Cannulation. Portal vein (PV) cannulation. (A) The PV is identified easily with the pancreas split open. (B) The confluence of the superior mesenteric vein and splenic vein to become the PV is seen. (C) The PV is cut open and cannulated quickly with a soft 18-Fr cannula or cut T-tube to allow cold perfusion to the liver.

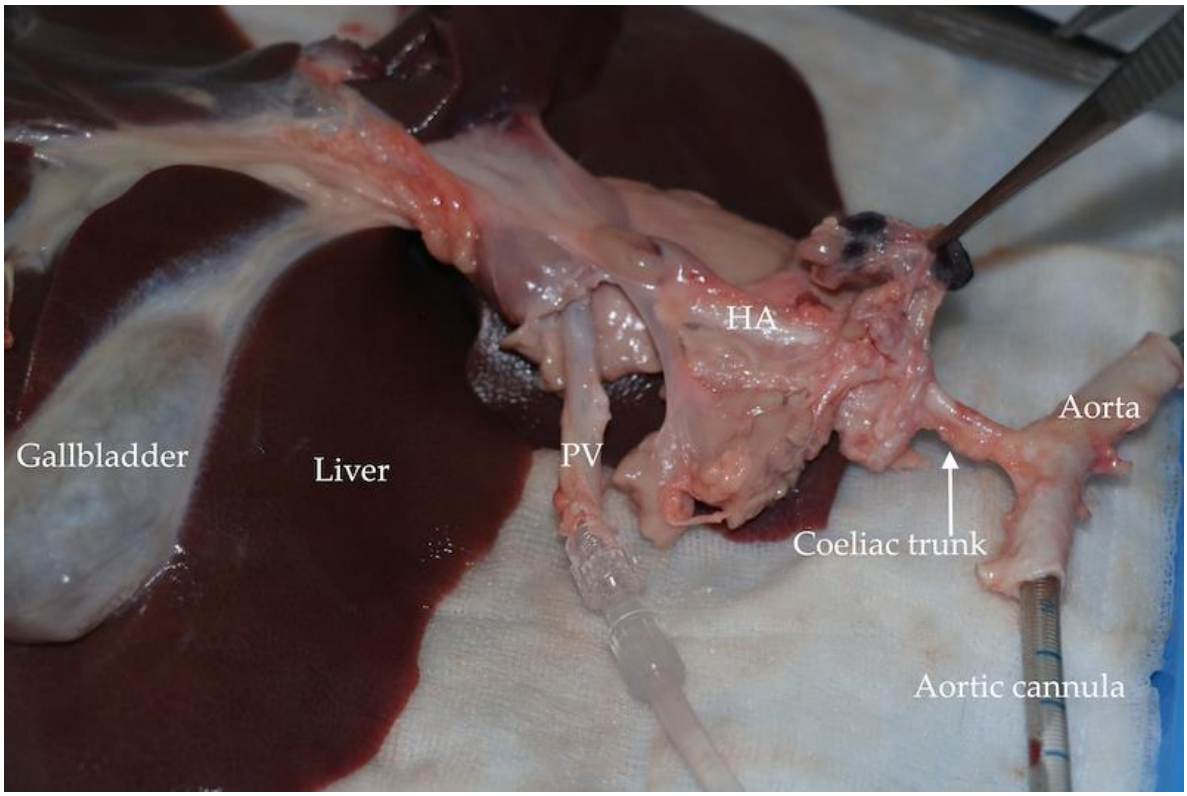


Figure 0.6: Arterial perfusion. Cannulated aorta segment containing the coeliac axis, which is the arterial inflow to the liver from aorta. This aortic cylindrical segment can be fashioned into a coeliac arterial patch by splitting the posterior wall longitudinally. Abbreviations; HA (hepatic artery), PV (portal vein).

1.30.6 Packing and transport of all procured and perfused organs: Static cold storage

Once the back-table perfusion and inspection of organs has been completed, the liver is placed in a sterile PVC intestinal bag (Bunzl, Leicester, UK) containing 1-1.5 litres of cooled preservation solution, UW® solution in our experiments, and placed in an icebox for transfer back to the laboratory.

1.30.7 Back at Base: Benchwork, cannulation & perfusate preparation

1.30.8 Back-table organ preparation

Meticulous back table preparation of the liver is an important step in achieving reliable perfusion results. Briefly, we start by dissecting the portal vein into the hilum, we proceed to dissect the hepatic artery, also going into the hilum, with care being taken to avoid taking out early branches to lobes. Lymphatic tissue is dissected away, this can become oedematous and then bleed later, so we performed an extensive dissection (depending on the design of the experiment, this can be adapted). The supra-hepatic inferior vena cava is oversewn with a 4.0 prolene (Ethicon) and the phrenic veins identified and ligated. The cannulation is performed in a standard fashion (see General Methods).

1.30.9 Blood work & machine priming

Heparinised whole blood is collected during the procurement as described above. This blood is prepared for perfusion based on the individual experiment. Whole blood reperfusions do not require additional preparation beyond being filtered for debris using a giving set with a microaggregate filter (normally 170-260 micron) to facilitate removal of any small clots and other debris that may be present as we deliver the blood into the device for priming. For leuko-depleted blood perfusions (red cells and plasma) which formed the majority of experiments during the NMP phase, we filtered the whole blood via a leukocyte filter (Haemotenics® RS1 Filter) before priming

the circuit. The final option is the use of packed red cells only, which involves leukocyte filtration and centrifugal separation and removal of plasma leaving the red cells that are used to prime together with a colloid solution. We routinely perform ABO blood typing using point of care tests (Eldon® biologicals cards). The OrganOx metra® device is set up and primmed using the selected perfusate for the specific experiment and phase.

1.30.10 Simulated transplant reperfusion:

Allogenic whole blood matched for blood type (ABO) can be collected from a different animal at the abattoir, typed and stored in preparation for an allogenic reperfusion on the device in a simulated transplant reperfusion. In these experiments, following a period of NMP, the OrganOx metra® device was emptied and reprimed with allogenic blood matched for ABO group. The liver was flushed with 2L of crystalloid and then reconnected to the reprimed device ahead of allogenic whole blood reperfusion.

1.31 Results

1.31.1 Procurement results:

With colleagues from the lab group, I performed more than 35 liver procurements using the described methodology to produce successful and reproducible normothermic machine perfusion experiments. In our experience the time from exsanguination to the initiation of cold perfusion (i.e. the warm ischemia time, WIT), which includes the period of time the animal is in the hot bath, takes between 12-20 minutes (**Figure 0.7**). We managed to achieve a time less than 30 minutes in 100% of cases, which is often regarded the acceptable upper limit of WIT in DCD liver transplantation in the published literature, albeit this is changing in the context of novel technologies such as NRP and NMP (18). Furthermore, previously published work in the context of porcine transplant models, has demonstrated that a WIT of less than 60 minutes has high survival rates, especially when grafts are preserved using NMP (238).

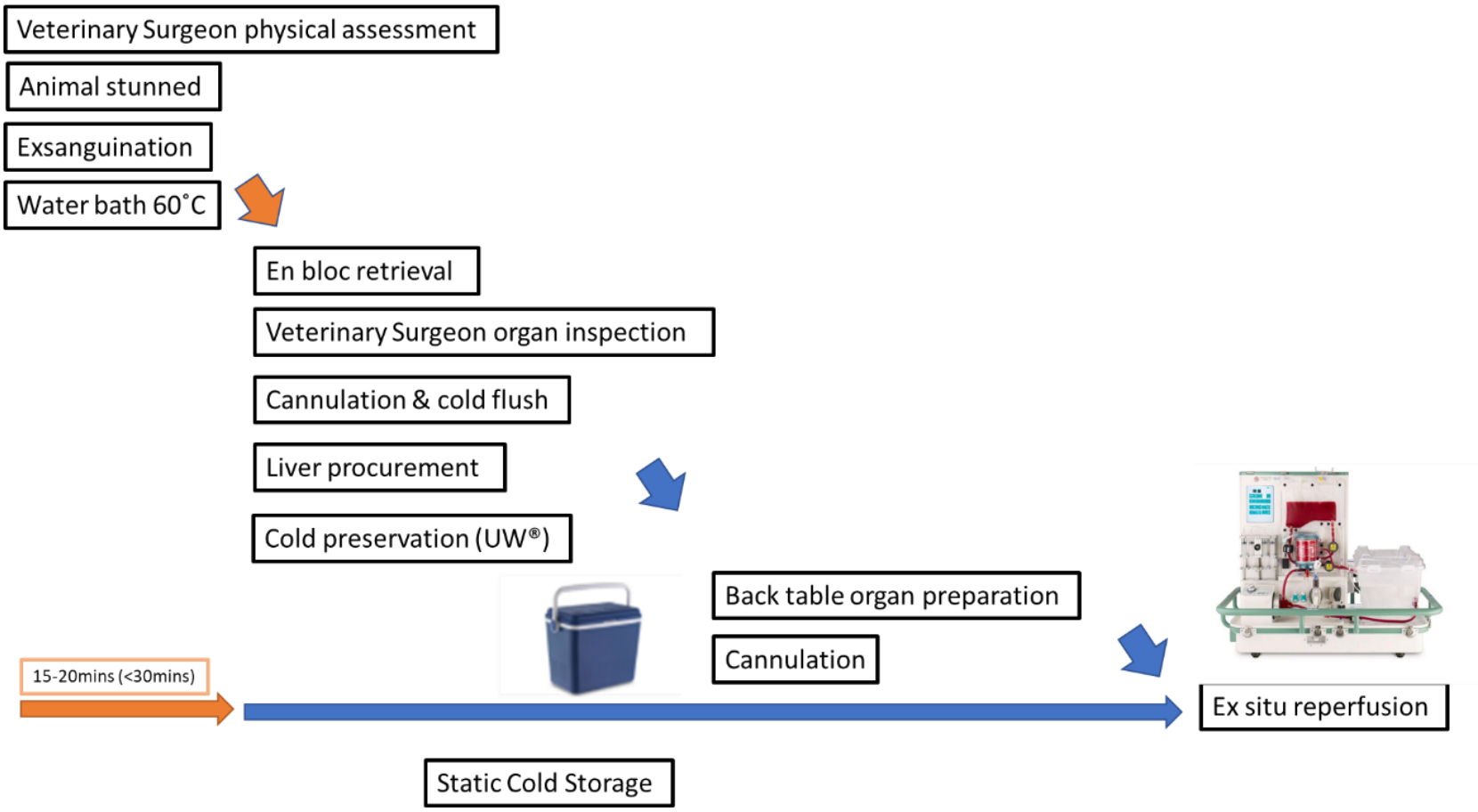


Figure 0.7: A diagrammatic overview of the multiorgan procurement. The process is initiated with Veterinary surgeon assessment followed by the sequence of stunning, exsanguination and hot bath hair removal. The suspended animals are eviscerated and the thoracic and abdominal organs transferred to the back table for liver isolation and procurement. After flushing, organs are cold stored and transported to the laboratory where further back table preparation and cannulation occurs.

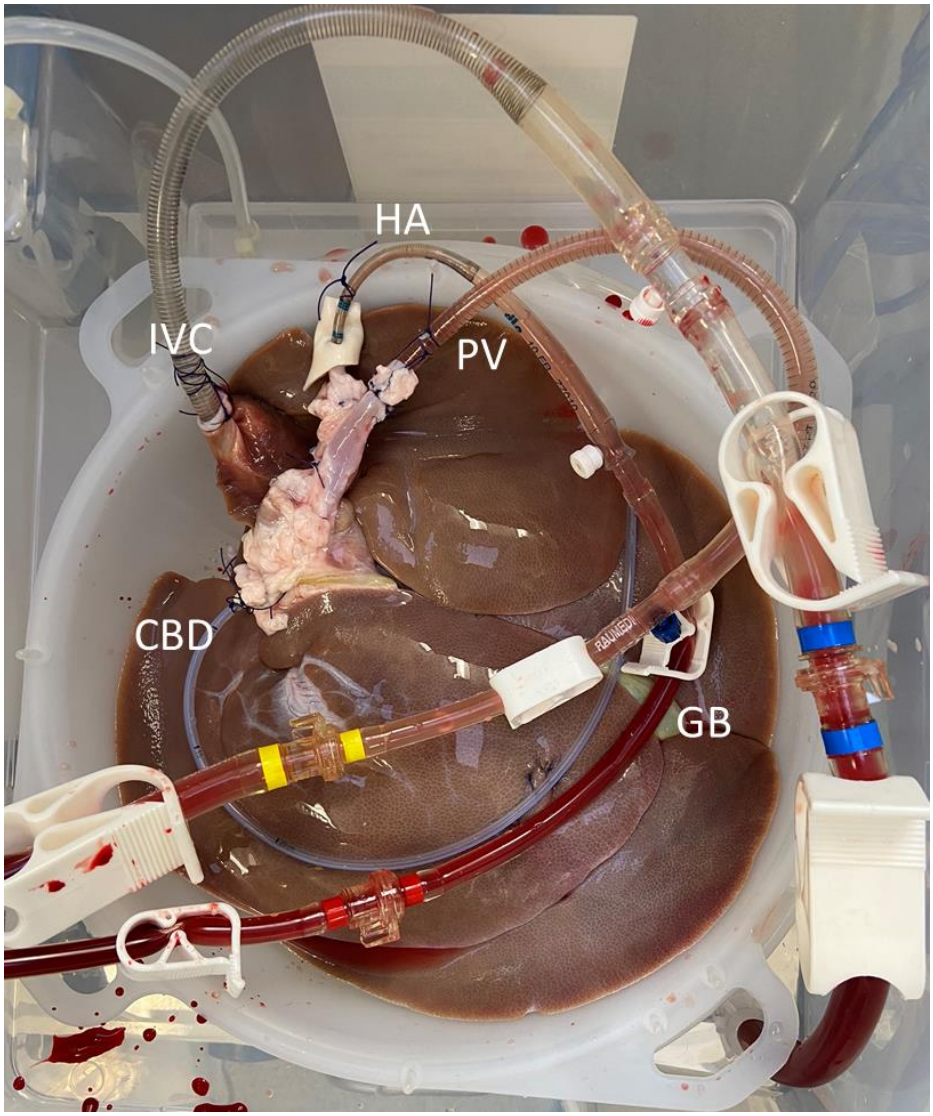


Figure 0.8 Image of cannulated porcine liver ready for the commencement of NMP.

The inferior vena cava (IVC), portal vein (PV), hepatic artery (HA) and common bile duct (CBD) are all cannulated. The gallbladder (GB) is also present and has been closed.

Table 0.1: Blood preparation (Table of different perfusate combinations and types of experiments)

Perfusate	**Whole blood**	**Leuko-depleted**	**RBC based**
RBCs	Yes	Yes	Yes
Leukocytes	Yes	No	No
Plasma	Yes	Yes	No (Gelofusin/Albumin)*
Autologous experimental applications	Drug delivery Pharmacokinetics Ischaemia reperfusion Liver regeneration	Normothermic machine perfusion (autologous)	
Allogenic experimental applications	Simulated Transplant Reperfusion Ischaemia reperfusion injury	N/A	N/A

1.31.2 Normothermic machine perfusion & simulated allogenic transplant reperfusion

The perfusion characteristics, including haemodynamic and blood gas analysis were collected in real time by the OrganOx *metra* device and stored for analysis. All experimental perfusions demonstrated technically stable haemodynamic parameters (see appendix I). Perfusate biochemistry was analysed using point of care tests a clinical biochemistry and this was found to be reliable and reproducible. Bile biochemistry measurement and measurement of volume was also feasible: indeed, every perfused liver porcine liver produced bile (Figure 0.9).

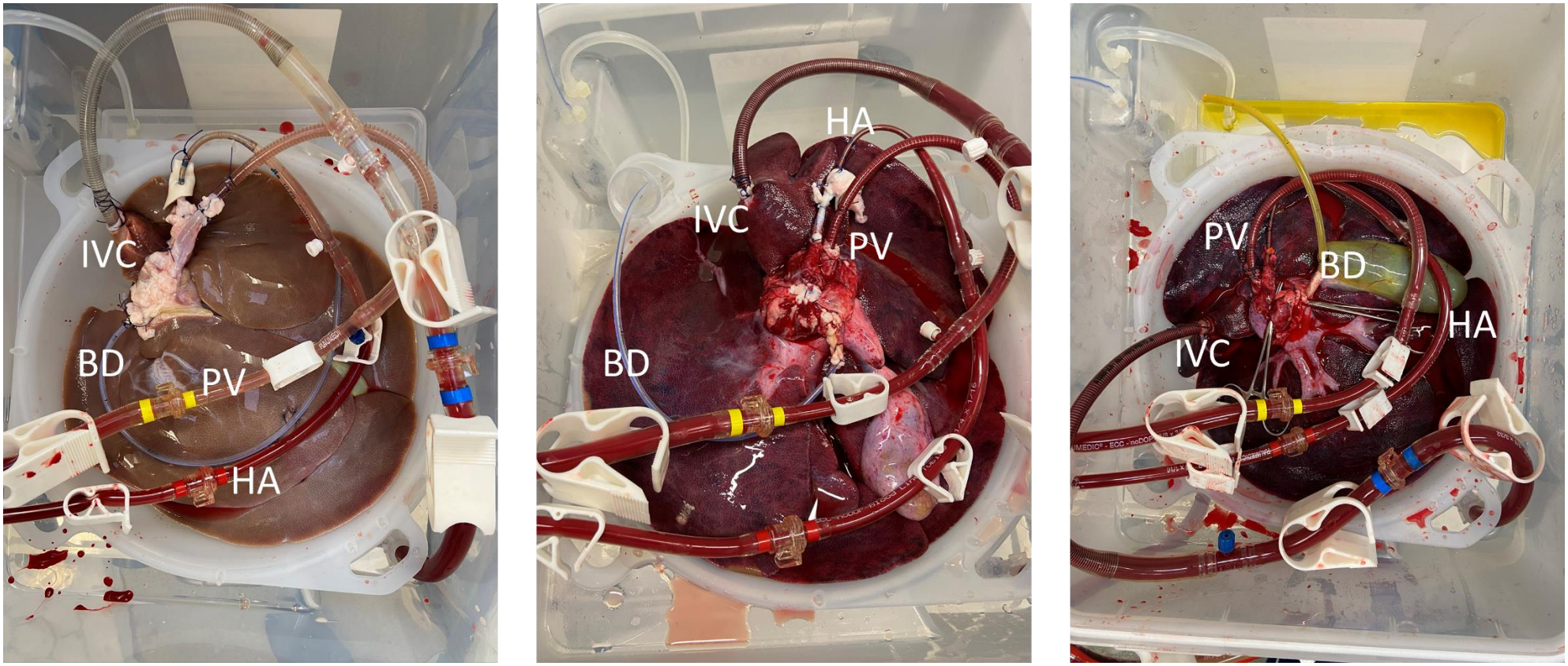


Figure 0.9: Livers during phases of perfusion. (Left) Cannulated porcine liver, pre-perfusion. (Middle) Porcine liver immediately after initial normothermic machine perfusion. (Right) Porcine liver at the end of simulated reperfusion demonstrating bile production. HA = hepatic artery cannula (red), PV = portal vein (yellow), IVC = inferior vena cava (blue), BD = bile duct (Silicone tubing).

1.32 Histology

Serial biopsies were obtained in the majority of perfusions and were suitable for histopathological analysis. Importantly, we are able to show that following the slaughterhouse protocol for procurement, there is almost no detectable injury related to procurement alone: injury begins to manifest upon ex situ reperfusion (**Figure 0.10**)

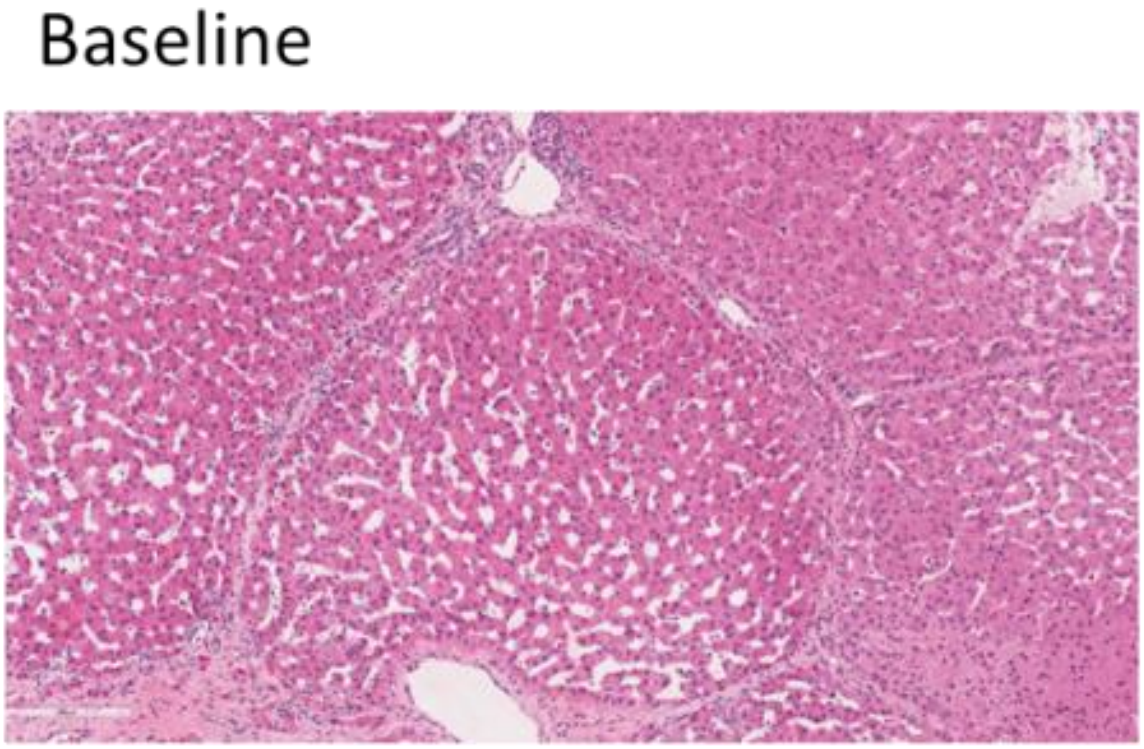


Figure 0.10 (a): Histology following slaughterhouse procurement. There is minimal injury at the baseline histological assessment of livers procured with the slaughterhouse protocol.

