Novel approaches in optimising steatotic livers for transplantation

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by

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Abstract

Normothermic machine perfusion (NMP) has the potential to transform liver transplantation by facilitating the safe and reliable transplantation of higher-risk livers, thereby reducing the number of waiting list deaths. However, it poses logistical challenges and the evidence to support its potential beneficial effects in steatotic livers remain to be elucidated in human studies. This thesis aims to investigate whether the preservation of steatotic livers can be enhanced by NMP resulting in improved post-transplant outcomes through a reduction in ischaemia-reperfusion injury (IRI) and by reducing the amount of intra-hepatic fat. I have demonstrated that static cold storage (SCS) prior to NMP is safe and feasible with comparable clinical outcomes and markers of graft injury to those in continuous NMP and significantly improved compared to SCS. This is likely to facilitate clinical adoption by improving logistics and reducing the cost. I have provided evidence to support NMP's role in steatotic liver transplantation where early biochemical function is improved compared to cold stored steatotic livers. Furthermore, IRI characterised histologically and systemically is attenuated in steatotic NMP compared to SCS livers. Finally, I have demonstrated that in conjunction with de-fatting adjuncts, the *ex situ* function of steatotic livers can be improved and the amount of fat within the liver can be reduced by 45%. This is achieved through altering liver fat metabolism to favour improved/enhanced fatty acid oxidation with a concomitant reduction in *de novo* lipogenesis.

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3-HBDH	3-hydroxybutyrate dehydrogenase
3-OHB	3-hydroxybutyrate
4-AAP	4-aminoantipyrene
4-HNE	4-hydroxynonenal
ALD	alcoholic liver disease
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANOVA	analysis of variance
ApoB	apolipoprotein B
AST	aspartate aminotransferase
ATP	adenosine triphosphate
AV	arterio-venous
BMI	body mass index
cAMP	cyclic adenosine monophosphate
CBD	common bile duct
CE	cholesterol ester
CIT	cold ischaemia time
COPE	Consortium for Organ Preservation in Europe
CPT-1	carnitine palmitoyl transferase-1
CVA	cerebrovascular accident
DAMPs	danger-associated molecular patterns
DBD	donation after brain-stem death

DC	dendritic cell
DCD	donation after circulatory death
DLI	donor liver index
DNL	*de novo* lipogenesis
DPX	distyrene plasticizer xylene
DRI	donor risk index
EAD	early allograft dysfunction
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ESPA	N-Ethyl-N-(3-sulfoponyl)-m-anisidine
ET-1	endothelin-1
ET-DRI	Euro-transplant donor risk index
FA	fatty acid
FAS	fatty acid synthase
FFPE	formalin-fixed paraffin embedded
FWIT	functional warm ischaemia time
GB	gallbladder
GC	gas chromatography
GDA	gastroduodenal artery
GGT	gamma glutamyl transferase
GUI	graphical user interface
H&E	haematoxylin and eosin
H_2O_2	hydrogen peroxide
HA	hepatic artery
HAT	hepatic artery thrombosis

HCC	hepatocellular carcinoma
HI	haemolysis index
HTK	histidine-tryptophan-ketoglutarate
ICH	intra-cerebral haemorrhage
IFN-γ	interferon gamma
IL	interleukin
INR	international normalised ratio
IRI	ischaemia reperfusion injury
IVC	inferior vena cava
K	potassium
KC	Kupffer cell
LD	lipid droplet
ld-MaS	large droplet macrovesicular steatosis
LDH	lactate dehydrogenase
MAP	mean arterial pressure
MaS	macrovesicular steatosis
MEAF	model for early allograft dysfunction
MELD	model for end-stage liver disease
MHC	major histocompatibility complex
MiS	microvesicular steatosis
Na	sodium
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis

NEFA	non-esterified fatty acid
NF	non-function
NHSBT	NHS Blood and Transplant
NKC	natural killer cell
NMP	normothermic machine perfusion
PAS	periodic acid Schiff
PBS	phosphate buffered saline
PNF	primary non-function
PPARα	peroxisome proliferator-activated receptor alpha
PRS	post reperfusion syndrome
pSCS-NMP	post-static cold storage normothermic machine perfusion
PV	portal vein
RCT	randomised controlled trial
ROS	reactive oxygen species
RRT	renal replacement therapy
SCS	static cold storage
SD	standard deviation
sd-MaS	small droplet macrovesicular steatosis
SPE	solid phase extraction
TC	total cholesterol
TCA	tricarboxylic acid
TG	triglyceride
TNF-α	tumour necrosis factor alpha
TPN	total parenteral nutrition
TRIS	trisaminomethane

UK	United Kingdom
UW	University of Wisconsin
VLDL	very low density lipoprotein

Chapter 1

Introduction

1.1 Liver Transplantation

Since the first successful clinical case in 1967 (2), liver transplantation has developed into an established and standardised procedure which aims to cure acute and chronic liver disease. For patients with liver failure, techniques for supporting liver function provide only limited and temporary benefit as a bridge to transplantation. The outcomes of liver transplantation have greatly improved in recent decades with patient survival rates of over 85% at one year and 75% at five years being routinely achieved for elective liver transplantation (3). Thus, liver transplantation has become the mainstay of treatment for an increasing spectrum of patients with chronic liver disease, metabolic liver disease, acute liver failure and some liver cancers.