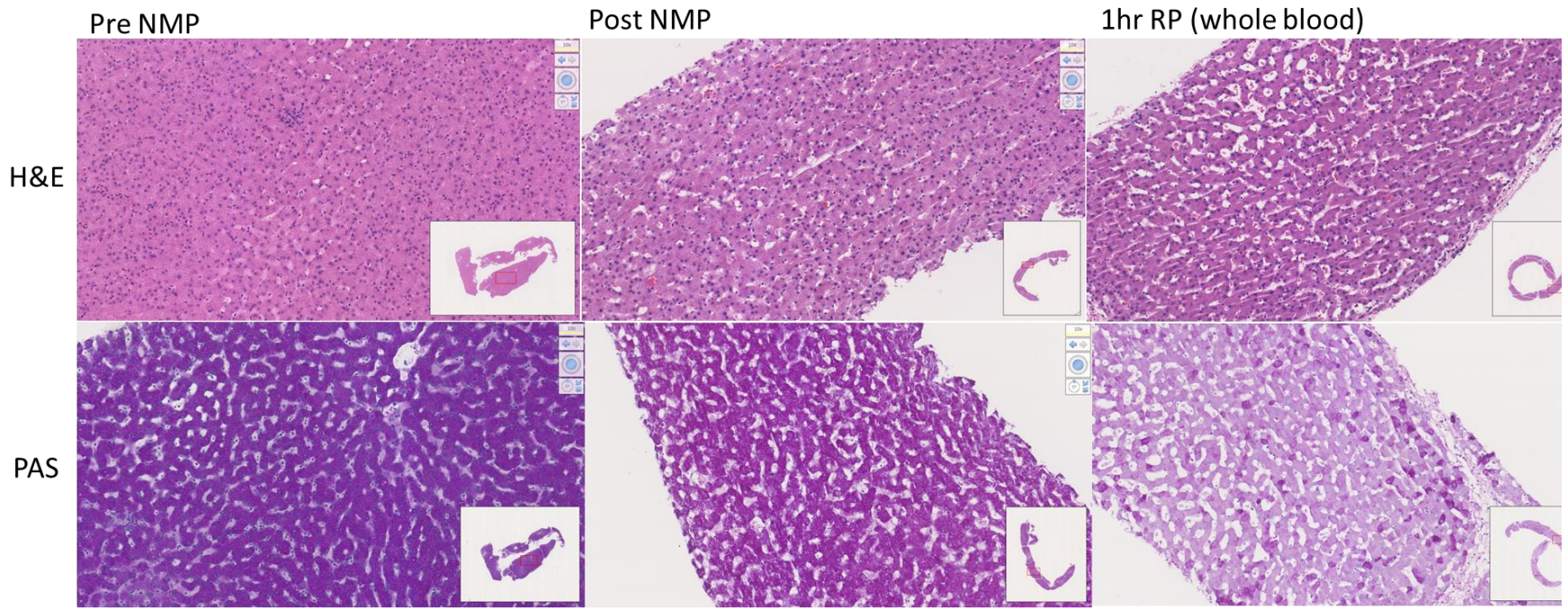


Figure 0.11: Histological changes during Normothermic Machine Perfusion and Reperfusion. Illustration of the histological changes during a perfusion, with normal baseline tissue, then progressive injury /inflammation with the post reperfusion biopsies (allogenic whole blood) showing significant glycogen depletion and some neutrophil infiltration.

1.33 Blood typing:

We implemented a duplicate blood typing system into the procurement process to facilitate our reperfusion experiments. We found that a significant proportion of pigs were blood group A and that this posed a theoretical risk of impacting the allogenic reperfusion phase of our experiments and affect our reperfusion read out.

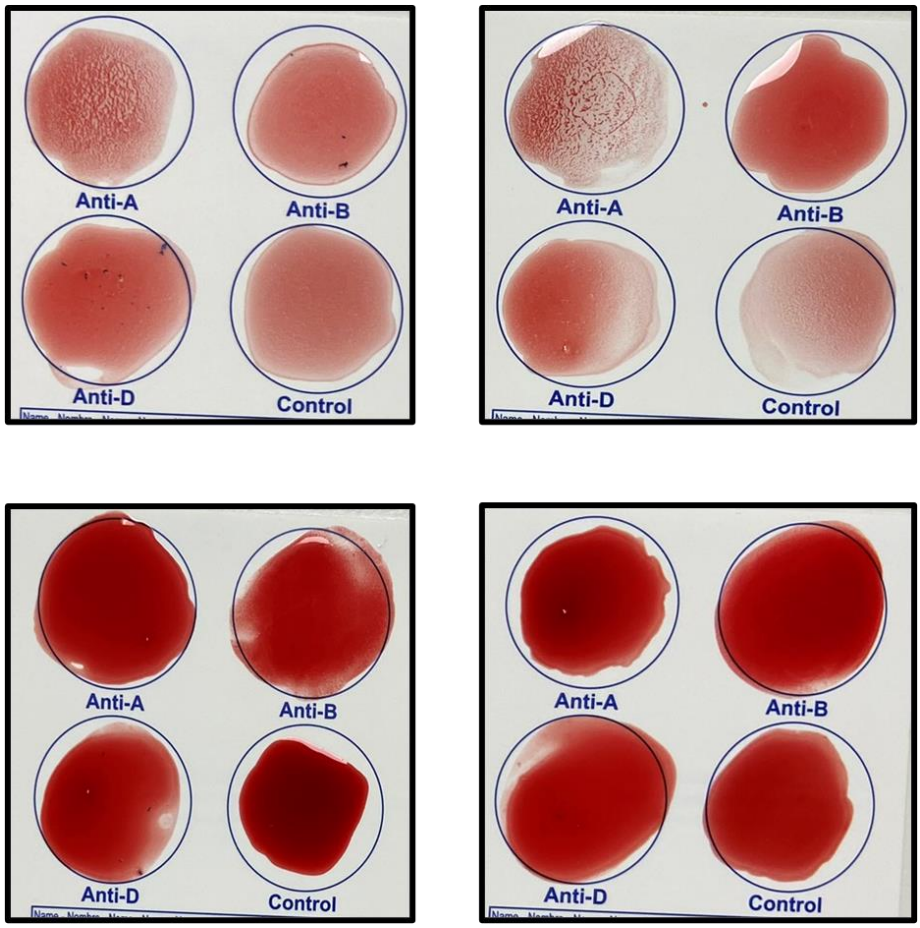


Figure 0.12: ABO compatibility testing for porcine liver NMP experiments (Duplicate Blood typing).Duplicate analysis of blood type of porcine pigs demonstrating blood group A pig (top row), showing agglutination with anti-A, and O pig (bottom), showing no agglutination. This is important in the context for an allogenic simulated transplant reperfusion experiment. Eldon card test kits were used in all experiments and performed in duplicate for reperfusion experiments.

1.34 Discussion

In this chapter, I have described the development of a large animal model for ex situ liver perfusion using slaughterhouse animals. We have been able to dramatically reduce the cost of experiments (by more than £2000 per perfusion), comply with the 3Rs ethical framework and consistently perform perfusion experiments that are reproducible and reliable. Furthermore, we have a model that has construct validity, owing to its similarity to donation after circulatory death, which represents one of the most important frontiers in transplant research.

DCD organs are, in many countries, numerically the fastest growing type of donor organs for transplant and have doubled in the UK over the last decade (239,240). As with donation from older donors and those with steatosis, DCD increases the risk profile of livers and can result in inferior clinical outcomes, predominantly related to the susceptibility of these grafts to ischaemia reperfusion injury (118). Investigating these organs is critically important to being able to continue to expand the donor pool, reduce the discard rate and improve outcomes from marginal donors. The dual impact of sequential warm and cold ischaemia is a key element of the injury that manifests at reperfusion: developing a model that can mirror these processes is particularly valuable. Many research groups have been able to perform perfusion studies using discarded human organs and provided critically important insights into reperfusion injury as well as facilitating the use of a greater proportion of higher risk grafts. However, the number of discarded organs for research is facing a sharp contraction, with the advent of clinical machine perfusion reducing the number of livers available for research and resulting in the offering of organs that have often already been perfused or undergone interventions or failed ex situ assessment. The resulting organs available for human pre-clinical perfusion studies in the UK are therefore unpredictable, often severely injured or compromised to the extent that they are deemed unfit for ex situ assessment. The utility of such heterogenous organs in certain types of scientific research is therefore limited.

This has resulted in some groups using animals bred specifically for research, to provide the consistency and reliability necessary for scientific investigation (15). However, the majority of groups have been able to use farm reared animals, but these animals are used for research purposes. This has been very successful and in some cases instrumental in translating different techniques and ex situ perfusion paradigms into clinical practice such as NRP, prolonged NMP, novel perfusates and is also being used to investigate novel therapies and interventions (241–244). However, these experiments are financially costly and resource intensive. Avoiding the use of pigs bred exclusively for large animal research in the laboratory, instead using animals that are already being used in the food industry, adheres to the core ethical principle of reduction. This is clearly preferable when considered in the context of the '3Rs' (Reduction, Refinement, Replacement) ethical principles that have been developed to underpin animal research in the past 50 years.

The slaughterhouse liver does have some subtle yet important differences to deceased donor human organ perfusion experiments and thus clinical NMP. The use of autologous or "fresh" blood is a point of difference between (all) animal and human studies, whereby human liver studies typically depend upon aged packed red cells (often expired units) and units of variable age are used in clinical NM. The impact of this on the starting biochemistry of the perfusion (high potassium, high lactate, low calcium) is significant and alters the expected profile of certain functional assessment markers during the perfusion. As opposed the characteristic fall in lactate from a high level seen in human liver NMP when livers are functioning well or the persistently high or rising lactate when livers are considered unsuitable for transplantation, we observe a slightly different picture. We observe either a progressive fall, as seen in human livers (albeit from a substantially lower baseline, typically <10 mmol/l)(238), or a progressive rise from this lower lactate level to a peak between 4-6 hours and then a fall as the liver recovers from the ERI. Interestingly, in the human liver literature, initial clearance followed by a progressive rise in lactate levels has been considered an ominous sign and these livers have been discarded (245). This model has not shown

this pattern, with livers consistently following one of the two trajectories of immediate clearance or progressive injury followed by clearance of lactate beyond the 4–6-hour time point. The published human lactate-based assessment metrics in terms of their specified time cut offs for assessment of "viability" are therefore less translatable to the model, as in the case of injured livers, the peak lactate occurs later and the clearance curve is thus different. Interestingly, we observed that low hepatic arterial flow was associated with higher lactate, supporting a widely accepted concept that poor haemodynamic parameters exacerbate ex situ injury (appendix), although further experiments are required to fully elucidate the direction of causality as the converse could equally explain our current observations. In terms of glucose metabolism, we find a more similar picture to that of human liver NMP, of grossly elevated glucose levels in the perfusate immediately following reperfusion and a progressive fall over the course of perfusion(appendix)). The metabolism of glucose on the circuit is increasingly being used as a predictor of poor outcome, with the Cambridge group protocolising glucose challenges to grafts that fail to mount a high perfusate glucose concentration, attributing this to glycogen depletion and thus severe preservation injury. Whether the observations from human glycogen depleted livers are similar to porcine slaughterhouse livers, is yet to be determined (246).

The use of bicarbonate supplementation varied between livers, and may be an interesting avenue to explore when quantifying ERI, but in the course of our experiments, the pH readings were not interpretable given the liberal use of bicarbonate to maintain this in the physiological range. Our approach in the development of the model was to maintain a physiological perfusate pH in order to assess liver function with as many aspects optimised as possible (temperature, oxygenation, antibiotics, nutrients, vasoactive medications and biochemistry). Furthermore, cholangiocyte functional parameters were insightful, with good bile production in all livers. Bile biochemistry and cholangiocyte functional assessment as a separate compartment in organ assessment ex situ requires specific attention. This cellular compartment differs from the traditional hepatocellular

markers, is increasingly important to assess separately in order to avoid biliary complications that would not be apparent when only assessing hepatocellular function (247,248) (Op Den Dries *et al.*, 2014; Matton *et al.*, 2019). We found that consistently, our livers met the biliary pH criteria set out in the major viability assessment criteria.

Although the ethical and economic advantages are clear, there are important scientific trade-offs that must be considered. One of the key variables, that represents an unknown entity is the impact of the 60˚C hot water bath during the initial warm ischaemia. In other groups, unpublished and anecdotal experiences have suggested that aspiration of the hot water can render the lungs unsuitable for ex situ perfusion studies and may in turn cause an injury to abdominal organs that is unquantified and does not occur during human DCD procurement. Although this is an uncontrolled variable, we have managed to demonstrate that rapid cannulation and cooling is feasible and there is minimal histological evidence of damage in our baseline liver samples (see **Figure 0.10**). Other limitations include the sterility of the working environment within the slaughterhouse, where we will not achieve clinical standards of sterility and are therefore at risk of developing occult infective complications that could impact our experimental readouts. Despite these limitations, our data suggest that this is a good pre-clinical model and is a valuable advance that will underpin organ perfusion-based research. This model is already supporting new research opportunities within our research group, reducing the high cost barrier to research.

This model was developed and has been used in order to facilitate the experimental work that constitutes much of this thesis, which focuses on organ preservation and reconditioning, but also has the potential to support studies in drug delivery, cellular therapeutics, decellularization and bioengineering.

Porcine ERI

"Far better an approximate answer to the right question, which is often vague, than an exact answer to the wrong question, which can always be made precise."

John W. Tukey, (1962), "The future of data analysis."

1.35 Summary: observations from human studies

During human liver NMP, ex situ reperfusion induces immune cells to efflux into the circulating perfusate: with levels that peak early and then plateau as perfusion progresses. Cytokines, by contrast, seem to rise progressively through perfusion. Nuclear DAMPs (nucleosomes), released into the perfusate are detected at high concentrations by the 1-hour timepoint and maintained at high levels thereafter. Collectively, this creates an inflammatory circulating perfusate that impacts the molecular and proteomic profile of the graft in what we have called ex situ reperfusion injury/inflammation or 'ERI'. To explore this, we set up a large animal experimental model of liver NMP and subsequent simulated (allogenic) transplant reperfusion, creating a model of preservation reperfusion injury or 'PRI'. My objective was to investigate the interplay and mechanistic underpinnings of ERI and or 'PRI' in this model, with the intention that this would enable us, in due course, to devise, test and deploy interventions to mitigate these processes.

1.36 Introduction

Although we had previously developed a successful experimental NMP model, there were several outstanding issues which had been identified using samples stored from the clinical trials of NMP, that needed to be addressed prior to investigating interventions and therapies.

It was important, first, to establish whether the efflux of cells seen upon ex situ reperfusion of human organs would be consistently replicated in our porcine model, providing confidence in the model's ability to recapitulate our prior human observations. The second objective was to identify the fate of the effluxed immune cells: the rapid decline in cells after an initial peak indicated that the cells were either rapidly consumed (including being trapped or lysed) or returning to liver. Determining the fate of these cells holds significance in understanding the trafficking of cells between the circuit and graft during NMP, and has potential therapeutic implications. The third objective related to cytokine production: in the previous clinical trials total cytokine concentration in the perfusate progressively increased and the levels of nucleosomes (nuclear DAMPs) peaked after an hour of NMP, with extremely high levels detected throughout. These observations required validation in our porcine model if this was to be used as the model for designing interventional studies and for the interpretation of immunological readouts from future experiments.

Interestingly, during a clinical liver perfusion in the COPE liver study, there was a serendipitous observation, in which a donor liver in the immunology sub-study was exposed to a period of warm ischaemia during NMP due to a technical perfusion issue: upon re-establishment of perfusion to graft, the liver experienced a second efflux of immune cells into the perfusate (Figure 0.1).

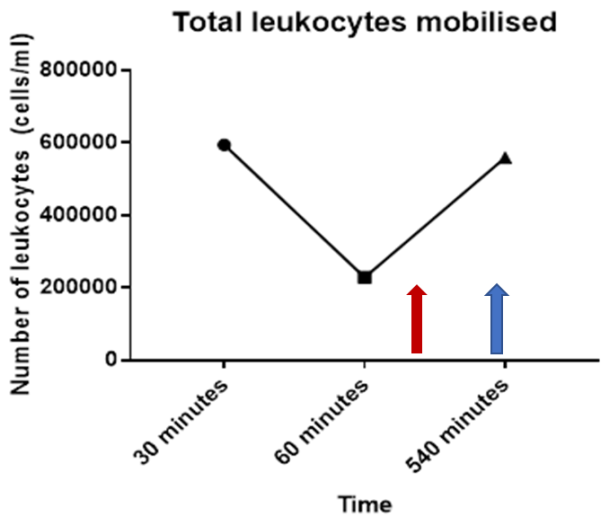


Figure 0.1: **COPE liver technical incident - Cells effluxed into the circuit following an ischaemic period.** During a clinical perfusion, a period of warm ischaemia was inadvertently created by machine malfunction for 20 mins (red arrow). Following this ischaemic period, the graft was reperfused (blue arrow) and the number cells detected peaked again. (Courtesy of David Nasralla & William Critchley,)

This single observation suggested that ischaemia, even for a very short period of time as is the case prior to continuous NMP, or related to a machine malfunction during NMP (as observed in the above liver) could *induce* an efflux of cells from the graft into the perfusate upon reperfusion. The specific immune pathway responsible is not understood, but has been proposed to be an innate process of mobilising the "marginated immune system" (249) in response to ischaemic insult. This (Figure 0.1) observation supported the concept of ex-situ ischaemia-reperfusion being an inflammatory post-ischaemic event: we decided to explore this further and determine the pattern of cytokines and DAMPs alongside the immune cells to gain insight into the underlying molecular processes occurring during reperfusion on the device.

Furthermore, given that ex situ reperfusion triggers the efflux of cells into the perfusate, and that these may have an important role in allorecognition and the immunogenicity of the graft, their removal during NMP has been proposed as a modulatory intervention including in kidney (181), lungs (250) and heart (251). Similarly, cytokines and nuclear DAMPS, as triggers of the immune/inflammatory cascade, are potential therapeutic targets for immunomodulation of livers

during preservation: this is supported by data from other organs preserved with ex situ perfusion (184,252). In this chapter, we sought to determine the extent to which we could recapitulate the human liver NMP immunological events in our porcine model.

1.36.1 Chapter Aims

In this chapter I describe a series of experiments with three aims: First, to determine the extent to which our observations from human liver perfusate analysis regarding ex situ reperfusion immune responses (immune cell efflux, nuclear DAMP levels & circulating cytokines) were replicated in our experimental porcine model. Second, to systematically address the questions that emerged from the human data, such as the fate of effluxed immune cells, the role of preceding ischaemia on the phenomenon of immune cell efflux, quantity and composition of circulating cytokines and nuclear DAMPs in the circuit. Third, to explore approaches to immune cell depletion during NMP.

1.37 Methods

1.37.1 Liver Procurement

Livers were procured, flushed, stored in SCS for transport and established on NMP back at the perfusion laboratory as previously described. Briefly, Sus domesticus (white landrace) pigs (50-70kg) were electrically stunned, exsanguinated, briefly cleaned in a hot water bath at 60˚C (140^{0}F) and eviscerated via thoracoabdominal incision. The liver was isolated from the viscera, flushed and preserved in UW® solution for transport. Third party (allogenic) - whole blood was collected into heparinised containers for storage and preparation at the laboratory.

1.37.2 Liver NMP

All livers were perfused using the OrganOx metra® device in a standard fashion after backbench preparation, cannulation and further flushing of the liver via the secured cannulae. The perfusate composition was autologous packed red cells resuspended in gelofusin® or re suspended in autologous plasma, following leuko-depletion in all cases. Allogenic ABO compatible whole blood was used during simulated transplant reperfusions. We modified the OrganOx metra circuit to integrate a leukocyte filter (leukogaurd®) to explore passenger leukocyte depletion (**Error! Reference source not found.**)

1.37.3 Samples

Liver characteristics, weight, blood group (A/O), macroscopic appearance were recorded; alongside procurement and perfusion times, including WIT, CIT, NMP time and simulated transplant reperfusion time (**Table 0.1**)

1.37.4 Porcine pSCS-NMP sampling

Perfusate and bile samples were obtained prior to initiation of NMP (during priming), and then serially at various intervals following initiation of NMP. Samples were collected for analysis via haematology (Sysmex XN-1000™ Hematology Analyzer), clinical biochemistry and point of care testing blood gas analysis as well as for storage and later analysis, as previously described (Methods). Liver tissue and bile duct biopsies were collected at specific timepoints during 'preservation perfusion' and simulated 'transplant reperfusion' and handled as previously described (Methods).

1.37.5 Porcine pSCS-NMP perfusate immunological assessment

1.37.6 Flow Cytometry

Immune cell populations were identified and quantified using flow cytometry performed on a BD LSR II flow cytometer (Becton Dickinson, Oxford, UK). Briefly, leukocytes were identified (CD45+) and their viability assessed using Zombie viability dye (Biolegend, UK). Leukocyte subsets were determined using a panel of antibodies. Innate immune cells including immature neutrophils (6D10 + 2B2-), mature neutrophils (6D10 + 2B2+), mature eosinophils/basophils (6D10-2B2+) and NK cells (CD16+, CD56+) were measured. Monocytes were also included in the panel to include classical (CD14 + CD163-) and non-classical (CD14/CD163+) phenotypes as well as macrophages (CD203a+). Adaptive immune cells/lymphocytes, including T cells (CD4+, CD8+) and B cells (CD3-CD21+), were quantified, as well as NKT cells (CD56+ CD16-) and T cells with a potentially regulatory phenotype (CD4 + FoxP3+), the latter were identified by assessing intracellular expression of FoxP3. Cells were resuspended with PBS and divided into tubes for erythrocyte lysing (BD Biosciences, UK), washed twice and resuspended in 300 µl staining buffer. Cells were quantified a represented as cells/mL. This was performed at the

University of Manchester by our collaborators in the Fildes Lab where an experienced porcine immunology team designed the panel and conducted these specialised protocols and workflow.

1.37.7 Cytokine Luminex analysis

The concentration of cytokines and chemokines within the perfusate was assessed using plasma samples collected at baseline, 60 minutes, and 360 minutes. A commercially available porcine 13-plex magnetic bead panel (Merck Millipore, Billerica, MA, USA) was used, following the manufacturer's protocol. The plate was read using a Bio-Plex 200 system (Bio Rad, Hertfordshire, UK).

1.37.8 : Damage Associated Molecular Patterns: Nucleosome Measurement

Nucleosome levels were measured using Nu.Q™ ELISA assays (Belgian Volition SRL, Isnes, Belgium). An Automated device in the central Volition Laboratories was ultimately used and measured samples in triplicate after multiple dilutions to achieve detectable levels. Initial analysis using the commercial Nu.Q™ ELISA assay (Belgian Volition SRL, Isnes, Belgium) was performed in-house (myself), but the levels were well above the standard range for detection of the kit despite attempted dilutions, the samples were then sent to the University of Manchester for repeated analysis by our collaborators in the Fildes Lab, who encountered the same issue. They were able to do more dilutions and used an extrapolated standard curve to calculate the levels after multiple dilutions. In subsequent experiments samples were sent to Belgium for analysis in the central Volition laboratory due to issues with measurement above the ELISA kit range.

1.37.9 Statistical analysis:

Descriptive statistics were reported as mean and standard deviation or median and inter-quartile range according to normality. A Student t-test was used to analyse parametric, continuous variables, and the Mann-Whitney U test was used for non-parametric continuous variables.

Fisher's exact test was used for categorical variables and a p-value of 0.05 was considered statistically significant.

Experiments:

We conducted three main sets of experiments, with different perfusion protocols. These protocols varied from perfusions without the liver on board to liver perfusions with multiple ischaemic insults followed by whole blood reperfusion. All liver perfusions involved an initial NMP phase with autologous RBC based leuko-depleted perfusate. The reperfusion phase (RP) refers to the allogenic whole blood simulated transplant reperfusion that occurs at the end of NMP after a brief period (30mins) where the liver is flushed with cold preservation fluid or crystalloid, this mirrors the clinical setting where livers are flushed prior to implantation and in terms of "warm ischaemia" simulates the 'anastomotic time' ahead of reperfusion in the recipient, which is a period where the liver is out ice and being implanted before revascularisation is achieved.

1.37.10 Experiment 1: Immunological and molecular events during porcine liver NMP

We conducted standard liver perfusions after a period of SCS, focusing on the early events following ex situ reperfusion. We serially sampled the perfusate and measured the number of cells at specific timepoints through perfusion and then characterised the cell types using flow cytometric analysis. (**Figure 0.2**)

1.37.11 Experiment 2: Fate of effluxed cells – perfusions without liver.

We previously observed that a large proportion of cells effluxed into the perfusate following ex situ reperfusion are no longer detected in the perfusate as the perfusion progresses, with the greatest changes occurring early after reperfusion. In order to determine if the loss of cells was due to rupture or damage related to the circuit itself (oxygenator, pump, tubing or reservoir), we conducted perfusions with no liver on the device to assess the rate of attrition of circulating leukocytes attributable to the circuit as opposed to the organ. (Figure 0.3)

1.37.12 Experiment 3: Role of ex situ warm ischaemia on reperfusion immune events.

We conducted these experiments, to determine the extent to which the observed ERI was fundamentally related to preceding ischaemic injury. The typical NMP profile of cells/DAMPS/cytokines in the perfusate was largely observed at the start of NMP (i.e after a period of cold ischaemia) but whether or not further episodes of ischaemia reproduced similar responses was previously not known. We set out to establish if repeated ischaemic insults resulted in a repeated and consistent efflux of cells (Figure 0.4).

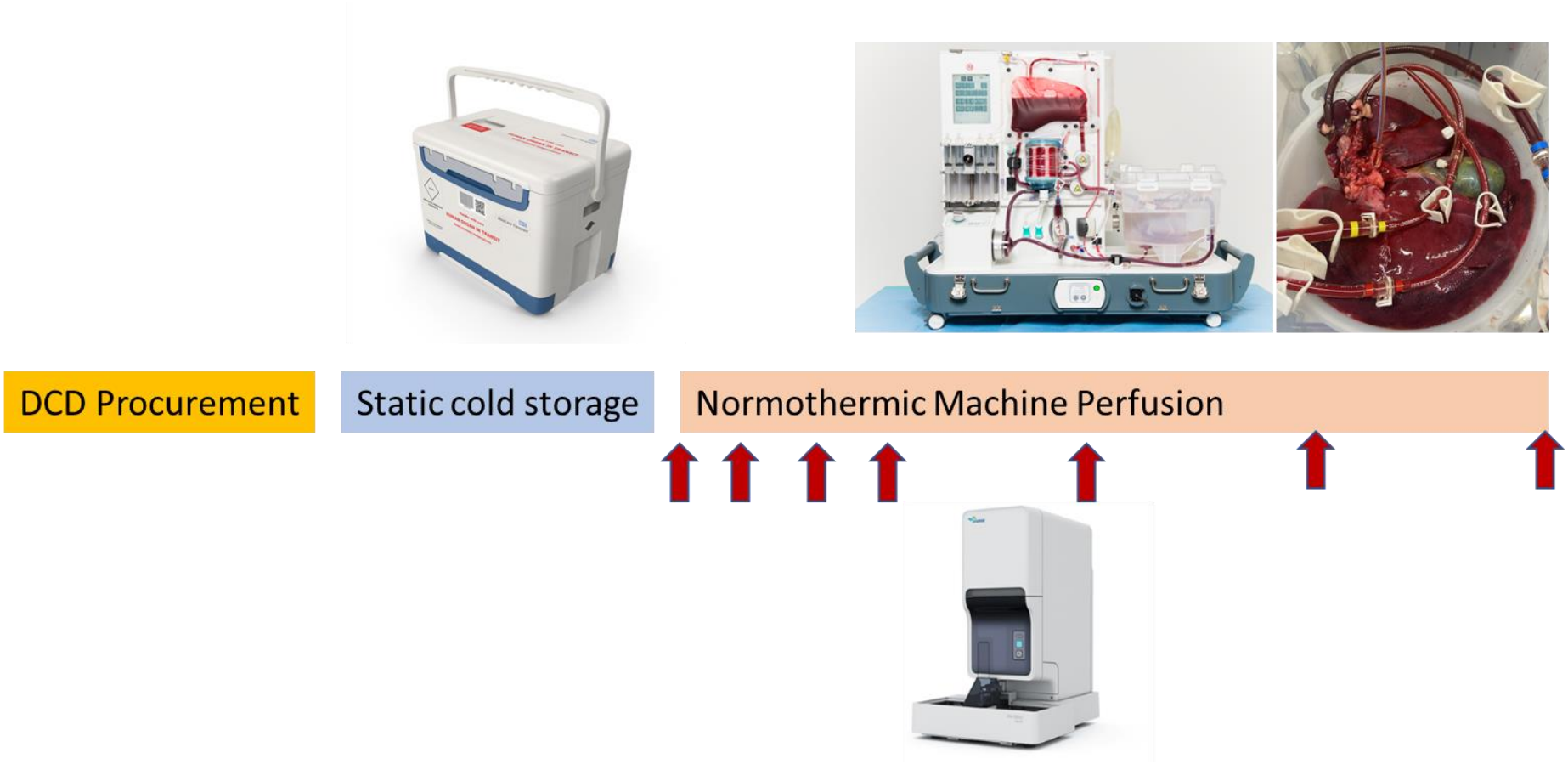


Figure 0.2: Experiment 1, Porcine pSCS-NMP model: Following DCD procurement and transport to the lab under SCS conditions, livers we perfused on the OrganOx metra device. Serial perfusate samples were obtained for analysis of the cells present within the perfusate following reperfusion. A higher frequency of samples were obtained early in the perfusion, then with deceasing frequency, the samples were analysed using the Sysmex NX-1000 Analyzer™. DCD = donation after circulatory death, NMP = normothermic machine perfusion, pSCS = prior static cold storage.

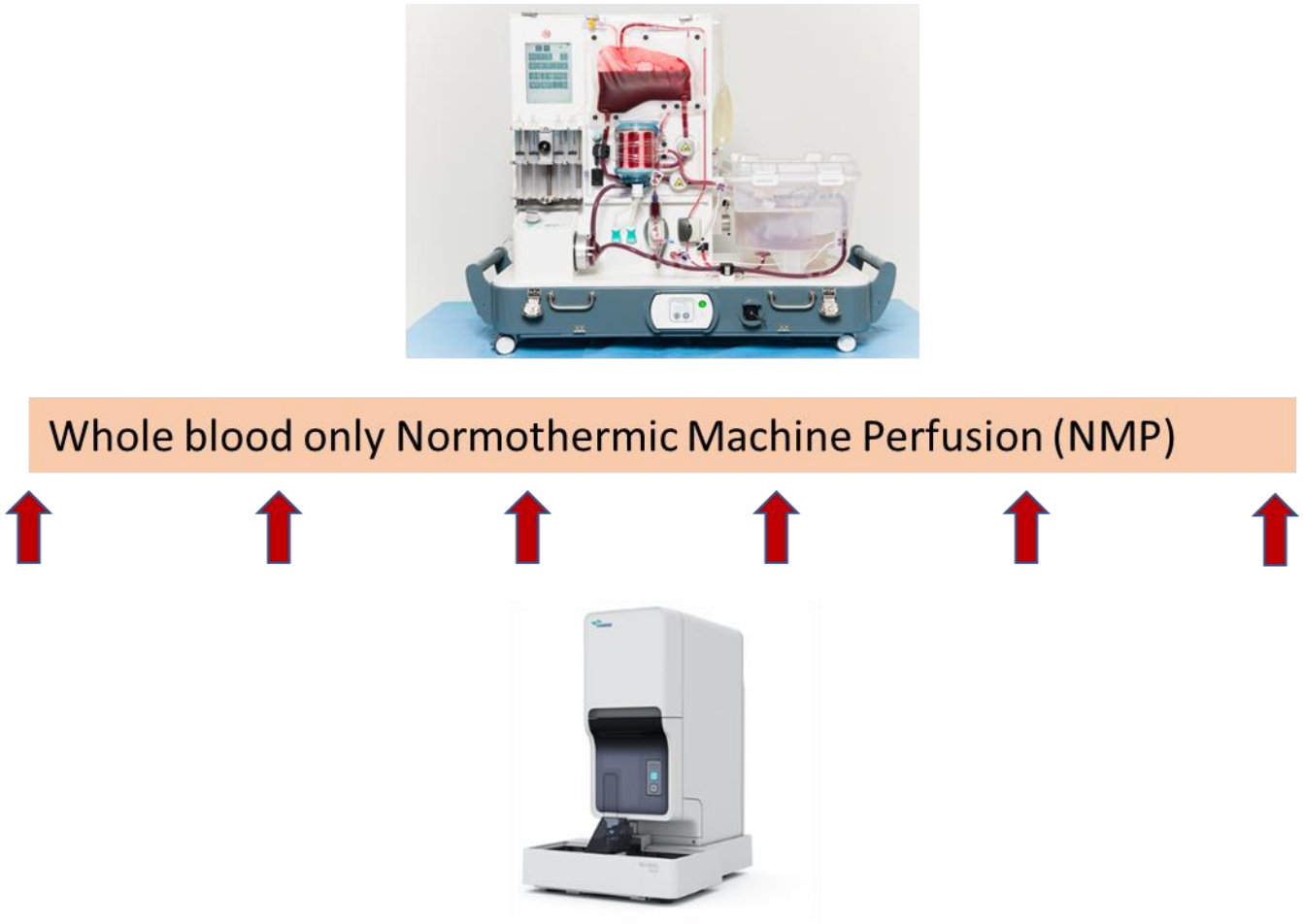


Figure 0.3: Experiment 2, Fate of effluxed cells. Whole porcine blood only perfusion on the OrganOx metra device. After priming with whole blood and starting the perfusion, serial samples of perfusate were taken to measure the cells present in the perfusate using the Sysmex NX-1000 Analyzer™. NMP = normorthermic machine perfusion.

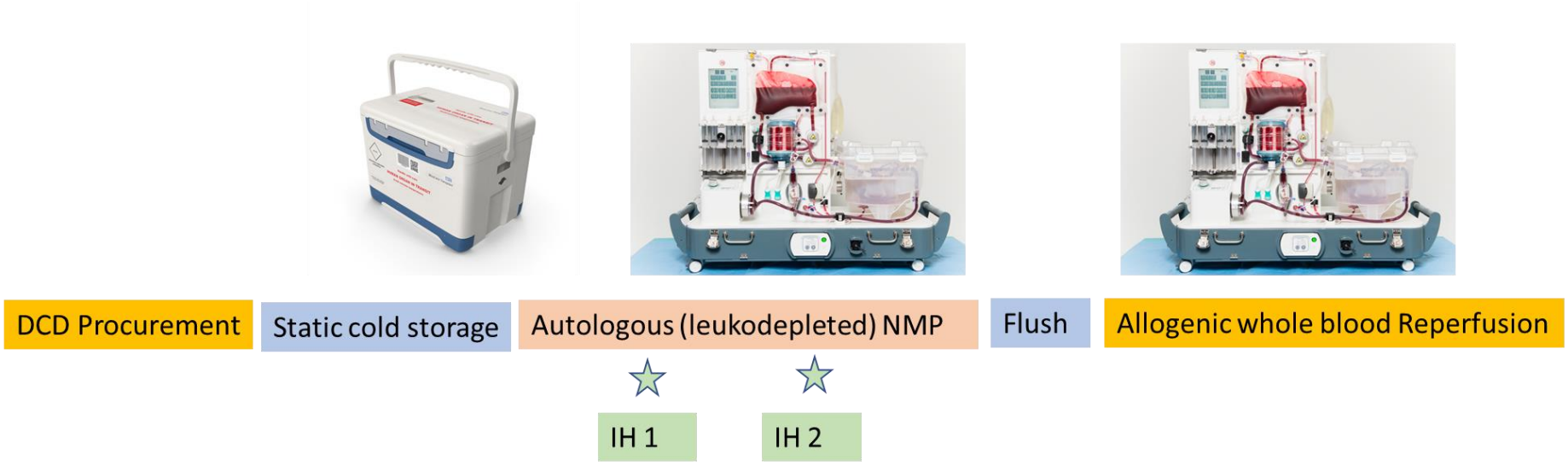


Figure 0.4: Experiment 3, Ischaemic insults during NMP: Following DCD procurement and transport to the lab under SCS conditions, livers we perfused on the OrganOx metra® device with autologous RBCs and colloid solution (gelofusin®). During this period of NMP, livers were subjected to ischaemic hits (IH1 and IH 2) lasting 20 minutes alternating with resumption of NMP for 30 minutes. 30 minutes after IH2, livers were flushed with 2 litres of crystalloid while the device was reprimed with allogenic blood group-matched whole blood: livers were then reperfused in a simulated transplant model. Serial perfusate samples were obtained for analysis of the cells, cytokines and nucleosomes present within the perfusate during NMP and reperfusion. DCD = donation after circulatory death, IH = ischaemic hit, NMP = normothermic machine perfusion, pSCS = prior static cold storage, RBC = Red blood cells.

1.38 Results

1.39 Experiment 1: Immunological and molecular events during porcine liver NMP

All livers in the first set of experiments were retrieved and established on the OrganOx metra® device without complications. All livers had stable haemodynamics and gas exchange during perfusion (see appendix for machine data sheets).

1.39.1 Immune cells: porcine liver ex situ reperfusion is associated with an immune cell efflux, similar to that observed in human livers.

We found that similar to our observations from the human liver perfusion clinical studies, porcine ex situ reperfusion was associated with a substantial immune cell efflux into the perfusate, occurring upon reperfusion (**Figure 0.5)**. The overall pattern followed a consistent pattern of an early peak, rapid fall and plateauing of detectable immune cells which was very similar to our human observations.

1.39.2 The peak in effluxed cells occurs very early after ex situ reperfusion.

In the clinical trial perfusions, the earliest timepoint we were able to assess was at 30 mins and this was therefore regarded as the "peak cell efflux", however, with earlier sampling in the porcine liver NMP laboratory experiments, we have demonstrated that the peak occurs earlier, indeed at the earliest measured (10 minute) sampling timepoint. We therefore, analysed perfusate samples taken from a set of livers (n=3) where we sampled the perfusate at even earlier timepoints (1 minute, 5 mins, 10 mins, 20 minutes and 30 minutes). We found that by 30 mins, the timepoint we had previously considered to be the peak, the total number of cells detected had fallen by 55% (at 5mins) and 59% (at 10 mins) from their 1 min peak. 598627.28 (SD 339208.72) = 5 mins, 651828.78 (SD 271680.61) down to 270183.92 (SD 78045.86) at 30 mins.

During the initial efflux of cells, the predominant cell type was CD4+ T lymphocytes, with CD8+ cells and NKT cells also present in high numbers; low levels of B cells and Regulatory T cells were also detected. In terms of the innate cell populations, monocytes, NK cells and non-neutrophil granulocytes were detected in low numbers. Neutrophils (immature) were present in more substantial numbers with a very early peak at 10 mins, but rapidly decline within the first 30 mins, which may explain the lack of neutrophils in the human liver studies, which only started sampling at 30 mins.

1.39.3 Porcine pSCS-NMP is associated with a progressive increase in circulating cytokines

We found that the levels of cytokines detected in the perfusate increased throughout perfusion. The highest concentration cytokines were IL1-Ra (which is an anti-inflammatory cytokine), IL-6 and TNFa, both of which are well characterised pro-inflammatory cytokines. The remaining cytokines were detected at very low levels in the perfusate, with anti-inflammatory IL-10 comprising a small proportion of the detected cytokines (**Figure 0.8**).

1.39.4 Nucleosomes were detected at high levels during NMP.

Nucleosome levels were detected at very high levels in the perfusate during NMP, starting from a relatively low baseline. These extremely high levels were maintained throughout the perfusion and detected in the perfusate using the NuQ.H1.3 ELISA kit. This was similar to our findings from the human liver perfusate, although with levels that were even higher. These levels were maintained during the simulated transplant reperfusion, despite the liver being flushed and circulating perfusate being exchanged between the NMP and RP stages of the experiment.

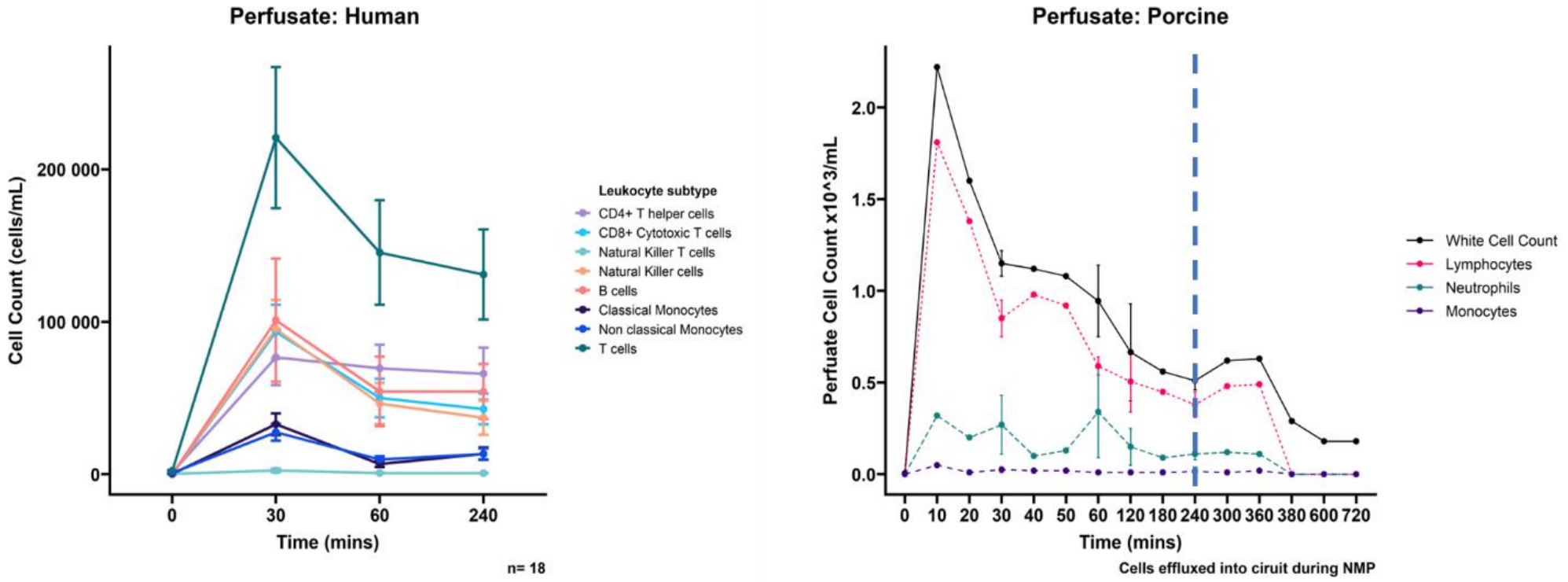


Figure 0.5: Leukocytes detected in perfusate following human clinical NMP (from COPE liver trial) and porcine ex situ reperfusion. Left: Human liver NMP, cells detected during perfusion in the perfusate over the first 4 hours, with the cellular sub types. Human liver immune cells were quantified using manual isolation and flow cytometry in the research lab, this was represented in cells/mL. Right: Porcine liver perfusion, cells detected during perfusion, dashed blue line at the 4 hour timepoint for comparison with human data. Cells were quantified using a clinical grade Sysmex NX-1000 Analyzer™, with cells represented in cells $x10^3$/uL (= $x10^9$/L) for the porcine livers. Similar overall pattern of efflux is observed with human and porcine perfusion. NMP = normothermic machine perfusion.

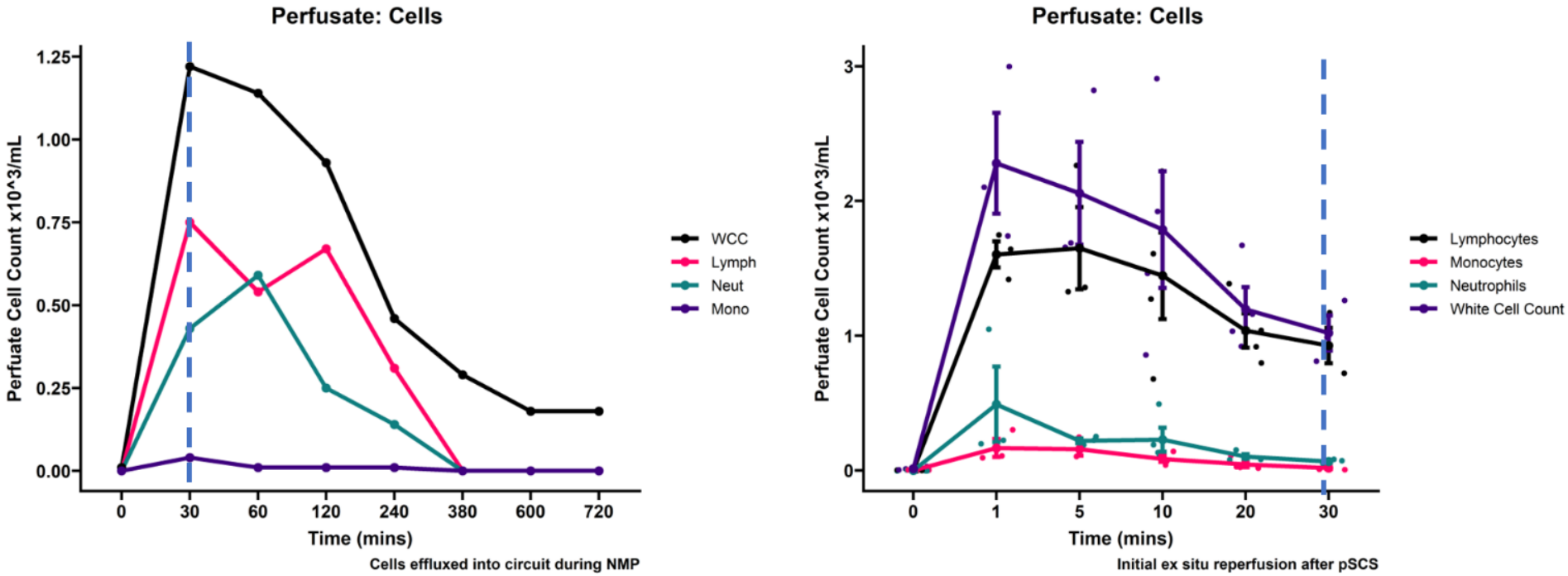


Figure 0.6 Cellular efflux from graft into perfusate after ex situ reperfusion. Left: Overall pattern of cellular efflux following ex situ reperfusion of the liver (porcine data). Right: Earlier timepoints measuring cellular efflux after reperfusion. A large proportion of cells already lost or re-entered the graft by the 30 minute timepoint (dashed blue line) previously thought to be the peak efflux of cells based on human data, where earlier timepoints were not measured. Cell numbers are represented as cells $x10^3$/uL (= $x10^9$/L) and measured on the Sysmex XN-1000™ Haematology Analyzer.

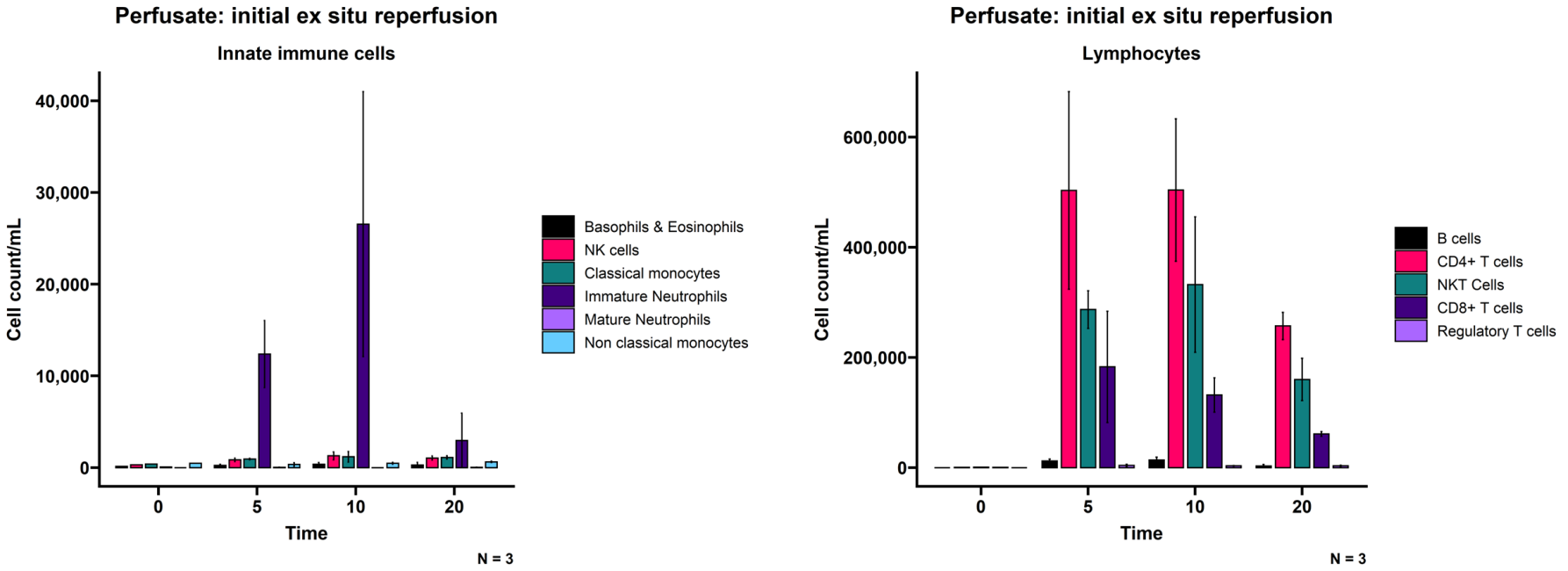


Figure 0.7: Ex situ reperfusion leukocyte efflux subpopulations: Following a period of SCS, porcine livers were reperfused on the OrganOx metra® device. Serial samples were obtained for analysis using flow cytometry to determine the immune cells populations effluxing into the perfusate. Starting with leuko-depleted blood, leukocytes were undetectable, but immediately after ex situ reperfusion cells effluxed into the circuit. (Left)Lymphocytes were present in the greatest numbers with CD4+ Tcells being the most abundant, CD8+ T cells were also detected, but very low levels of B cells and Treg were present. (Right) innate immune cell populations were also detected in the perfusate with immature neutrophils (6D10+, 2B2-) peaking early and sharply falling to low levels by the 20-minute timepoint. NK = natural killer. cells

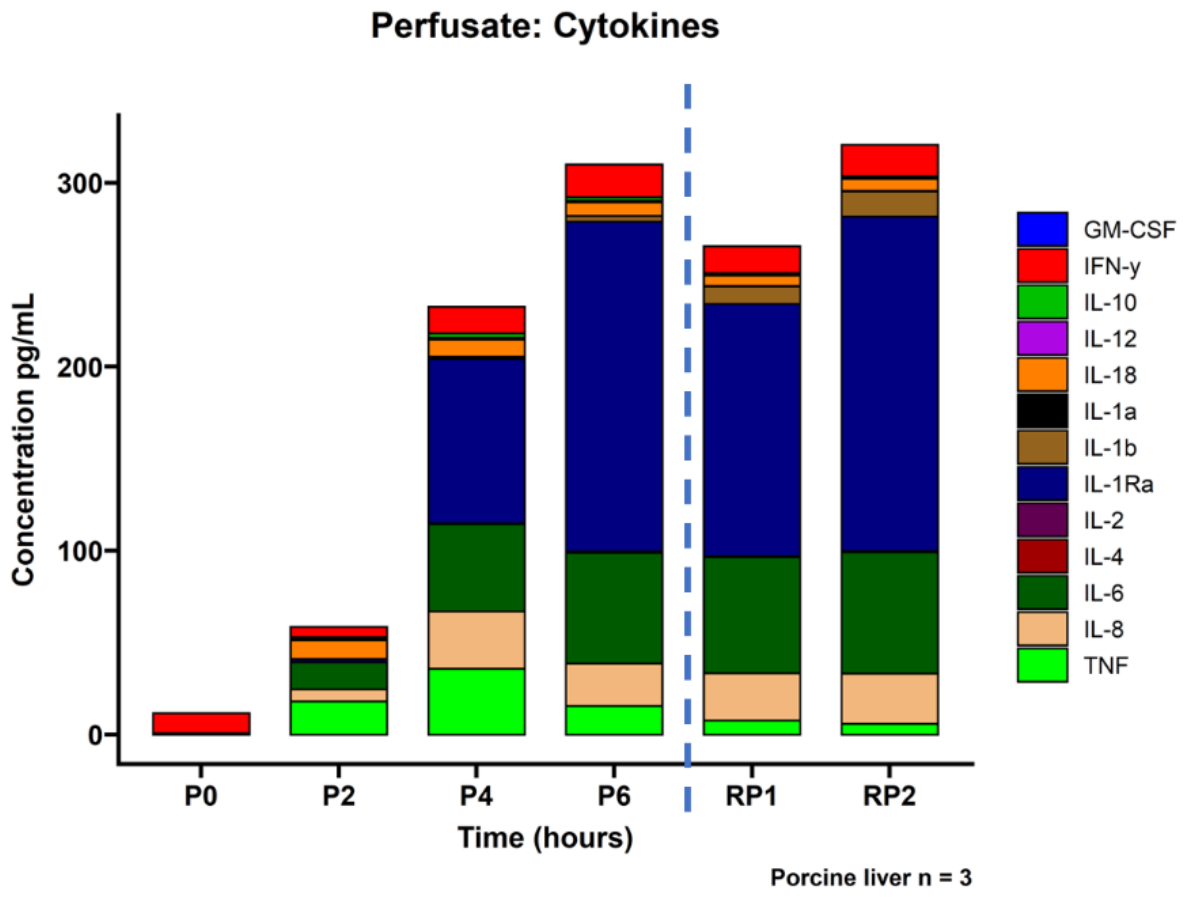


Figure 0.8: **Perfusate cytokine levels during standard pSCS-NMP and RP**. Perfusate concentrations of cytokines rise progressively through the NMP phase "P". Within an hour of allogenic whole blood reperfusion (RP) cytokines are elevated to levels seen at after 4 hours of NMP. Dashed blue line denotes the end of NMP and beginning of RP during which the liver is flushed with crystalloid and the circuit is reprimed with allogenic whole blood prior to the simulated transplant reperfusion. NMP = normothermic machine perfusion, RP = Reperfusion with allogenic whole blood (simulated transplant model).

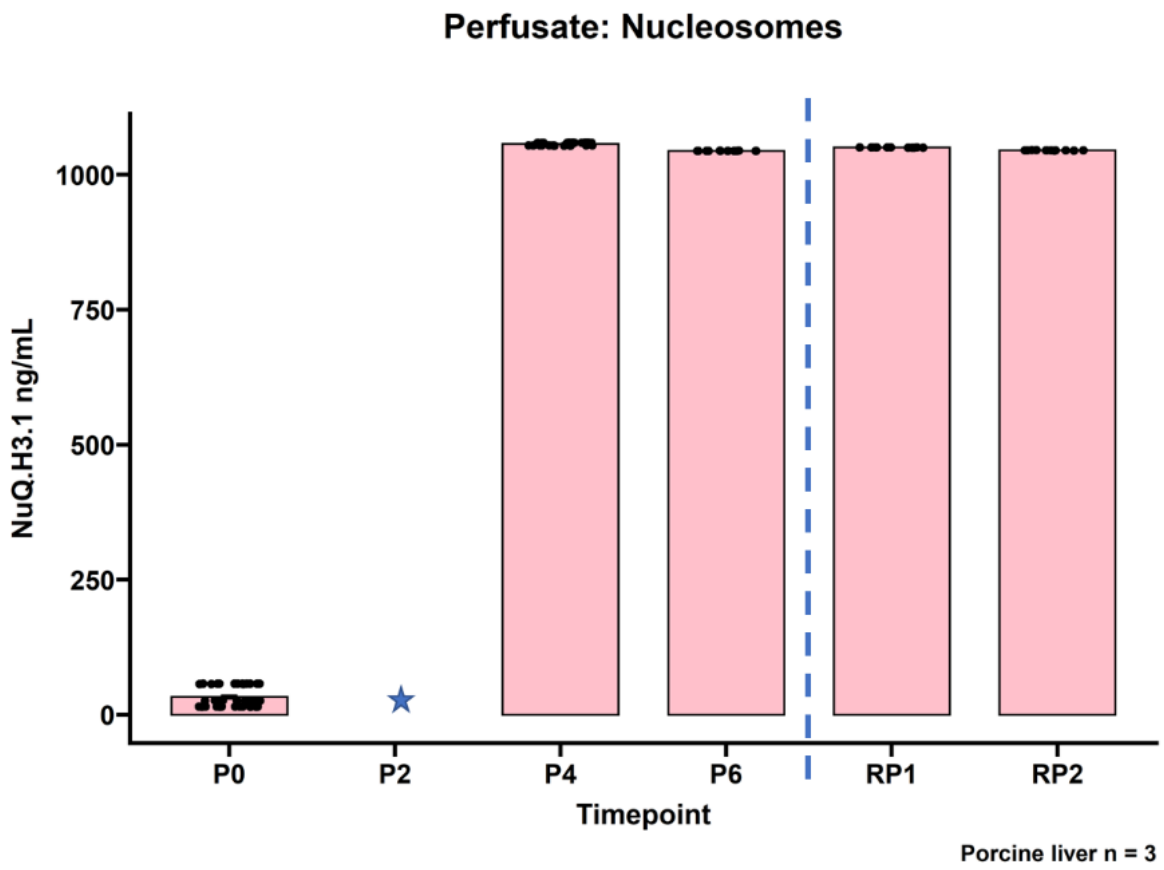


Figure 0.9 Perfusate nucleosomes during pSCS-NMP and RP. Baseline levels of nucleosomes are very low and rise to very high levels by the 4-hour timepoint and remain elevated at this level for the remainder of perfusion and also in the RP phase. Nucleosomes are measured using the using Nu.Q™ ELISA assay. Dashed blue line denotes the end of NMP and the beginning of RP during which the liver is flushed with crystalloid and the circuit is reprimed with allogenic whole blood prior to the simulated transplant reperfusion. NMP = normothermic machine perfusion, RP = Reperfusion with allogenic whole blood (simulated transplant model) P2: No sample was remaining for the further dilution analysis (unrecordably high on first measurement).

1.40 Experiment 2: Determining the fate of effluxed cells following ischaemia-reperfusion

1.40.1 Effluxed cells re-enter the liver during NMP

A large proportion of cells effluxed into the perfusate following ex situ are no longer detected as the perfusion progresses, with the greatest changes occurring within the first 5 minutes after reperfusion. In order to determine if the apparent transience of these cells was due to components of the circuit itself (oxygenator, pump, tubing or reservoir), as opposed to an effect of the liver. We conducted experiments with no liver on the device to assess the rate of attrition of circulating leukocytes. The number of leukocytes within the perfusate was stable over many hours, with no observed loss of cells through perfusion related to damage or the circuit. This was in contrast to the large changes observed during ex situ perfusion **(Figure 0.10)**. This supported the idea of cells returning to the liver as opposed to being consumed by the circuit itself.

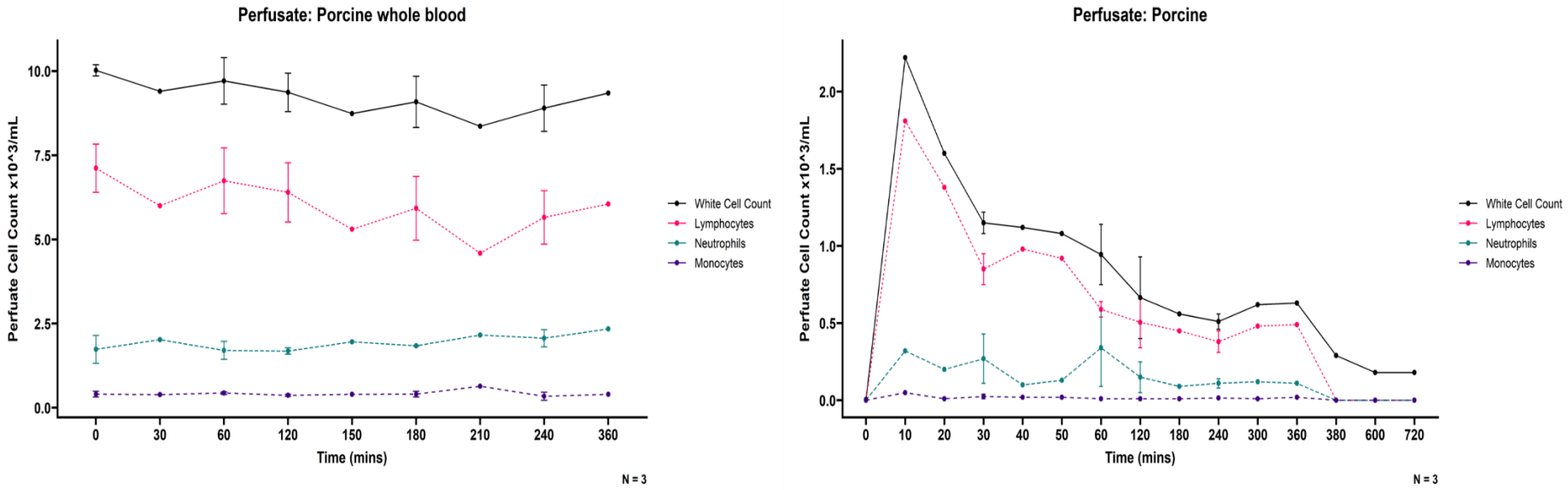


Figure 0.10: Perfusate leukocyte count during NMP with and without liver on board. (Left) NMP of whole blood without a liver on board, and serial samples of perfusate were taken for analysis. Right: pSCS-NMP with a liver on board demonstrating the efflux of cells and rapid decline in cells detectable in the perfusate thereafter. Al samples were analysed with a clinical grade Sysmex NX-1000 Analyzer™ NMP = normorthermic machine perfusion, pSCS = prior static cold storage. (NB: following leukodepletion, the number of cells falls to very low levels, the scale on the y-axis is therefore altered to reflect this in the two panels).

1.41 Experiment 3: The impact of repeated ischaemia-reperfusion on the cellular and molecular profile of the perfusate during NMP

1.41.1 Ischaemia reperfusion induces an efflux of immune cells into the perfusate during NMP

Repeated ischaemic insults during NMP reproducibly resulted in an efflux of immune cells into the perfusate. This effect was present following both cold ischaemia (initial ex situ reperfusion) and warm ischaemia (induced on the device) and consistently comprised mostly lymphocytes, with innate immune cells playing a small role (**Figure 0.11)**

Cytokine levels rose progressively during NMP with repeated ischaemic insults, however in small increments over the relatively short perfusion time (2 hours). Reperfusion was associated with a greater concentration of cytokines, which rose further to a peak at the final measured timepoint.

Nucleosome levels in the perfusate increased following ex situ reperfusion and remained elevated throughout these experiments, with no discernible pattern related to repeated ischaemic insults. The levels were marginally lower than those observed in NMP without ischaemic insults, although in both cases the values were at the highest detectable levels of the assay (**Figure 0.13).** Circulating cells enter the liver upon allogenic reperfusion

During the reperfusion phase, where the circuit was reprimed with allogenic whole blood (restoring the circulating leukocyte levels), we found that there was a large influx of these cells into the graft (76% fall in circulating leukocytes), within the first 10 minutes of reperfusion. The levels of cells continued to fall up to the 60 min timepoint (**Figure 0.14**).

1.41.2 Donor liver characteristics

As outlined in the previous chapter, blood group compatibility was ensured. Female pigs were used as organ donors, from which the liver was procured and male pigs were used for blood collection that would be used during the whole blood reperfusion phase. The ischaemic times were also recorded with the warm ischaemia and cold ischaemia times being similar.

Table 0.1: Donor and Recipient pig characteristics ischaemic hits experiments with reperfusion phase : preservation characteristic, WIT = Warm ischaemic time, NMP = normorthermic machine perfusion, CIT = Cold ischaemic time, RP = allogenic whole blood reperfusion, BG = blood group. Recipient (whole) blood is used to prime the device for simulated transplant reperfusion. Donor liver = Sex of the donor liver being perfused, Reperfusion blood = allogenic blood used in whole blood reperfusion.

PIG	WIT	CIT	DONOR LIVER (SEX)	REPERFUSION BLOOD (SEX)	D-BG	RB-BG
1	00:19	09:24	F	M	O	A
2	00:17	06:45	F	M	O	O
3	00:17	09:34	F	M	O	O

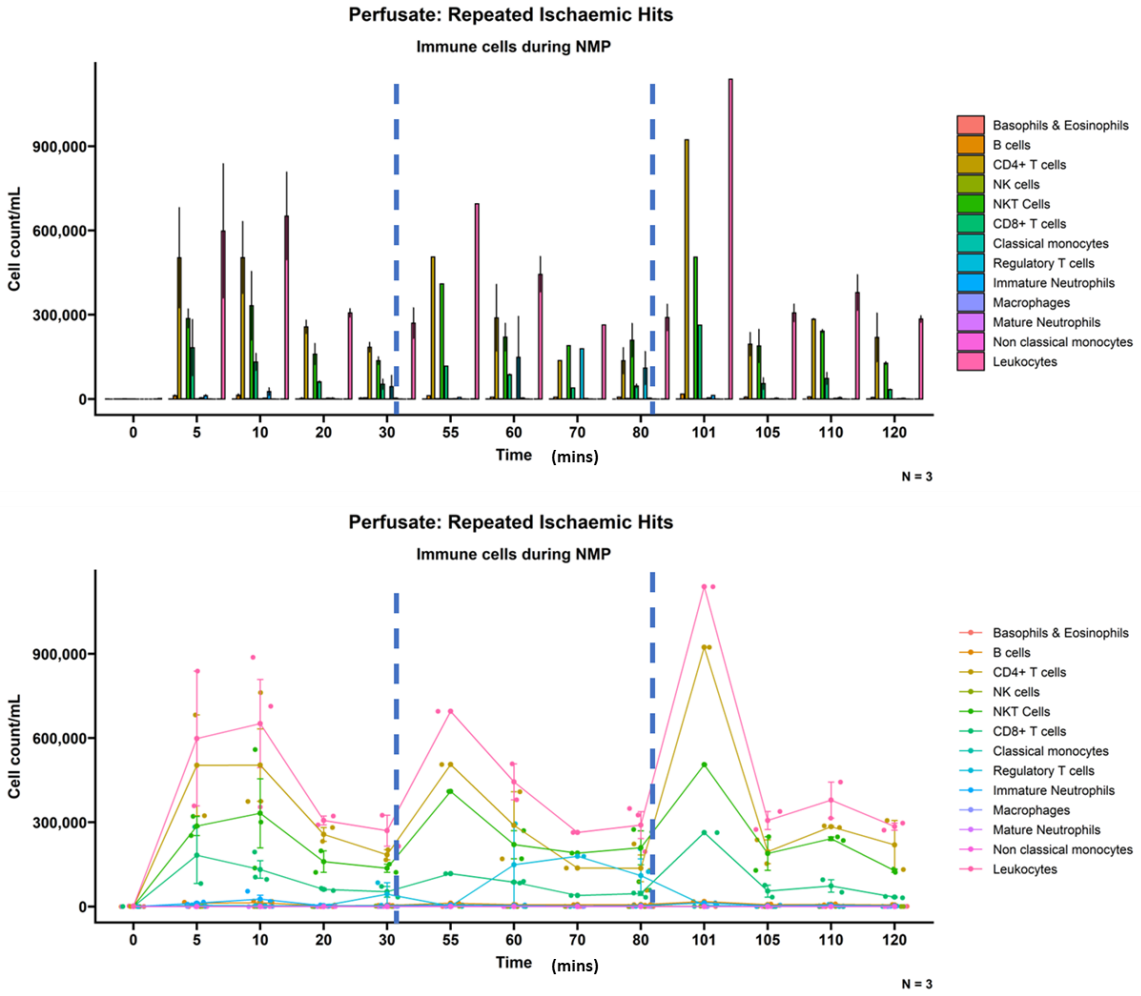


Figure 0.11 Immune cell efflux during pSCS-NMP with repeated ischaemic hits. Livers were reperfused ex situ after a period of SCS and then subjected to repeated 20 minute 'ischaemic hits' during NMP. Serial samples were obtained for analysis of the effluxed cells into the circuit by flow cytometry. Following each ischaemic hit, ex situ reperfusion was characterised by an efflux of predominantly lymphocytes, into the circuit and then a rapid decline over the next 30 minutes of NMP. NMP = normothermic machine perfusion, pSCS = prior static cold storage

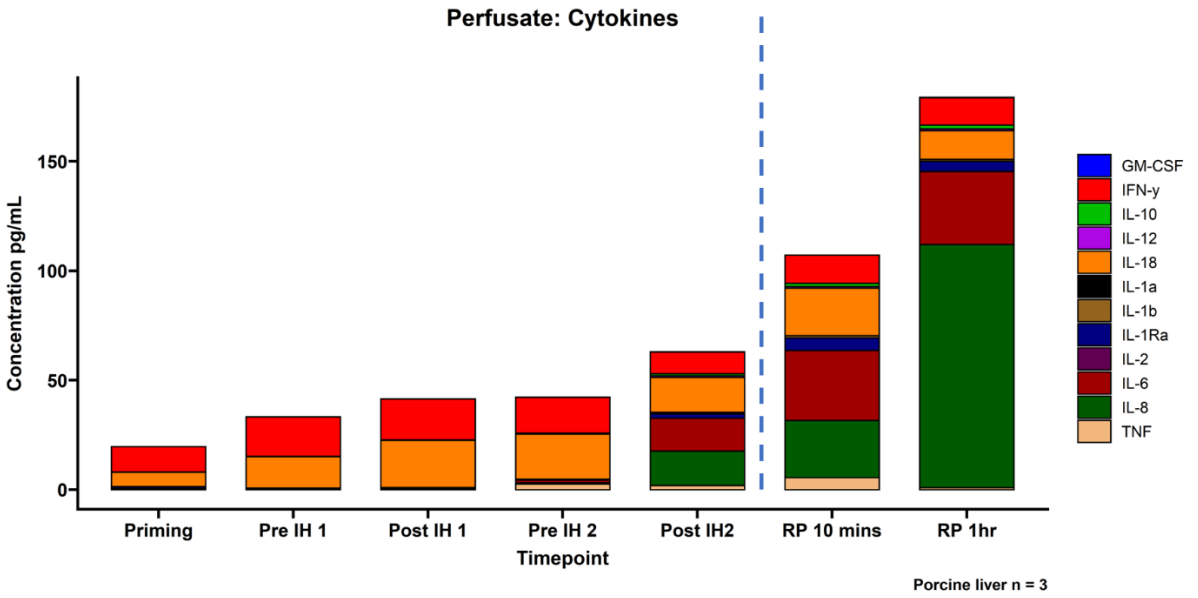


Figure 0.12 Perfusate cytokine levels during pSCS-NMP with repeated ischaemic hits. There is a progressive rise in cytokines detected in the perfusate as NMP progresses with repeated ischaemic insults. During RP there is a further increase in cytokine levels with the highest levels seen at the last timepoint measured. Cytokines were measured using a porcine specific antibody panel on a Luminex device. The most abundant cytokines during NMP are TNF and IL-18, whereas during the RP phase the highest concentration were IL-8, and IL 6. Dashed blue line denotes the end of NMP and beginning of RP during which the liver is flushed with crystalloid and the circuit is reprimed with allogenic whole blood prior to the simulated transplant reperfusion. NMP = normothermic machine perfusion, RP = Reperfusion with allogenic whole blood (simulated transplant model) pSCS = prior static cold storage.

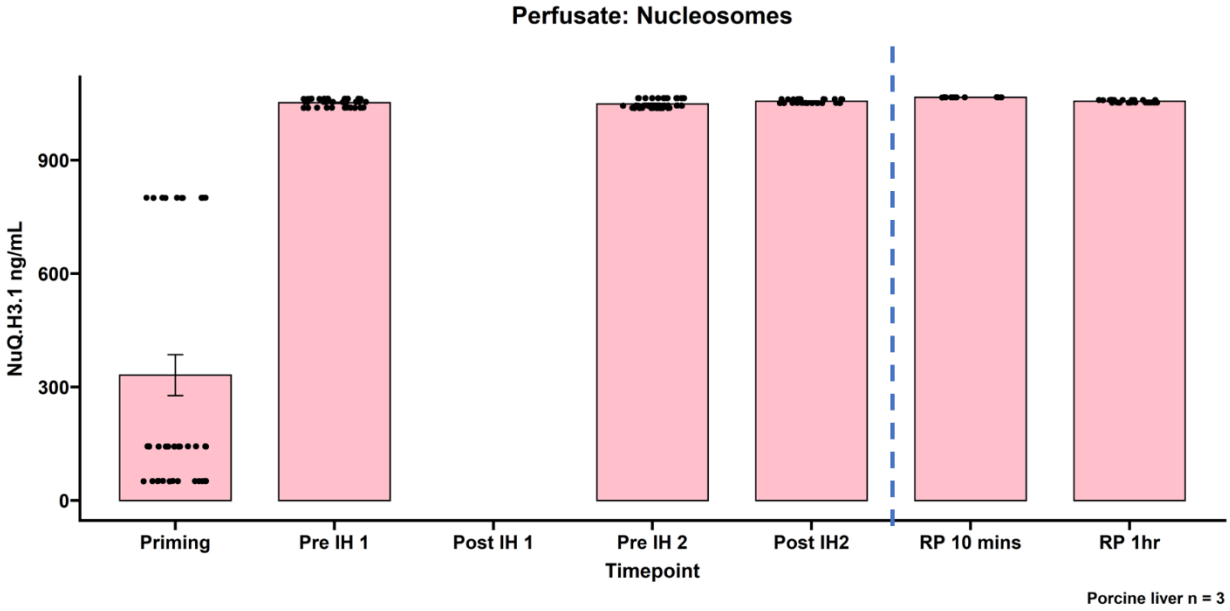


Figure 0.13 Perfusate nucleosomes during pSCS-NMP and RP. Baseline levels of nucleosomes are elevated and rise to very high levels prior to the first ischaemic hit (IH). The levels of nucleosomes remain elevated through NMP and RP. Nucleosomes are measured using the using Nu.Q™ ELISA assay. Dashed blue line denotes the end of NMP and beginning of RP during which the liver is flushed with crystalloid and the circuit is reprimed with allogenic whole blood prior to the simulated transplant reperfusion. NMP = normothermic machine perfusion, RP = Reperfusion with allogenic whole blood (simulated transplant model. (Post IH 1 value is missing - No sample was remaining for the further dilution analysis (unrecordably high on first measurement)).

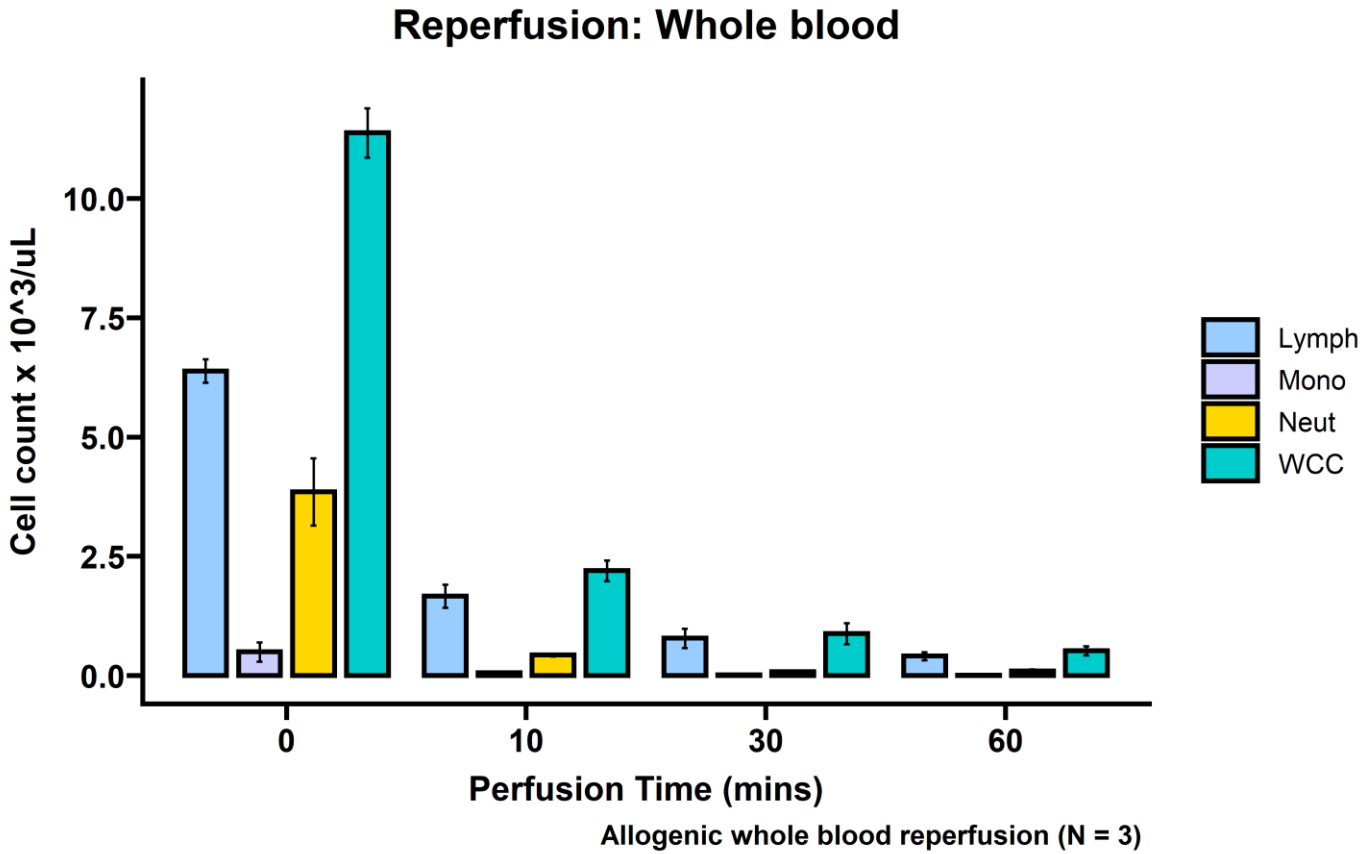


Figure 0.14: Leukocyte influx upon reperfusion. Following allogenic whole blood reperfusion in the simulated transplant, there is a rapid infiltration of the graft with circulating cells. The circulating levels of leukocytes cells remain low thereafter. (lymph = lymphocyte, Mono = monocyte, Neut = neutrophil, WCC = Total white cell count).

1.42 Discussion

In this chapter, we have been able to investigate in our porcine model observations from the human studies of ex situ reperfusion. We have demonstrated that upon reperfusion there is an efflux of immune cells into the perfusate, a progressive rise in cytokine levels, and high levels of nucleosomes detected throughout. We have characterised the previously unexplored very early phases of this phenomenon, specifically identifying the predominance of lymphocytes and identifying a substantial neutrophil efflux that occurs early and is absent at later timepoints. We found that the device and circuit were not responsible for the loss in circulating cells, thereby suggesting that leukocyte trafficking between the liver and perfusate is responsible for the observed changes in perfusate cell numbers during NMP. We were able to show that the sequence of ischaemia and reperfusion, triggers the efflux of cells and is followed by a repatriation of effluxed cells into the graft. This pattern was reproducible with repeated ischaemic insults during NMP. A similar pattern was not seen with cytokine and nucleosomes levels, which rise progressively or increase and remain elevated respectively.

The liver has a highly specialised immune system with organ resident (non-hepatocyte) immune cells that are released from the graft following injury or inflammation (253). In this study we have been able to demonstrate this with a porcine DCD model, supporting the results from the human liver NMP studies presented earlier. In almost every case of liver NMP, there is a preceding period of SCS, which varies from very short periods (approx. 90 minutes in cNMP (140)) to much longer periods as seen in clinical pSCS-NMP of several hours (170,171). These periods of SCS, when prolonged, are associated with greater injury, and this can be detected in the perfusate by evaluation of its cellular composition (253). De Vries and colleagues have shown that structural liver specific cells (hepatocytes) are also released into the perfusate and preservation solution of donor livers

and that they increase with greater cold ischaemic insult(253). They postulate that IRI is associated with the release of proteolytic enzymes that cause extracellular matrix injury and the release of structural and parenchymal cells into the perfusate and preservation solution.

More recently, Lee & colleagues (167) conducted paired sampling of tissue and perfusate during NMP of declined for transplant liver grafts in the United States, evaluating the immune cell compositions through perfusion. They found that predominant cell type present in both tissue and perfusate to be neutrophils, which decreased in frequency over their evaluation period from 55.6% of all detected cells at the start, to 36.4% by six hours. They also found an increase in eosinophils detected, rising from 7.7% to 32.6%, with B-cells also increasing significantly from 1.5% to 6.0% over the six hours. Other cell types, particularly of lymphoid lineage remained largely stable through NMP including Tregs which had previously been reported to increase in proportion to cytotoxic T- cells during liver NMP (163).

In our study, we found a low proportion of neutrophils present in the perfusate. This was true across all perfusions, measured by Sysmex clinical instrument and traditional cell surface antibody labelling and flow cytometry, performed in two different labs, in a blinded fashion. Furthermore, our data corroborated the human data observed in our previous work (with n=18) clinical liver perfusions, on a highly reliable device and with a circuit we have shown to not impact the cellular composition of perfusate. The observed decrease in tissue and perfusate neutrophils was explored by Lee et al, with the NETosis, degranulation and release of NETS into perfusate alluded to but not explicitly mentioned as an explanation for this decrease in the detectable cells. This suggestion may be more in keeping with our Nucleosome findings, where we hypothesis that NETosis is an important source of nuclear DAMPs, albeit our NET-specific assay (NuCitH8) demonstrated that the majority of extracellular chromatin detected in the perfusate was unlikely to be of NET origin (See Chapter 3:Ex situ reperfusion).

It has previously been demonstrated that reperfusion in the recipient is associated with a rapid infiltration of leukocytes which play an important role in immune priming and graft rejection (53). In this study, we found that circulating leukocytes present in the perfusate prior to [simulated] transplant reperfusion, rapidly infiltrated the graft upon reperfusion (**Figure 0.14**). The infiltration of grafts with recipient leukocytes, is well recognised in transplantation (254), with the magnitude of this infiltration associated with post-transplant outcomes and used in histological evaluation of post-reperfusion biopsies to determine the severity of IRI (255,256). This is important, as although donor-derived cells may efflux into the perfusate, they may also represent a small proportion of the overall pool of immunologically significant cells within the graft. Moreover, upon reperfusion in the recipient where a large number of recipient cells will infiltrate the graft, the significance of returned cells previously effluxed during NMP is unclear. That said, donor-derived effluxed cells within the recipient following transplantation (i.e. in situ) have an important role in direct allorecognition and downstream adaptive immune responses after transplantation (257,258) and therefore cannot be dismissed.

We found that our previous estimations of the number cells effluxed into the perfusate, based on the initial human liver perfusion immunological studies (see Chapter 3: Ex situ reperfusion) and therefore subsequently returning to the liver over time, are likely to be substantial underestimates. As we found that most of the trafficking between the liver and perfusate occurs within the first few minutes of ex situ reperfusion, the peak efflux of cells is easily missed with any delay in sampling the perfusate. As a consequence, more cells than previously thought are exposed to the inflammatory circulating perfusate upon reperfusion prior to trafficking back to the liver. This deeper understanding of the early events occurring following ex situ reperfusion provides novel insight into molecular and immunological events occurring and how they may affect the graft microenvironment during NMP. Further work is required to fully appreciate the extent to which

transient exposure to an inflammatory perfusate alters or impacts the phenotype of lymphocytes trafficking back to the liver. Taken together, we have demonstrated that our porcine model is a valid model for ERI, closely resembling the human NMP findings and that it can facilitate the investigation of specific aspects of ex situ preservation.

We found that cytokines are present at high concentrations during porcine liver perfusion. This matched studies in other organs, where cytokine absorption is being investigated to mitigate the effects of high concentrations of pro-inflammatory cytokines (252,259,260). Interestingly, the most abundant cytokine was IL 1Ra, an anti-inflammatory cytokine. This was unexpected based on our previous human NMP observations, but does fit well with the proposed underlying mechanism of NMP previously reported by Jassem et al (261) where NMP inhibits pro-inflammatory signalling in comparison to SCS. Importantly, the observed cytokine profile suggests that not all cytokines are having a negative impact and they may have an important contributory role in the underlying mechanism of NMP (261).

Transplant reperfusion in the recipient typically occurs after a second ischaemic period (during anastomosis) in clinical liver transplantation, regardless of the preservation modality adopted(177). Upon reperfusion, donor derived cells, comprised of immunogenic antigen presenting cells and T lymphocytes, migrate from the graft into the recipient circulation and draining lymphatic system where they initiate immune responses via the direct and semi direct allorecognition pathways (258,262,263). Paradoxically despite this migration of donor origin cells, there is massive graft infiltration after transplantation, with circulating leukocytes rapidly entering the graft at transplant reperfusion as part of the preservation reperfusion injury(177). We propose that our findings support the concept of bidirectional trafficking of cells occurring within the recipient, which influences the immunogenicity of grafts: this phenomenon requires further investigation to determine the extent to which it holds potential as a therapeutic target.

Unexpectedly, we found that when we subjected livers to repeated ischaemic hits, they perfused well with good functional assessment parameters despite sustaining significant (planned) injury on the device. The detected cytokine concentrations were also relatively low, compared to standard NMP without ischaemic hits. Liver preservation and ischaemia reperfusion is associated with parenchymal injury and the release of damage-associated molecular patterns that amplify the inflammatory response and can be directly injurious in themselves (64,70,264). We have previously shown that ERI is associated with an increase in detectable DAMPS, in particular nucleosomes, which have a well-characterised pro-inflammatory effect. In this study, the measured nucleosomes were also slightly lower in the ischaemic hit livers compared to standard NMP livers. These results should be interpreted cautiously as these groups of livers were from separate experiments, with (slightly) different perfusate compositions. However, when we consider the lower pro-inflammatory cytokines **Figure 0.12** and marginally lower nucleosomes **Figure 0.13**, there is a suggestion that ischaemic preconditioning may be occurring with repeated insults, resulting in less PRI than expected. Although this study was not designed with this in mind, this postulation comes on the back of extensive literature and sound mechanistic and scientific reasoning supporting ischaemic preconditioning in liver transplantation (265–267). ERI, therefore, may have a contributory role in the positive effect of NMP itself. In a standard pSCS-NMP clinical perfusion, there is a minimum of two ischaemia reperfusion events, ex situ on the device and the other at reperfusion in the recipient at the time of implantation. The degree to which these events are *positively* contributing to how an organ tolerates transplant reperfusion immunological events and whether it goes on to develop clinical syndromes associated with PRI is unknown and warrants systematic prospective evaluation.

The experiments in this chapter have several limitations. A small number of livers were included in each experimental set, limiting the strength with which we can extrapolate the results. Although we had a broad immune panel for the flow cytometry, we were limited to a subset of leukocytes

with available porcine antibodies and well-characterised identification strategies and did not evaluate the liver-specific structural and parenchymal cells that have well recognised immune function. It is unclear what effect returning cells have on the liver parenchyma, partially because we have no understanding of the phenotypic changes that may occur upon exposure to inflammatory perfusate, but also, we are not clear on what the potential role a (relatively) small proportion of cells can have on a large pool of immune cells resident in the liver. That said, most cell therapy protocols are predicated on the delivery of a similarly small number of cells in relation to the total pool within the graft (58,268,269). The perfusion durations were also relatively short, making the trajectory of different constituents of the perfusate, such as cytokines and DAMPs difficult to interpret. In recent studies of prolonged preservation, using injured (ECD livers), a subset of livers managed to demonstrate reductions in the inflammatory cytokines and DAMPs detectable in the perfusate after 24-48 hours of perfusion (243). Although in this experimental set-up haemodialysis is integrated into the circuit and it is not clear whether DAMPs of different molecular sizes are partially or completely dialysed out or maintained within the perfusate.

This chapter has corroborated findings from human liver NMP, confirming that within a porcine perfusion model, immune cells efflux into the perfusate in response to ischaemia-reperfusion and a large proportion subsequently return to the graft. Conversely, cytokines progressively rise through NMP and nucleosomes levels are elevated throughout. The cells entering the perfusate consisted predominantly of lymphocytes (particularly NKT, CD8 and CD4 cells) and a very early peak of neutrophils not previously appreciated in the human NMP data. The cellular efflux occurs almost immediately upon initiation of NMP, occurs following periods of both cold ischaemia (prior to NMP) and warm ischaemia (occurring during NMP), robustly linking the efflux phenomenon to the sequence of ischaemia-reperfusion, as opposed to a passive washout effect of organ storage. Transplant reperfusion with allogenic whole blood, initiated a massive leukocyte infiltration, occurring as donor derived cells are effluxed further confirming the bidirectional

trafficking capability of immune cells during the transplantation process. Crucially, the inflammatory components of ERI, cytokines and nucleosomes are consistently present at elevated levels through perfusion, presenting themselves as important targets for immunomodulation.

DAMP removal during NMP

"Seek simplicity, but distrust it," Alfred North Whitehead, the mathematician and philosopher, once advised his students.

Siddhartha Mukherjee, The Gene: An Intimate History

In the previous chapter we were able to recapitulate our initial observations from human liver NMP (Chapter 3) of an efflux of immune cells into the perfusate associated with a rising cytokine concentration and high levels of detectable nuclear DAMPS (specifically nucleosomes) in an experimental porcine liver perfusion model. Through this model, we were able to link this phenomenon to ischaemia-reperfusion, demonstrating that reperfusion events on the device can induce these processes and that they are key catalysts to the generation of injurious inflammatory molecules.

1.42.1 DAMPS in reperfusion injury

DAMPs are known propagators of inflammation and play a crucial role in innate immune responses as they are necessary for the rapid recruitment of immune cells to sites of injury and pathogens (61,270). This alarm function, can however, paradoxically cause harm via driving an exaggerated immune response and lead to injury (37,264). This has been well established in many disease processes, including in transplantation where DAMPs play a critical role in the propagation of reperfusion injury and sterile inflammation (48,61,271). Nuclear DAMPs (histone, cfDNA, nucleosomes/NETS and HMGB1) are particularly implicated in sterile inflammation as a result of ischaemia reperfusion in the liver, driving a self-propagating and escalating cycle of inflammation that results in graft injury (64,272) .

1.42.2 NucleoCapture

NucleoCapture technology leverages the characteristics of the naturally occurring H1.3 protein (linker histone), which in normal physiology is a critical protein in the packaging of chromatin within the nucleus by powerfully binding DNA/chromatin. Recombinant H1.3 protein can be conjugated to porous polymer beads in a column, enabling the column to powerfully bind free chromatin present in plasma flowing through it. This unique extracorporeal technology has been developed for use in sepsis where extra cellular chromatin is similarly deleterious, driving systemic inflammatory responses via mechanisms analogous to the ERI we observe in reperfused organs during NMP.

1.42.3 Hypothesis

Central to my thesis, is the hypothesis that circulating nucleic DAMPs (nucleosomes, NETs cfDNA and histone) generated through ex situ reperfusion injury that occurs during NMP, are not only markers of damage, but are in themselves injurious and propagate an inflammatory environment within the perfusate that impairs function and may alter the overall immunogenicity of the graft. Thus, in this chapter, we explore an approach that attempts to mitigate ex situ reperfusion injury by targeting nuclear DAMPs. We aimed to integrate NucleoCapture into the OrganOx metra perfusion circuit, assess the efficiency of the column in removing nuclear DAMPs generated by ex situ reperfusion and determine if this impacts functional parameters during NMP.

1.42.4 Aims:

1. Assess the ability to integrate NucleoCapture technology into the NMP circuit.

2. Determine if nuclear DAMPs can be removed from the perfusate during NMP

3. Establish if the removal of DAMPs reduced the magnitude of ex situ reperfusion injury

1.43 Methods

1.43.1 Animals

Livers were procured, flushed, stored in SCS for transport and established on NMP back at the perfusion laboratory as previously described. Briefly, Sus domesticus pigs (50-70kg) were electrically stunned, exsanguinated, briefly cleaned in a hot water bath at 60°C (140^{0}F) and eviscerated via thoracoabdominal incision. The liver was isolated from the viscera, flushed and preserved in UW® solution for transport. Autologous and third party (allogenic) whole blood was collected into heparinised containers for storage and preparation at the laboratory.

1.43.2 Livers

Liver characteristics, blood group (A/O), macroscopic appearance was recorded; alongside procurement and perfusion times, including WIT, CIT, NMP time and simulated transplant reperfusion time.

1.43.3 Liver NMP

All livers were perfused using the OrganOx metra® device in a standard fashion after backbench preparation, cannulation and further flushing of the liver via the secured cannulae. The perfusate composition was autologous leuko-depleted blood (RBCs + plasma) during the initial NMP phase in all cases. At the end of NMP, all livers were flushed with 2L of crystalloid while the circuit was reprimed with allogenic (3rd party) ABO compatible whole blood, that was used during simulated transplant reperfusions, this resulted in 30 min ischaemic interval between NMP and simulated transplant reperfusion analogous to the anastomotic time in clinical transplantation.

1.43.4 NucleoCapture integration into the circuit.

Integration of NucleoCapture into the circuit is outlined below (**Figure 0.1**). Briefly, a connection was made from the bypass line (in line blood gas sampling line) of the metra circuit using a 3-way tap system allowing a line to go into the Terumo Spectra Optia system for centrifugal plasma separation and subsequent flow through the NucleoCapture column before reconstitution of perfusate and re-entering the circuit back into the bypass line distally. This configuration was additionally advantageous as this permitted pre-and post NucleoCapture sampling of the plasma as well as systemic circulating perfusate sampling.

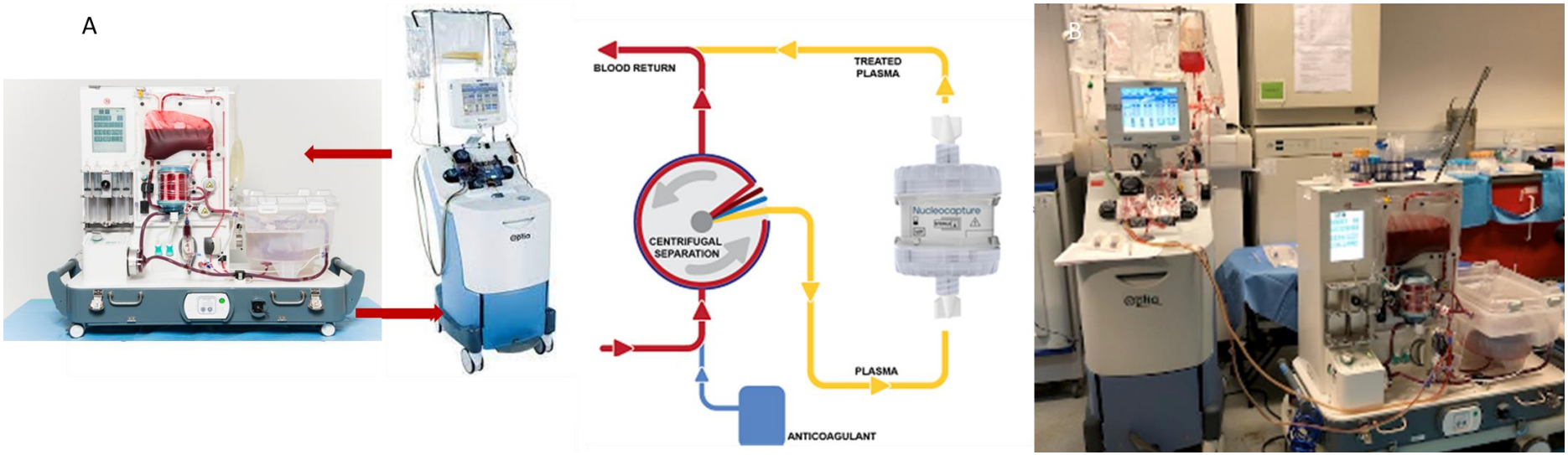


Figure 0.1: **Integration of NucleoCapture into perfusion circuit.** A) The OrganOx metra circuit bypass line is connected to a 3-way tap system allowing a line to go into the Terumo Spectra Optia system (with additional anticoagulation introduced) for centrifugal plasma separation and subsequent flow through the NucleoCapture column before reconstitution of perfusate with plasma added back to the red cell based perfusate and re-entering the circuit back into the bypass line distal to the outflow port. Left: OrganOx metra Device Middle: Terumo Sectra Optia Right: Schematic of flow of perfusate. B) Photograph of set up it in the lab with the Spectra Optia (left) and OrganOx metra(right).

1.43.5 NucleoCapture

NucleoCapture (Santersus AG, Zurich Switzerland) is an adsorbent column containing porous, spherical agarose beads with covalently linked human recombinant N,N-bismethyonyl-histone H1.3 as ligand (via its N-terminus) **Figure 0.2**. The column binds cell free DNA, histones, intact nucleosomes in the plasma due to the high affinity of the analogue of linker histone H1 family protein to DNA.

1.43.6 Porcine pSCS-NMP sampling

Perfusate and bile samples were obtained prior to initiation of NMP (during priming), and then serially at various intervals following initiation of NMP. Samples were collected for analysis clinical biochemistry and point of care testing blood gas analysis as well as for storage and later analysis, as previously described (see General Methods). Liver tissue and bile duct biopsies were collected at various timepoints through preservation and simulated transplant reperfusion and handled as previously described (see General Methods).

1.43.7 Damage Associated Molecular Pattern measurement

Nucleosome levels were measured using Nu.Q™ ELISA assays (Belgian Volition SRL, Isnes, Belgium). Initial analysis using the commercial Nu.Q™ ELISA assay (Belgian Volition SRL, Isnes, Belgium) was performed in-house, but due to the levels being above the standard range for detection of the kit despite attempted dilutions, the samples were sent to Belgium for analysis in the central Volition laboratory. An automated device in the central Volition Laboratories was ultimately used and measured samples in triplicate after multiple dilutions to achieve detectable levels

The total amount of perfusate cell free DNA was quantified using a SYTOX® Green Dye fluorometric assay (Life Technologies, Cheshire, UK). Fluorescence was measured using a

BioTek® Synergy 2 fluorometric plate reader (NorthStar Scientific Ltd, UK) with excitation and emission set at 485 nm and 528 nm respectively. To determine cfDNA concentrations, a λ-DNA standard curve (Fisher Scientific, UK) was used. Extracellular histones were quantified by Western blotting using purified histones as a standard. This analysis was performed by Jeremy Scofield under the supervision of Dr Simon Abrams at the University of Liverpool.

1.43.8 2.4 Statistical analysis:

Descriptive statistics were reported as mean and standard deviation or median and inter quartile range according to normality. A Student t-test or repeated measures ANOVA was used to analyse parametric, continuous variables, and the Mann-Whitney U/Wilcoxon rank sum test was used for nonparametric continuous variables. Fisher's exact test was used for categorical variables and a p-value of 0.05 was considered statistically significant.

1.43.9 Histology:

Liver biopsies were taken and either snap frozen in liquid nitrogen (for storage in -80℃ or placed in formalin for fixation and embedding in paraffin (FFPE). Hematoxylin and eosin (H&E) staining was used for reperfusion injury assessment and periodic acid-Schiff (PAS) stain for assessment of glycogen depletion. All slides were scored/assessed blindly by Professor Alberto Quaglia, Professor of Liver Histopathology at University College London & the Royal Free Hospital NHS Trust

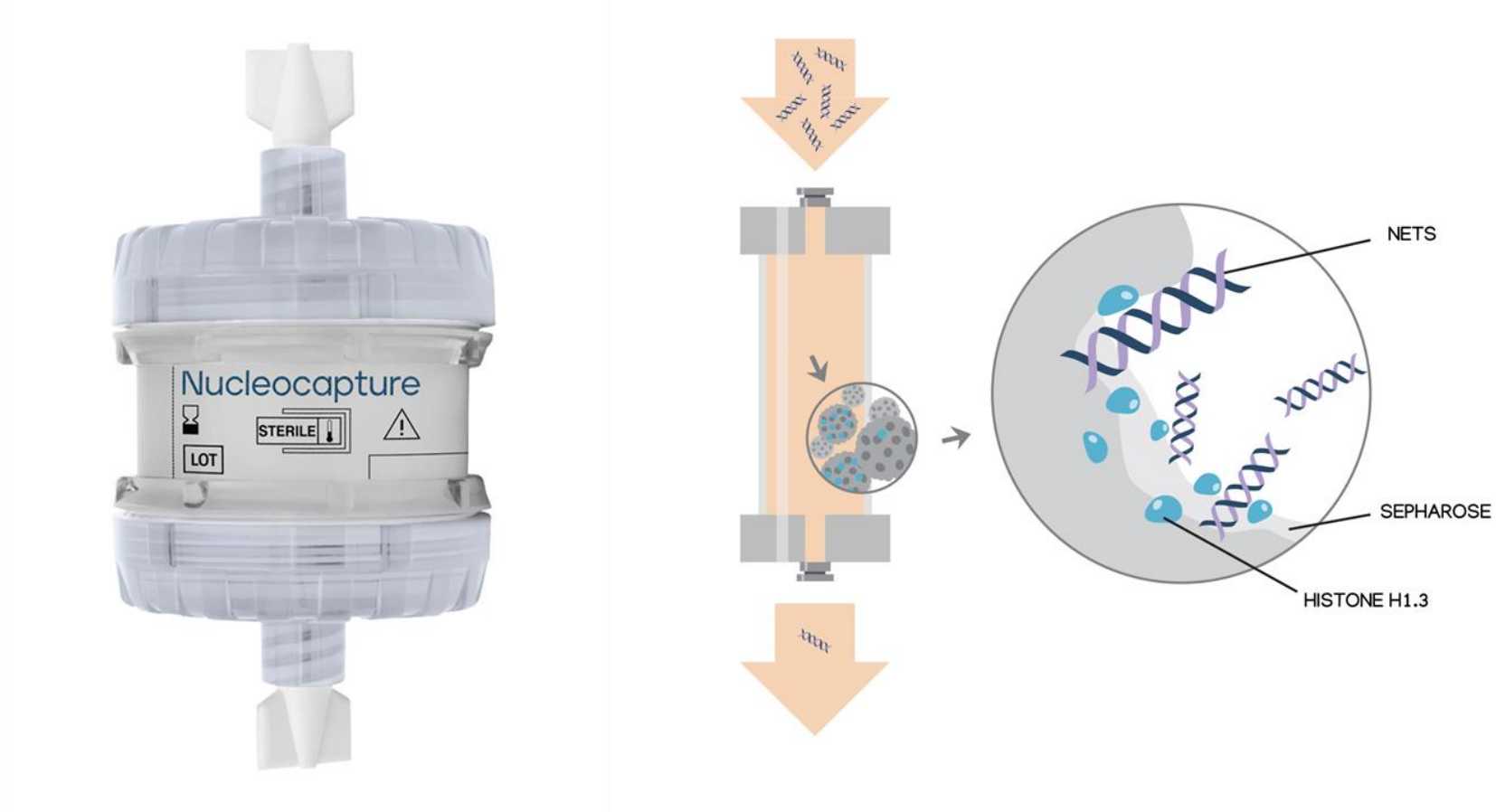


Figure 0.2: The NucleoCapture Column. Biocompatible, highly porous polymer (Sepharose) beads conjugated with human recombinant histone H1.3 protein (Santersus AG, Zurich, Switzerland). Plasma separated from the circulating red blood cell based perfusate by the Terumo Spectra Optia system is passed through the column coming into contact with the H1.3 conjugated beads allowing cell free DNA, histones and nucleosomes to be bound powerfully and removed from the circulation. Left: photograph of column, Right: schematic of column and beads with adherent NETS, cell free DNA and histone. NET = neutrophil extracellular traps.

1.44 Results

Table 0.1: **Preservation characteristics of porcine livers in NucleoCapture experiment**. Warm ischaemia time was considered the time from exsanguination to initiation of cold perfusion. SCS was considered the time from cold perfusion to initiation of NMP, or in the case of the SCS simulated transplant reperfusion (RP). NucleoCapture referred to livers that underwent NucleoCapture treatment during the NMP (but not during RP. SCS = static cold storage, NMP = normothermic machine perfusion.

Group	N (Pigs)	Warm Ischaemia Time (minutes)	Cold Ischaemia Time (minutes)
SCS	3	15 (15-16)	293(265-309)
NMP	6	17 (13-22)	273(260-327)
NucleoCapture	3	16 (15-18)	272(249-264)

1.44.1 NucleoCapture can be successfully integrated into the liver perfusion circuit.

All livers were procured, prepared and perfused without complication. The warm and cold ischaemic times were similar across groups (**Table 0.1**). All livers had acceptable perfusion parameters in both phases of machine perfusion, produced bile and were macroscopically homogenously perfused. Gas exchange (paO2 and paCO2), perfusate temperature and pH were stable throughout all perfusions, with the pH maintained in range with sodium bicarbonate as appropriate. The connection of the Terumo Optia and NucleoCapture column had no discernible effect on perfusion parameters or the performance of the OrganOx device. The NucleoCapture group consistently demonstrated higher flows, when compared to NMP alone. (Figure 0.3)

1.44.2 NucleoCapture depletes circulating cell free nucleosomes/NETS, histones and cfDNA during NMP

We found that The column efficiently at scavenged nucleosomes, cfDNA and free histone, with significant reductions across the column cfDNA p=0.0087(1hr) Histone p = 0.0087(1hr) Nucleosomes p=0.033(2hr). This resulted in a reduction of circulating DAMPs within the perfusate over the period of NMP when compared to NMP alone (Figure 0.5). In livers that underwent NMP without NucleoCapture, levels of circulating nucleosomes rise progressively, yet with the column in circuit, we saw a plateauing of the levels at 1 hour, remaining at these levels for the rest of the 6-hour perfusion (Figure 0.5). Free histones and cfDNA followed a very similar course within the groups with the livers perfused with NucleoCapture showing much lower levels of cfDNA and histone through perfusion (Figure 0.5).

1.44.3 Reperfusion is associated with an increase in cell free nucleosomes/NETS, histones and cfDNA

Upon reperfusion with allogenic whole blood in the simulated transplant model, where neither group is subjected to NucleoCapture, all livers exhibited a progressive increase in nucleosomes/NETs, cfDNA and histone. The exception was cfDNA in NucleoCapture livers, where the levels peak early and then fall by the final 6-hour timepoint. (Figure 0.6)

1.44.4 NucleoCapture significantly improves ex situ graft function during NMP

NucleoCapture significantly improved lactate clearance (0.5hrs p= 0.033 1hr p=0.013, 2hr p=0.043). These livers had significantly lower levels of perfusate lactate, had improved perfusion flows (Figure 0.3) and lower injury markers (Figure 0.9) when compared with NMP alone. Although all livers functioned during NMP and subsequent reperfusion, the magnitude of ex situ reperfusion injury was substantially lower in the NucleoCapture livers, with this effect also evident

in the allogenic reperfusion phase. Histological assessment of the livers revealed less neutrophil infiltration in the NucleoCapture livers but overall, very similar histology, with any form of NMP (i.e NMP with or without NucleoCapture) was associated with significant glycogen depletion upon whole blood allogenic reperfusion (**Figure 0.10**)

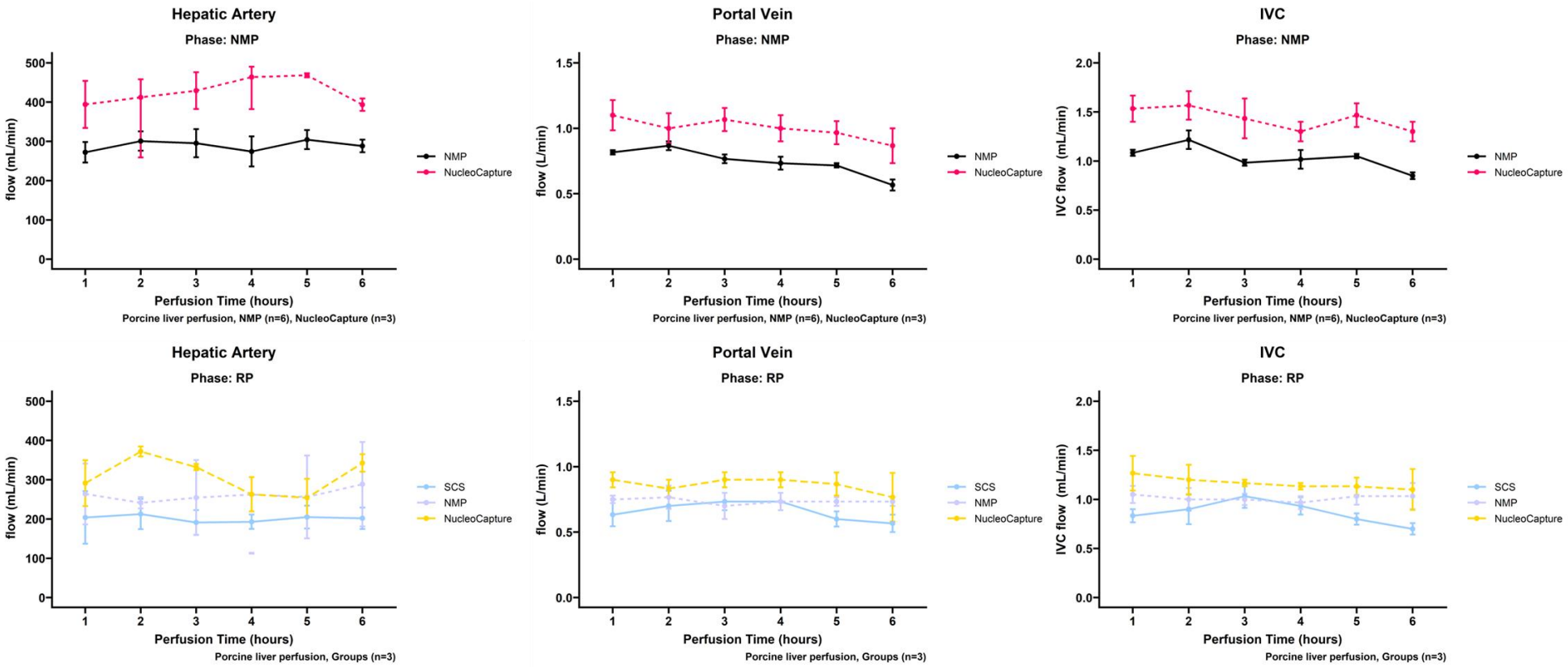


Figure 0.3: Perfusion parameters during NMP (Top) and allogenic whole blood reperfusion (RP)(Bottom). Top row: Haemodynamic data from the OrganOx metra during initial perfusion comparing the NucleoCapture livers (n=3) to standard NMP livers (n=6). The NucleoCapture consistently had higher flows through this phase of perfusion. Bottom row: Haemodynamic data from the OrganOx metra during the allogenic whole blood reperfusion phase (simulated transplant). All groups had similar perfusion characteristics during this phase. NMP = normothermic machine perfusion, SCS = Static cold storage, RP = reperfusion, IVC = inferior vena cava.

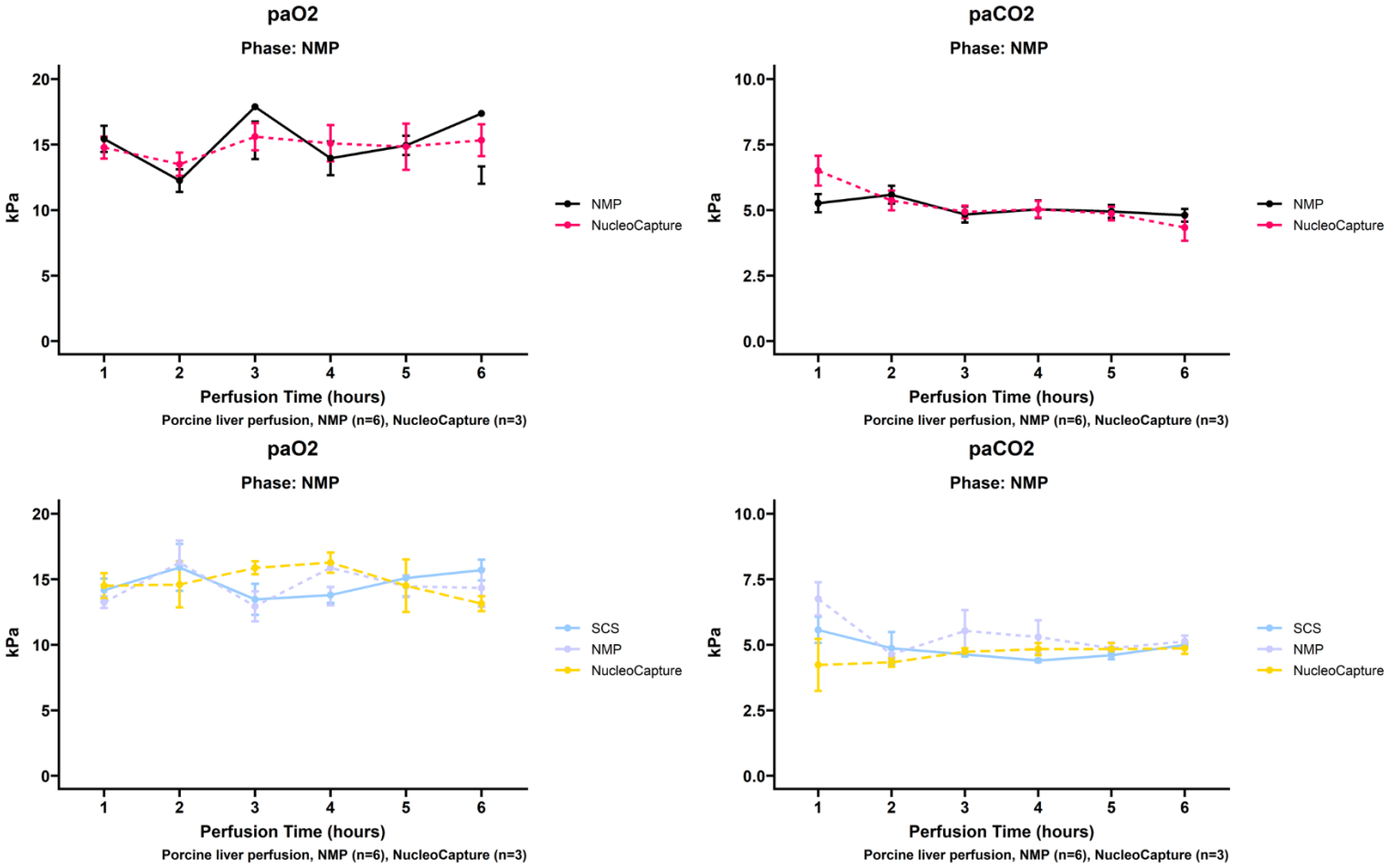


Figure 0.4: **Gas exchange during perfusion: Perfusate blood gas analysis during perfusion**. Top: Gas exchange during NMP. Bottom: Gas exchange during RP. There were no differences in gas exchange during both periods of perfusion, all groups had similar perfusion characteristics during both phases. NMP = normothermic machine perfusion, SCS = Static cold storage, RP = reperfusion, IVC = inferior vena cava.

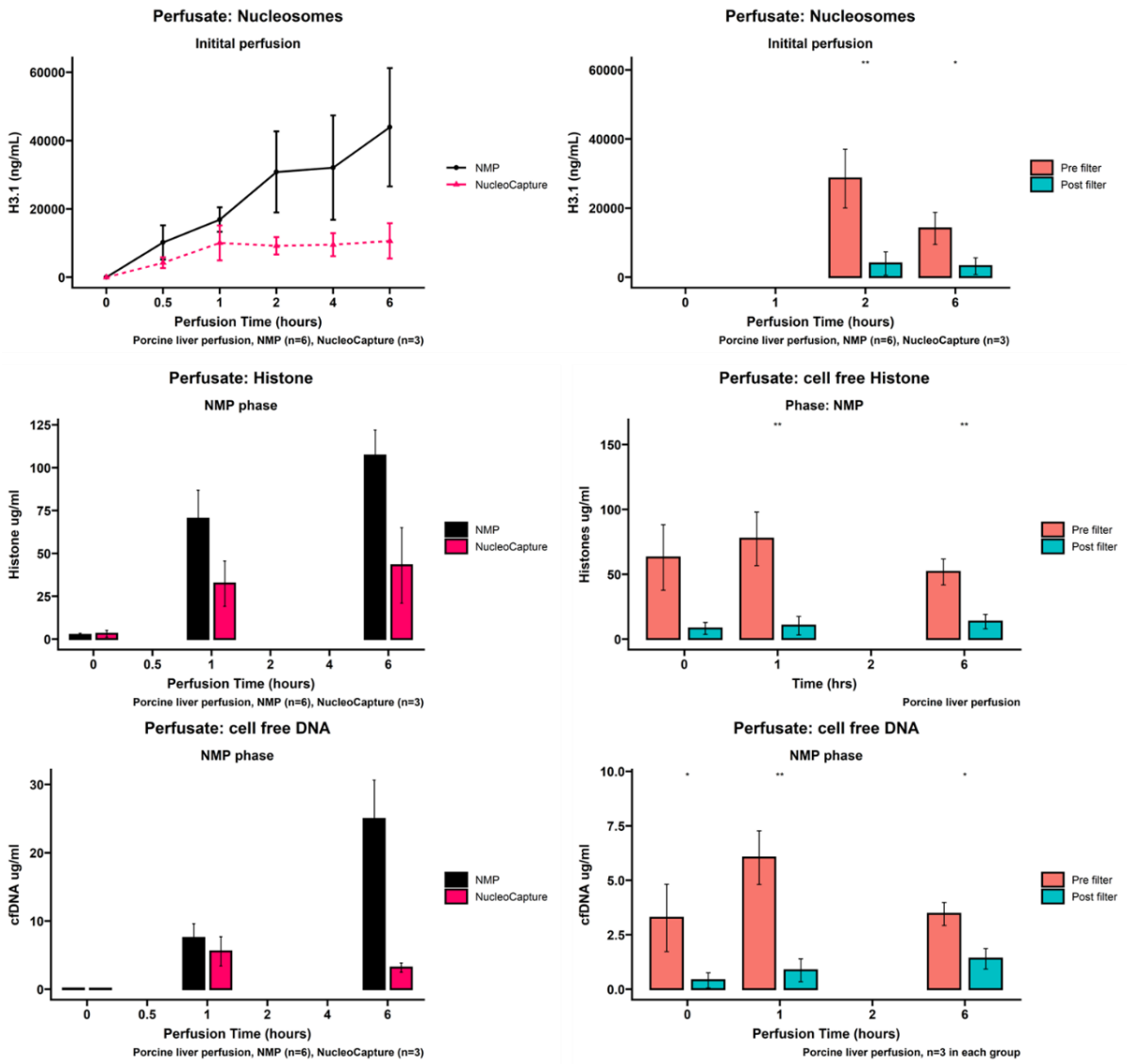
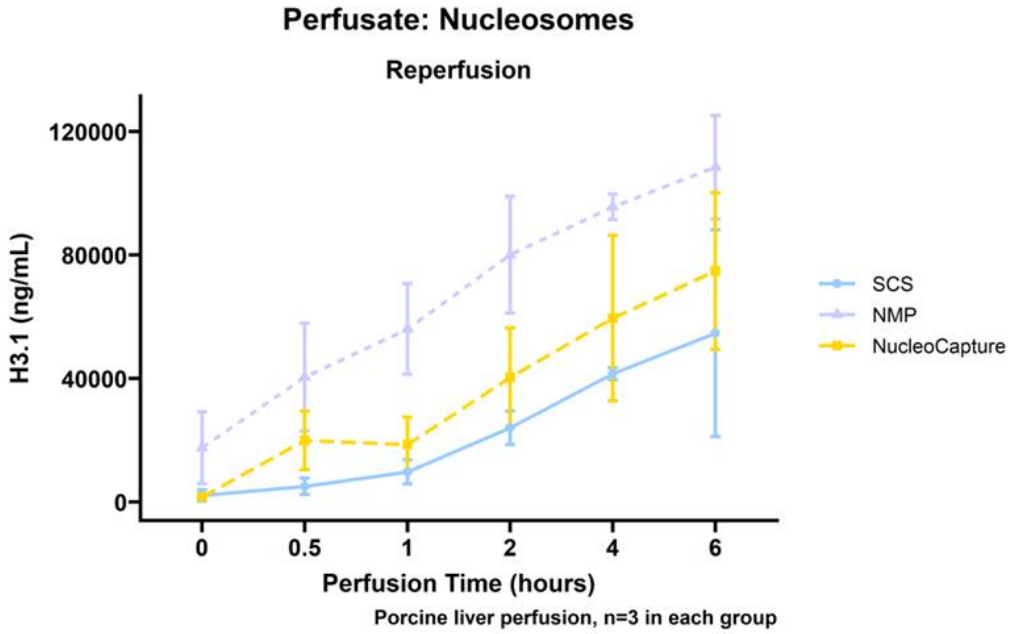
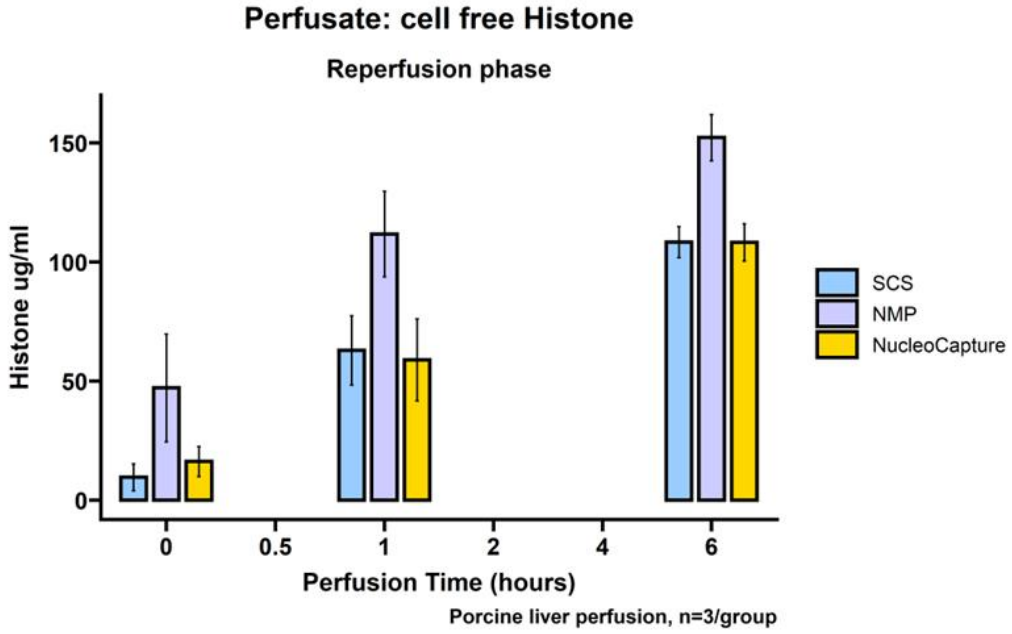


Figure 0.5: NucleoCapture during NMP depletes Nucleosomes, Histone and cfDNA. Top row: NMP alone is associated with a progressive rose in nucleosomes, yet with NucleoCapture we observe a plateauing of nucleosomes. Samples taken pre and post filter confirm NucleoCapture is removing nucleosomes, with significant reductions across the filter. Middle row: Cell free histone follows a similar course with rises through perfusion in the NMP group yet in the NucleoCapture group rises to lesser extent and efficient clearance by the column/filter. Bottom row: cell free DNA also rises during NMP in line with nucleosomes and histone, but actually falls when NucleoCapture is adopted, with significant reductions across the column at every timepoint. Nucleosomes/NETs are detected using the Nu.Q assay (Volition, Belgium), Histone was quantified using western blots (H3 antibody) and cfDNA quantified using a SYTOX® Green Dye fluorometric assay. NMP = normorthermic machine perfusion, cfDNA = cell free DNA.


Perfusate: Nucleosomes
Reperfusion
H3.1 (ng/mL)
120000
80000
40000
0
0
0.5
1
2
4
6
Perfusion Time (hours)
SCS
NMP
NucleoCapture
Porcine liver perfusion, n=3 in each group


Perfusate: cell free Histone
Reperfusion phase
Histone ug/ml
150
100
50
0
0
0.5
1
2
4
6
Perfusion Time (hours)
SCS
NMP
NucleoCapture
Porcine liver perfusion, n=3/group

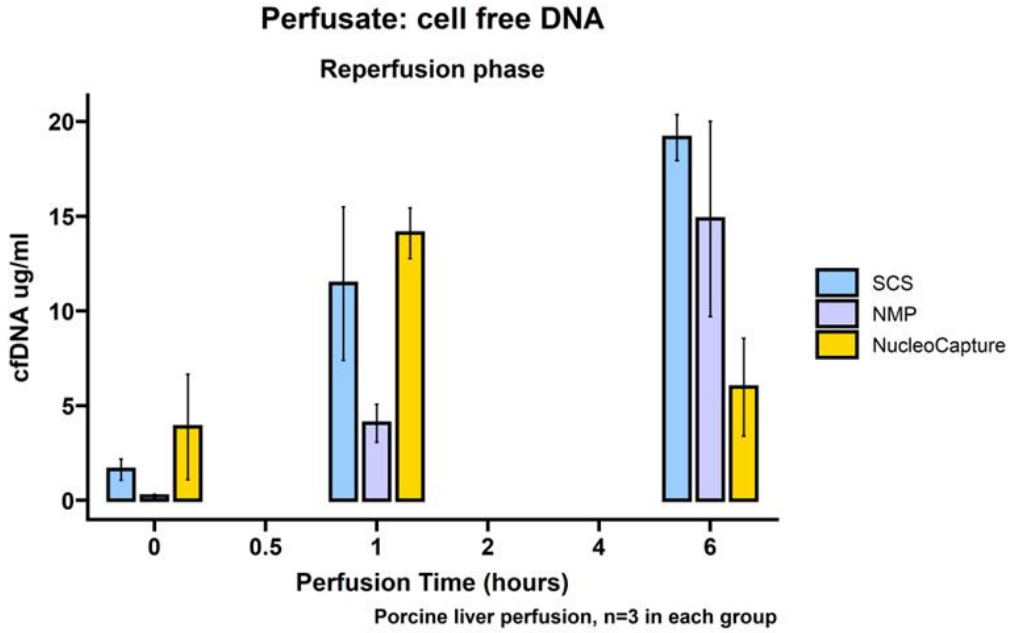
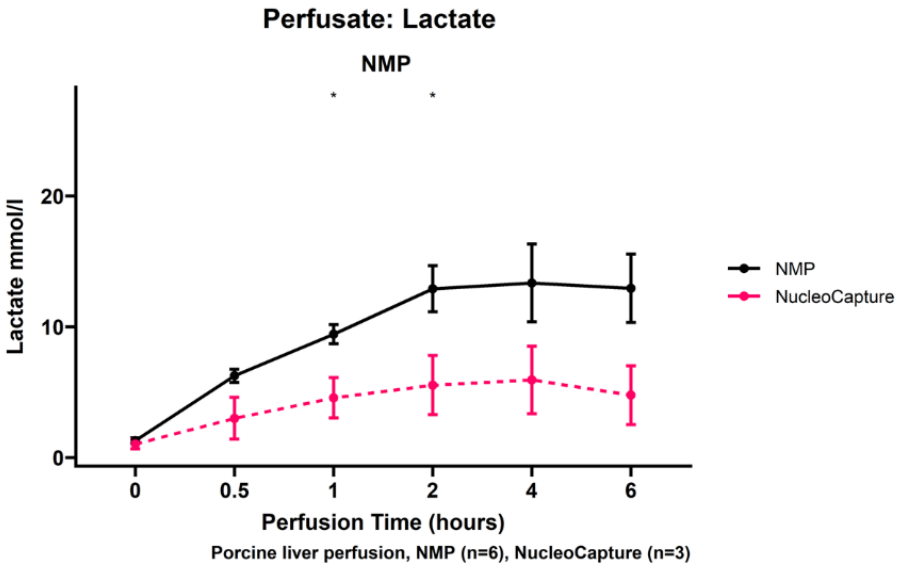
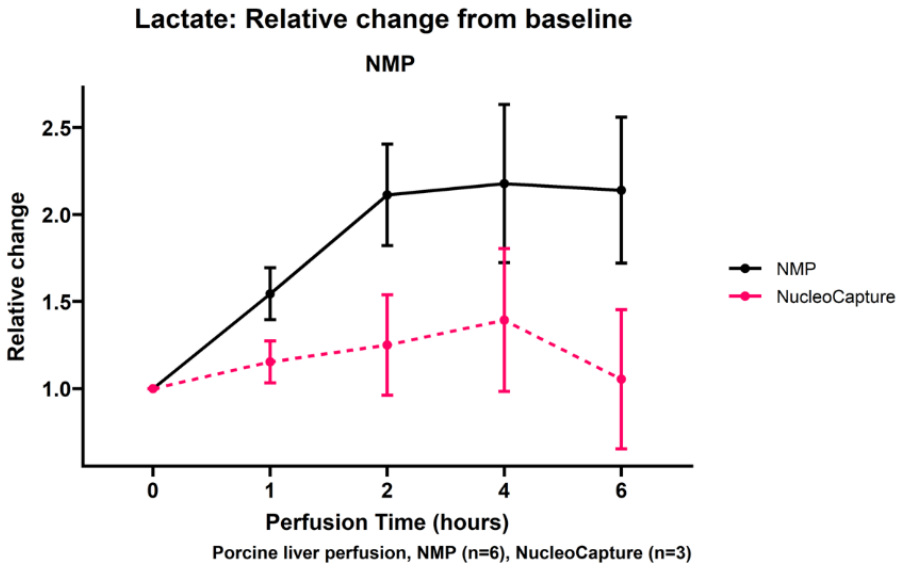
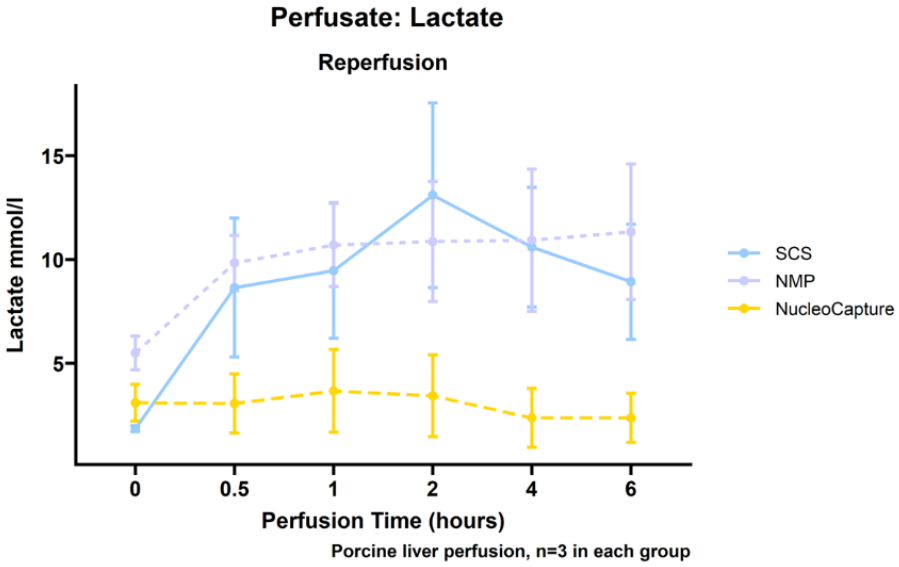

Perfusate: cell free DNA
Reperfusion phase
cfDNA ug/ml
20
15
10
5
0
0
0.5
1
2
4
6
Perfusion Time (hours)
SCS
NMP
NucleoCapture
Porcine liver perfusion, n=3 in each group

Figure 0.6: Nucleosomes/NETS, Histones and cell free DNA in the perfusate upon reperfusion. Top: All livers (SCS, NMP and those that had prior NucleoCapture during NMP) have a progressive rise in nucleosomes detected in the perfusate. Middle: Histones follow a similar course with progressive increase over the reperfusion phase. Bottom: cell free DNA peaks earlier and then falls by the final timepoint in the livers that had prior NucleoCapture during NMP, yet the other groups have rising levels of cfDNA through perfusion. Nucleosomes/NETs are detected using the Nu.Q assay (Volition, Belgium), Histone was quantified using western blots (H3 antibody) and cfDNA quantified using a SYTOX® Green Dye fluorometric assay.


Perfusate: Lactate
NMP
Lactate mmol/l
20
10
0
0
0.5
1
2
4
6
Perfusion Time (hours)
Porcine liver perfusion, NMP (n=6), NucleoCapture (n=3)
NMP
NucleoCapture


Lactate: Relative change from baseline
NMP
Relative change
2.5
2.0
1.5
1.0
0
1
2
4
6
Perfusion Time (hours)
Porcine liver perfusion, NMP (n=6), NucleoCapture (n=3)
NMP
NucleoCapture


Perfusate: Lactate
Reperfusion
Lactate mmol/l
15
10
5
0
0.5
1
2
4
6
Perfusion Time (hours)
Porcine liver perfusion, n=3 in each group
SCS
NMP
NucleoCapture

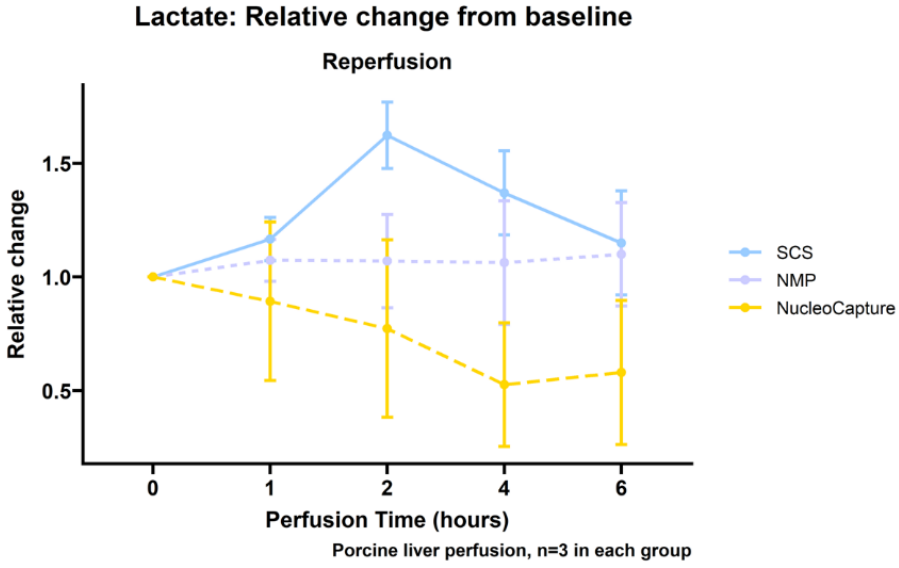

Lactate: Relative change from baseline
Reperfusion
Relative change
1.5
1.0
0.5
0
1
2
4
6
Perfusion Time (hours)
Porcine liver perfusion, n=3 in each group
SCS
NMP
NucleoCapture

Figure 0.7: Ex situ liver function (lactate clearance) during NMP and Reperfusion. Top: Perfusate lactate during NMP. NucleoCapture is associate with significantly lower lactate levels at 1 and 2 hours and with lower levels evident early after reperfusion. Top right: The relative change in lactate, taking the lactate level within the first 30 mins as a baseline, Bottom: Perfusate lactate during RP. NucleoCapture is with low levels of lactate throughout reperfusion. Bottom right: The relative change in lactate, taking the lactate level within the first 30 mins as a baseline. NMP = normothermic machine perfusion (n=6), NucleoCapture = NMP with the NucleoCapture column in circuit (n = 3) , SCS = static cold storage, RP = Reperfusion (allogenic whole blood reperfusion)

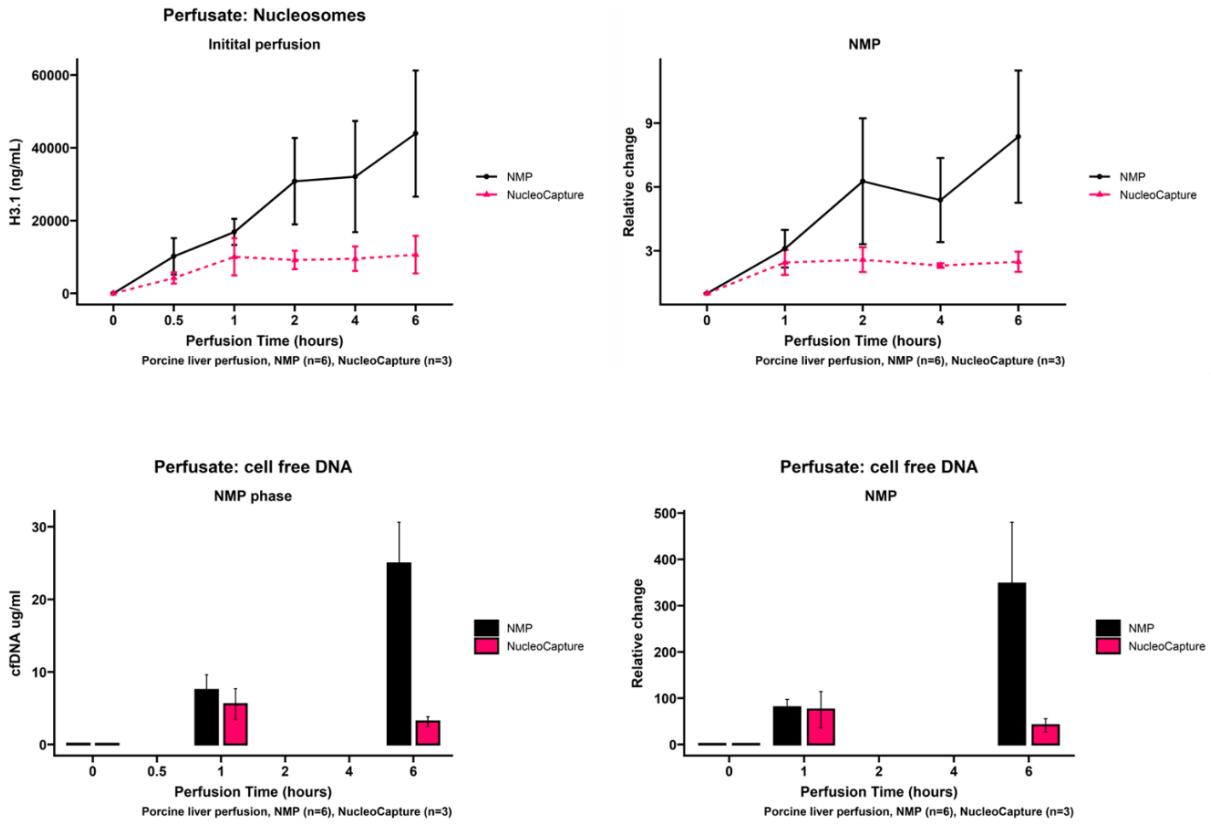


Figure 0.8: Ex situ reperfusion: Nucleosomes/NETs and cfDNA. Left: absolute levels of Nucleosomes and cfDNA following reperfusion of the donor liver on the OrganOx metra. Right: Relative change in nucleosomes/NETs following reperfusion of the donor liver on the OrganOx metra. Nucleosomes/NETs are detected using the Nu.Q assay (Volition, Belgium) and cfDNA quantified using a SYTOX® Green Dye fluorometric assay. NMP = normothermic machine perfusion (n=6), NucleoCapture = NMP with the NucleoCapture column in circuit (n = 3) , SCS = static cold storage, RP = Reperfusion (allogenic whole blood reperfusion)

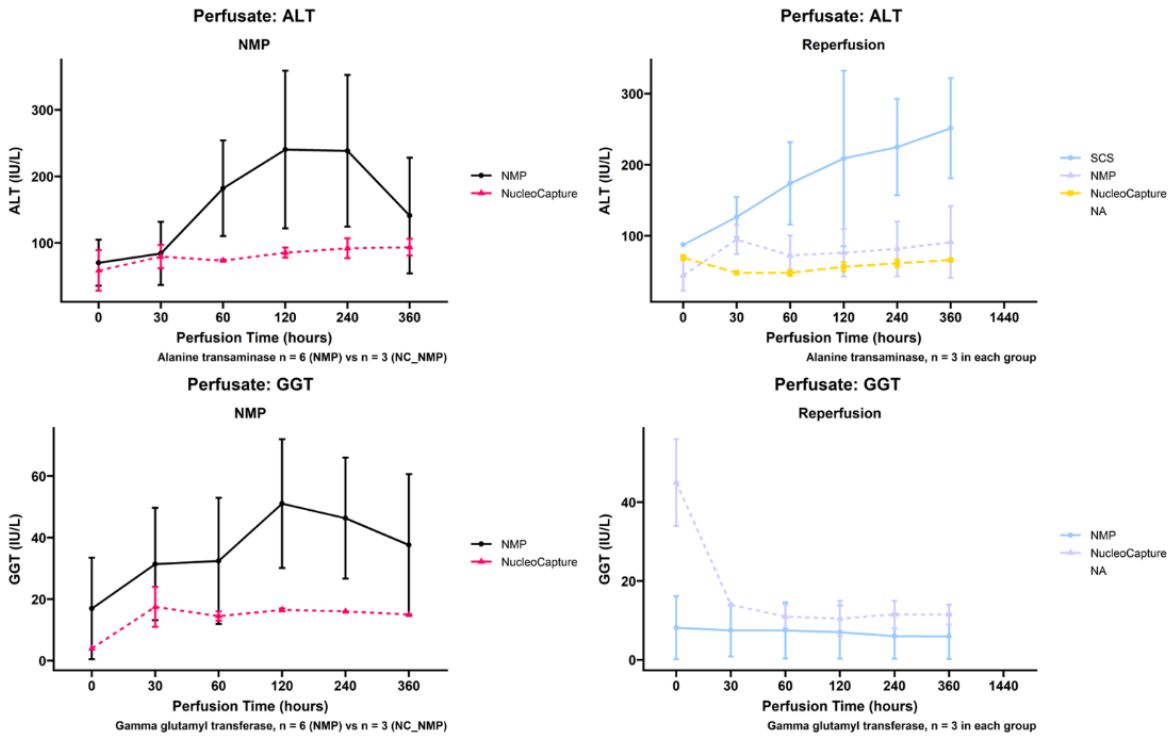


Figure 0.9: Hepatocellular injury markers: ALT and GGT measurements from the perfusate during NMP and RP. Top row: ALT levels in the perfusate during NMP and then RP. Bottom: GGT during perfusion and then subsequent reperfusion. GGT = Gamma-glutamyl transferase. ALT = Alanine transaminase. NucleoCapture. NMP = normothermic machine perfusion, SCS = static cold storage.

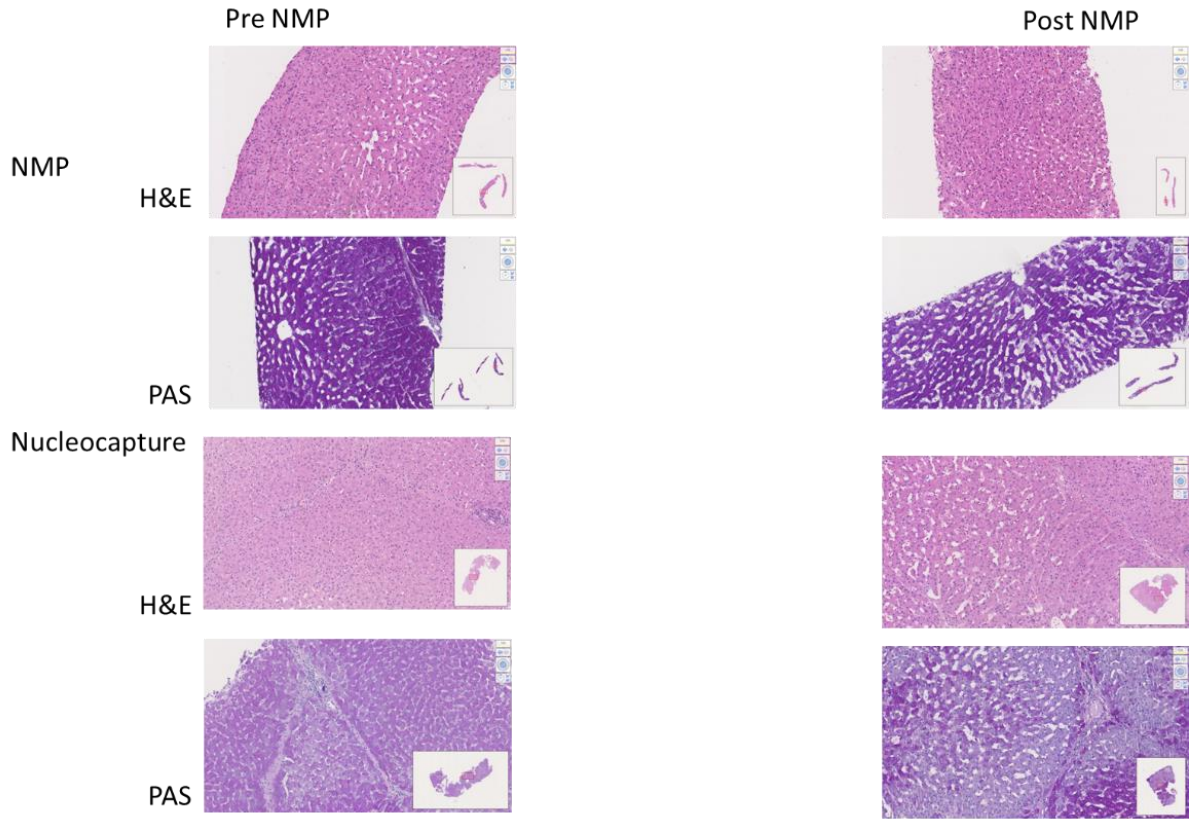


Figure 0.10 Histological assessment. Serial biopsies taken prior to NMP with or without NucleoCapture, post NMP and following reperfusion (at 1 hour). H&E confirmed that all livers were healthy at baseline but upon reperfusion injury markers were more evident. Glycogen depletion occurred in all livers perfused with NMP regardless of NucleoCapture. This was more marked at the reperfusion phase. NMP = normothermic machine perfusion.

1.45 Discussion

Throughout this thesis we have shown that NMP generates an exaggerated ex situ inflammatory response characterised by the efflux of immune cells, cytokines and nuclear DAMPs into the circulating perfusate causing injury to grafts and impairing ex situ function. In this Chapter we have shown that removal of inflammatory nuclear DAMPs by NucleoCapture during NMP improves ex situ function of injured DCD porcine grafts. We found improved lactate clearance, superior perfusion parameters and lower damage markers in livers where NucleoCapture was adopted.

Integration of NucleoCapture into the NMP circuit proved to be feasible and reproducible, with no impact on the flows/haemodynamic performance of NMP (**Figure 0.3**). The NucleoCapture column was effective and efficient across all the targeted/measured molecules including histones, cell free DNA and intact nucleosomes (**Figure 0.5**). This finding is critical as it underpins the proposed mechanism by which improved function on the device is achieved. The H3.1 protein that is conjugated to the Sepharose beads within the column has broad efficacy when it comes to removal of chromatin, this was evident in our findings.

Although cell free DNA, nucleosomes and histones are crucial DAMPS, High-mobility group box 1 protein (HMGB-1) is non histone nuclear DAMP that has been well established as a very important and potent propagator of inflammation and injury (68,273,274). (275) Beetz et al have recently shown that HMGB-1 and IL-18 are increased within the perfusate when livers are preserved with SCS for long periods prior to NMP, suggesting they are markers of cellular injury occurring during NMP. HMGB-1 may, therefore, be an important DAMP to measure and assess in this context, but was not included in our initial analysis. Although HMGB-1 is well established as a key DAMP implicated in liver disease and IRI (276,277), the measurement and interpretation is not straightforward. Significant proportions of HMGB-1 is bound (to IgG complexes, lipids or

DNA/chromatin fragments) and not detected without sample optimisation, furthermore, methodologies to characterise the specific iso-forms of HMGB1 present are expensive and complex but necessary to determine the inflammatory potential of HMGB-1 being detected (276). Moreover, HMGB1-nuclesome complexes, for example, are highly inflammatory, able to stimulate innate responses by stimulating pro-inflammatory cytokine release from T-cells, as well as adaptive mechanisms as they are able to mature dendritic cells (DCs) (in vitro) altering DC phenotypes to become more immunogenic antigen presenting cells (278,279). Thus, an appreciation of how much HMGB-1 is bound in nucleosome complexes or free HMBG1 and whether either or both are removed with NucleoCapture is an important and urgent consideration for future work.

We found that livers that underwent NMP preservation with NucleoCapture performed significantly better than control livers that had NMP alone. The perfusion parameters, such as vascular flow rates were improved, the clearance of lactate was superior and hepatocellular damage makers were reduced. As NMP remains an emerging technology, albeit rapidly being adopted, the majority of studies have historically focused on either demonstrating that NMP in and of itself is an effective preservation strategy/intervention with benefits in terms of reduced IRI or, more recently, focused on its potential as a platform technology for therapeutics(128,280,281). Very few investigators have sought to characterise the molecular and immunological events occurring *during* NMP (167) and even fewer have attempted to modulate these processes to improve graft outcomes ((241,242)).

The Toronto group (282) adapted their perfusion circuit and perfusate composition specifically seeking to address the inflammatory nature of perfusate. They identified specific aspects of whole blood that, in the literature, were associated with mechanisms of IRI: leukocytes, platelets and plasma cytokines. They employed inline leukocyte filtration (Pall LeukoGuard®, Cornwall, UK), dialysis filtration and used washed erythrocytes as the oxygen carrier suspended in 3-L Steen solution (XVIVO Perfusion, INC., Goteborg, Sweden) aiming for a 10-12% haematocrit and

avoiding the use of "cytokine rich plasma". This was done with the intention of avoiding the poorer IRI-related outcomes that had been observed in DCD pig liver models where SCS proceeded NMP and whole blood-based machine perfusion preservation was used. Knaak et al's adaptations and observations are important, however this group never directly quantified the cell free nucleic DAMP burden or characterised its composition either prior to the change in perfusate, or after adopting their modified circuit. This limits the scope of the conclusions we can reach regarding the impact of the circuit modifications instituted, particularly in relation to DAMPs and their effect on graft performance. More recently, in a study by (283) Obara et al, the authors compared the performance of DCD pig livers undergoing sub-normothermic machine perfusion (SNMP) with or without early perfusate exchange to attempt to remove the inflammatory cytokines and "humoral enzymes" immediately generated upon reperfusion (within 5 mins) that may drive IRI. In this case, aside from the fact that autologous whole blood was used, limiting the applicability for the reasons originally identified by the Toronto group (282) mentioned above, perfusate exchange has a significant dilutional effect, impacting the estimation of circulating damage *markers* directly. That said, a reduction in inflammatory *molecules* is also likely to have occurred due to loss of those generated upon reperfusion in the exchange process and the dilution of those present in the circuit, both of which may have contributed to the favourable outcomes. Interpretation of function based on damage marker concentration in perfusate is inherently confounded. It is impossible to uncouple improved damage marker concentration data obtained from a washout effect of diluting the perfusate, from a true reduction in the generation of injury[markers] attributable to perfusate exchange. In our study, the molecules we were assessing were not diluted and had a very short half-life of 4 mins (with Nucleosomes) to 15mins (with cfDNA) (67,284). This made our samples reflective of real time events occurring during NMP at/near the time of sample acquisition.

An alternative approach to perfusate optimisation has focused on cytokine filtration using the CytoSorb® cartridge (CytoSorb 300, CytoSorbents™ Europe GmbH, Berlin, Germany) to specifically remove circulating cytokines in lung (EVLP) and kidney NMP studies, with measurable improvements observed in both contexts (183,252,285). Limited data exists for liver NMP and cytokine filtration, however Karangwa et al (286) performed a DCD liver NMP (3.5hrs) using an acellular, plasma-free perfusion solution containing a mixture of a bovine haemoglobin-based oxygen carrier, HBOC-201 (Hemopure, HbO2 Therapeutics LLC) and then *autologous* whole blood reperfusion (2.5 hrs) randomising livers to Cytosorb® and NMP alone. They found that although it was feasible to connect the filter to the Liver Assist (Organ Assist, Groningen, the Netherlands) without complications impact graft function of performance, no differences in the circulating cytokine levels between groups were found and crucially no significant change in cytokine levels across the filter was observed. More broadly, in the context of the liver, an organ with a uniquely tolerogenic microenvironment that is strongly linked to an anti-inflammatory cytokine expression profile associated with a substantial post-transplant tolerogenic potential (107,287–289), a blanket/untargeted approach to removal of cytokines may paradoxically increase the immunogenicity of grafts.

In this Chapter, our approach significantly differs from previously published attempts at graft modulation via perfusate modification, primarily due to our combination of a novel target, circulating nuclear DAMPs, coupled with a highly efficient technological intervention able to achieve the desired effect. Unlike cytokines, DAMPs are unequivocally proinflammatory and injurious, yet they are highly preserved and part of the fundamental mechanics of innate immunity. These characteristics make them favourable targets for intervention. This study represents an initial strategy for immunomodulation during NMP, focusing on mitigating ERI via perfusate purification/optimisation.

This study had several limitations. Primarily, the low number of subject livers which were perfused. Although we found a large effect size that managed to overcome the low numbers, more data points would increase our confidence in the observed results. The lack of a true transplant model is a significant drawback as this would provide important post reperfusion/survival data. The other aspects of the inflammatory perfusate such as non-histone nuclear DAMPS (HMGB1) and cytokine measurement could also add to the overall picture. Histological assessment of liver tissue revealed that in all livers that underwent any period of NMP, glycogen depletion was evident, there were no differences between NMP groups, yet SCS livers had minimal glycogen depletion. Our perfusate composition did not include any insulin infusion as is standard on the Organ Ox metra perfusion protocol and this may have influenced this finding, alongside the fact that the SCS livers had much shorter total preservation times (i.e. 6 hours less than NMP groups). Our decision to minimise the additional infusions was to limit variables in what was an already complex experimental setup, but in future we would include an insulin infusion to support glycogenesis and avoid an exacerbated reperfusion injury.

Despite the complexity of the experimental set up, we had a highly reproducible model and the manged to execute the technical aspects of the experiment without confounding complications. Given the short half-life of chromatin fragments, the sampling represented events occurring close to the time of sampling, overcoming issues related to measuring transaminases and other traditional damage markers with long half-lives of 17-46 hours. Future experiments, with an inline whole blood compatible column (i.e. with an internal plasma separation filter: HaemoNucleoCapture (in development)) would reduce the complexity of the experiment and make translation more feasible. Progressing into human discarded liver perfusions would be advantageous to confirm the effects being observed in the porcine work, are transplantable to the human context. Although attractive, the heterogeneity of organs available for research perfusions is significant and often prohibitive

This Chapter has demonstrated that NucleoCapture technology reduces injury to grafts preserved with NMP. We have sequentially demonstrated that NucleoCapture technology can be integrated into the NMP circuit, performs its stated function efficiently and results in measurable improvements in ex situ liver performance. This has consolidated the concept of ERI being a targetable process in ECD liver preservation and provided a strategy that can be adopted to limit inflammatory injury on the device. This data supports a future where NMP will be adopted with consideration of the immune compartment and attempts to modulate it will be of interest not only for reconditioning and recovery of donor organs vulnerable to preservation injury, but to facilitate novel therapeutics. In conjunction with other adjunct interventions on the base circuit; (e.g. haemodialysis, thrombolysis and blood group manipulation), nuclear DAMP removal is an exciting step towards immunomodulation of donor livers during NMP.

Discussion & Conclusions

Historically, IRI has been conceptualised as occurring in the recipient or "in situ" where the full armamentarium of the host immune system is present, complete with innate and adaptive immune cells, complement and platelets (37,177). The response is a self-amplifying injurious cascade fuelled with inflammatory cytokines, mitochondrially generated ROS and DAMPs that cause organ damage upon resumption of oxygenation and cellular metabolism (46,290,291). The magnitude of this injury, as discussed earlier in the thesis, is determined by the interaction of multiple factors, including donor characteristics (steatosis, donor age and DCD) but also preservation considerations, such as warm and cold ischaemia times (34,292–294). Strategies to abrogate the effects of IRI have straddled these features, including a proliferation of machine perfusion technologies, supported by evidence that these have an impact on IRI related clinical outcomes (138,151,244). Ex situ organ perfusion, by its very nature, requires a broadening of our conventional concept of reperfusion. In this thesis I have explored the features of IRI in the specific context of normothermic machine perfusion, looking into the molecular mechanisms of reperfusion injury to understand the processes occurring during ex situ reperfusion. I have arrived at three central claims with associated suppositions that require further evaluation.

Firstly: **Ex situ reperfusion injury is an immunologically and molecularly distinct entity observed consistently across livers preserved with NMP**.

I have shown that donor livers that are preserved with NMP, experience reperfusion on the device in a fashion akin to implant reperfusion but distinguishable in several key molecular features. In characterising the distinguishing molecular processes, I have highlighted the important pathways and molecular networks linked specifically to the NMP environment and therefore consolidated ERI as a concept worthy of further investigation. In chapter 4, we demonstrated that NMP induces upregulation of protein translation and mitochondrial function while downregulating protein

degradation relative to SCS livers, but also revealed that in certain circumstances, such as those of prolonged prior cold ischaemia (pSCS-NMP), mitochondrial function is impaired despite NMP. Thus, the NMP modality (cNMP vs pSCS-NMP) and the risk profile of a donor organ (i.e its intrinsic sensitivity to IRI) collectively impact the outcome of liver NMP through augmenting molecular processes (related to ERI). These are factors that require consideration as we design and implement interventions during ex situ preservation.

Beyond IRI, liver transplantation is inherently intertwined with a second and equally critical immunological process: allorecognition (258,295). Distinct from IRI, yet interrelated, the pathways of allorecognition, described earlier in the thesis (chapter 3), have an immediate and in many cases lasting impact on the immunogenicity of grafts, influencing the functional capacity of transplanted livers (258,296). However, the mechanisms of how IRI and the constituents of allorecognition interact during NMP were relatively unexplored. This thesis has directly addressed this, using data from clinical human liver perfusions as well as from a large animal model developed and optimised through the course of my DPhil (297) (chapter 5). I have, therefore, generated a hypothesis based on a previously poorly characterised phenomenon: the impact of ex situ reperfusion injury on the immune compartment during NMP.

In this thesis, I have demonstrated that ex situ reperfusion is associated with distinct molecular processes, detectable at proteomic and transcriptomic levels and associated with organ injury. This phenomenon is consistent, occurs in the absence of leukocytes, complement and platelets, and has distinct characteristics that differentiate it from the reperfusion injury that occurs during implantation of the organ into the patient, albeit there are many similarities. The implications of this are potentially important: there is a clear need for organ reconditioning, modulation or protection of "at-risk" grafts, during preservation.

Secondly: **DAMPs, constitute an important element in ERI and alongside immune cells and cytokines propagate injury and inflammation potentially impacting the immunogenicity of the graft.**

The fact that donor organs that function well during normothermic preservation may still incur a considerable reperfusion injury implies that there are factors and mechanisms that we have not taken into account. I propose that through the observations and investigations presented in this thesis we can begin to unveil some of the underlying mechanisms that may be driving this injury and allow us to consider how these can be approached to improve outcomes.

In Chapter 3, the perfusate during NMP was examined and revealed the bidirectional trafficking of cells between the graft and perfusate at different stages of perfusion. This all occurs within an inflammatory milieu of cytokines and DAMPs or 'alarmins' (endogenous inflammatory molecules), that are generated upon ex situ reperfusion. The levels of cytokines (notably pro-inflammatory IL-6 and IFγ) rose persistently through perfusion maintaining the inflammatory environment within the perfusate in the hours flowing reperfusion. This pattern was reproduced within our subsequent porcine experiments in relation to both the cellular trafficking and cytokine profiles. However, in our assessment of standard criteria human liver grafts, that functioned well ex situ (and post-transplant), I found that the levels of DAMPs rose initially, peaking at 1 hour, but fell to lower levels by the end of perfusion. Although these levels were all well above the range recorded previously, even in patients with severe critical illness, the trend was consistent. The implication of this finding is that livers that function well ex situ, generate high levels of DAMPs during reperfusion, but as NMP progresses and the beneficial effects of NMP set in, the graft is able to clear these DAMPs and levels fall. Thus, the role of DAMP 'clearance' ex situ in functional organ assessment may be important and certainly warrants further investigation.

I found that in all livers that underwent NMP, Nuclear DAMPS such as extracellular chromatin (nucleosomes, histones and cell free DNA) were present in high concentrations within the perfusate. These molecules have well described inflammatory properties, and constitute an integral component of both reperfusion injury and innate immune activation (64,65). However, in the perfusion literature, they have been largely considered passive biomarkers of injury (298), with their role as active amplifiers of injury largely unrecognised until recently (48). In the setting of NMP, on a closed circuit, circulating nuclear DAMPs are implicated in the propagation of ongoing inflammatory injury during preservation. This opens up the possibility of targeted interventions, that unlike cytokine removal or indiscriminate cellular depletion/removal, targets a highly preserved (thus universally present) group of molecules that have a purely negative impact on grafts. There is an evolutionary logic to a rapidly amplified innate immune responses to unexpected circulating nucleic content derived from injured cells. Disordered cell death (non-apoptotic or unprogrammed) occurs upon reperfusion following rapid oxidation of accumulated metabolites during anaerobic cold storage (e.g. succinate) leading to ROS generation which causes immediate cellular injury, membrane rupture and DAMP release into circulation (290). This triggers an intense inflammatory response and immediate injury to the graft as well as (logically) an increase in longer-term immunogenicity (299). This is a process that with standard criteria grafts has no discernible clinical consequences (139,140,171) (i.e. it occurs under the limits of clinical detection). But as we push the boundaries of NMP with livers of a higher risk profile, an approach of "stress testing" organs by allowing unopposed ERI is deleterious. This may explain the emerging picture of poorer than expected IRI related outcomes of ECD livers preserved with NMP (172–174,212).

The recently published NAPLES study (212) assessed the outcomes of livers preserved with NMP to facilitate re-transplantation using higher-risk donor organs. The authors reported an unexpectedly high rate of acute T cell medicated rejection with 16/26(62%) compared to 9/31(29%) and 6/25 (24%) in historic and contemporary control (SCS) cohorts (p=0.010). This

may be the clearest clinical evidence of ERI induced immunogenicity in high-risk grafts undergoing NMP. The fact that ERI may have long-term implications in terms of the immunological risk of livers preserved with NMP provides further impetus for investigating approaches to mitigate it. This brings us onto the third and final claim of this thesis.

> Finally: **The removal of highly pro-inflammatory nuclear DAMPs from the circuit, during NMP of a porcine DCD liver, mitigates ERI and reduces the magnitude and impact of preservation injury.**

Bringing together the preceding works, the thesis concludes with an actionable intervention strategy for addressing DAMP driven inflammation during ex situ preservation. The NucleoCapture column combines specificity for unambiguously deleterious inflammatory molecules with a simple mechanism, leveraging the binding properties of a naturally occurring protein (linker histone). In the previous chapter, I presented the results of preclinical porcine liver perfusions (n=12) assessing the impact of damp removal on graft injury during NMP and functional parameters. NucleoCapture applied during NMP improved functional parameters (such as lactate clearance, hepatic artery flow and hepatocellular transaminase release) during NMP, while also maintaining the integrity of the cholangiocytes as assessed by bile pH and bile production. The implications of these findings are potentially, highly significant. If it is possible to create an immunologically stable and optimised perfusate that mitigates ERI, then it should be possible to use this "immuno-optimised" environment to facilitate the delivery of cellular and novel therapies. This might be used to achieve, for example, immunological tolerance of grafts, but also other therapeutic objectives dependent on immunological stability for optimal efficacy (300). Cell based therapies include Regulatory T-cells (Tregs)(301), Tolerogenic Dendritic cells (Tol-DCs)(302) as well as engineered cell products such as CAR-Tregs(58) and extra cellular vesicles (EVs) (303). A major concern regarding the delivery of these therapies is the risk of inadvertent transition to

immunogenicity, which may be heightened in the context of a highly inflammatory perfusate. The application of a technology that can reduce the pro-inflammatory status of the perfusate may increase the likelihood of success of this class of immunomodulatory therapy(304).

There are several limitations to the work and therefore conclusions presented in this thesis, many of which have already been discussed. Aside from the relatively low number of perfusions in each experiment which were limited by logistical (including COVID-19) and financial considerations, my extrapolations from pre-clinical animal perfusion studies are hindered by the lack of a transplant model to definitively evaluate clinically relevant endpoints. Furthermore, although the observations regarding ERI were validated in human liver perfusions, supporting conclusions coming from the pig model, I have not definitively proved a causal link between a more severe ERI and clinical complications. In the pre-clinical setting, this could be addressed with positive control experiments (human discarded liver and porcine DCD liver) perfusions, i.e. where DAMPs isolated using NucleoCapture are delivered to livers during NMP, in order to definitively demonstrate toxicity. Furthermore, the downstream effects of ERI related to the microcirculation have not been explored here, but are likely to be important features of injury. NETs have been extensively linked to thrombus generation in the context of liver IRI and more broadly in prothrombotic coagulation dysregulation (91). Their removal with NucleoCapture (as demonstrated in chapter 7) could influence this aspect of the pathophysiology of reperfusion injury, particularly in DCD liver transplantation where microcirculatory failure predominates(305).

Future work that is outside of the scope of this thesis is planned and ongoing. Evaluation of tissue and perfusate samples from the NucleoCapture experiments using transcriptomics and quantitative digital image analysis is planned. Alongside cytokine analysis and other injury markers (HMGB 1 ELISA) analysis, we will be able to develop a more complete picture of the impact of NucleoCapture on DCD pig livers. Furthermore, the role of NucleoCapture in the reperfusion phase of transplantation is being investigated by my colleague Hussain Abbas, with whom I will

soon embark on validation studies in both porcine livers and human discarded livers using the in-line HaemoNucleoCapture prototype. In these studies, we will place additional focus on the role of NETs on microthrombi formation during NMP looking at the microcirculation as an important compartment intrinsically related to ERI.

The data presented in this thesis and ongoing research in this programme of work will inform a clinical pilot study of NucleoCapture during NMP of DCD livers, aiming to minimise ERI and improve post-transplant preservation reperfusion injury related complications. This study will be another increment along NMP's remarkable journey from being a quasi-experimental technological development to a widely adopted platform upon which ex situ intervention and immunomodulation could become the standard of care for liver transplantation.

"True knowledge is to be aware of one's own ignorance"

— Rudolf Virchow

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