1.2 Indications for Transplantation

Liver transplantation should be offered to all patients with end-stage liver disease, where transplantation would prolong life beyond the projected life expectancy of the underlying condition (generally ≤ 1 year), or where the patient has an unacceptable quality of life due to liver disease (1). A liver transplant is indicated in patients with end-stage liver disease, hepatocellular carcinoma (HCC) or acute liver failure. For end-stage liver disease, the most common indication in adults is cirrhosis and patients should be referred for transplantation when complications such as variceal bleeding, ascites, hepatorenal syndrome or encephalopathy occur (1). Acute liver failure represents an urgent indication for transplantation and is most commonly caused by viruses (especially hepatitis A and B) or

drugs (paracetamol) (5). Indications for liver transplantation in Europe are summarised in Figure 1.1.

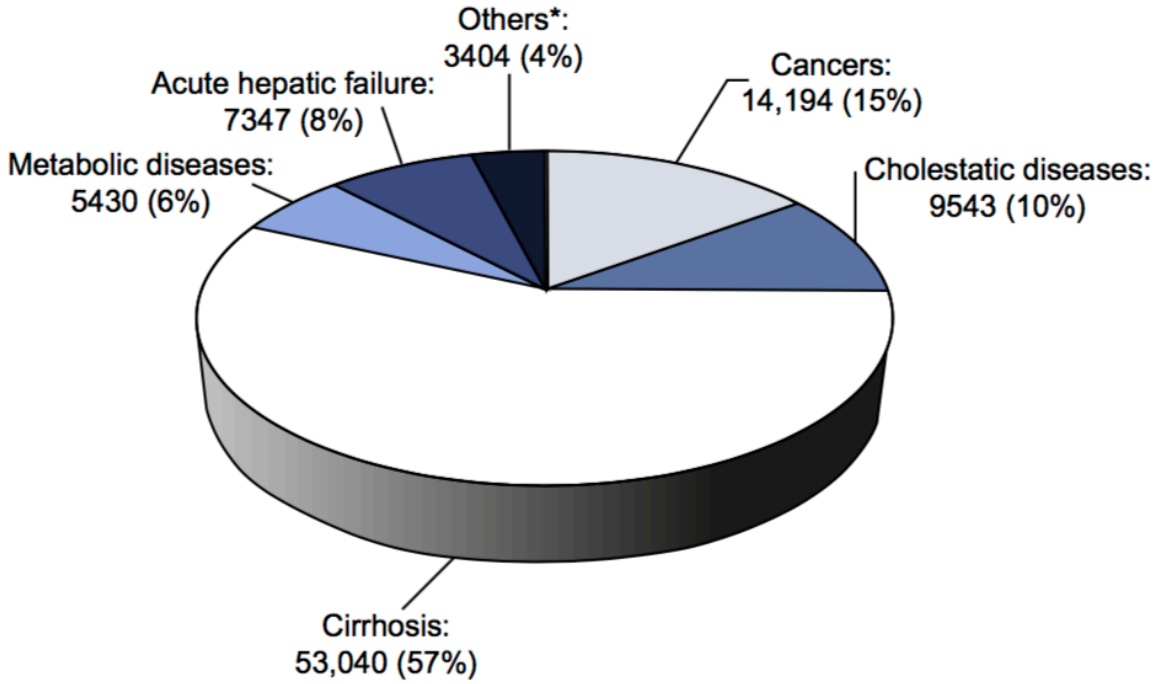


Figure 1.1. Primary diseases leading to liver transplantation in Europe as reported by the European Association for Liver Disease in 2016 (1). *Others: Budd-Chiari: 792, benign liver tumours or polycystic diseases: 1228, parasitic diseases: 80, other liver diseases: 1304.

With an increasing number of indications for liver transplantation, organ allocation schemes face a difficult task in ensuring that optimal patient selection and timing of transplantation result in the best outcomes. This is particularly difficult to achieve when, globally, there is a shortage of donor organs available (6). In order to help prioritise patients on the waiting list and ensure equitable access, the model for end-stage liver disease (MELD) score is used (7). This is based on objective measures such as creatinine, bilirubin and international normalised ratio (INR) and has been proposed for predicting 3-month mortality in patients with end-stage liver disease (7). MELD does not reflect the impact of complications of liver disease (ascites, encephalopathy, etc.) or the risk of HCC in assessing mortality without

transplantation; thus exceptions have to be considered to ensure appropriate patients have access to transplantation (8).

1.3 The Donor Organ Shortage

Improvements in organ preservation, surgical techniques, post-operative management and immunosuppression mean that liver transplantation outcomes have greatly improved since the procedure's inception. The demand for liver transplantation as a treatment for end-stage liver disease continues to increase and this is reflected in a doubling of the number of patients listed for a liver transplant in the UK over the last 10 years (9). However, the potential of liver transplantation to save the lives of patients with end-stage liver disease is limited by a shortage of donor organs. There are insufficient numbers of donor livers available to meet waiting list demands and as a result, around 15-20% of patients die before they can benefit from a life-saving transplant (9, 10). Although living donation can help bridge the gap between supply and demand for donor organs, this is not available in every country and does not help the large number of patients without a suitable living donor. Indeed, in the UK, this has made little impact on the number of liver transplants. Also, concerns are frequently voiced about the risks of serious complications and donor mortality associated with the donor procedure (11, 12). In many countries, greater focus has been directed to increasing utilisation of deceased donor livers. This involves pushing the boundaries of liver acceptance criteria, with a greater number of high risk or marginal livers being considered for transplantation (13). A marginal liver is defined as an organ with an increased risk of primary graft dysfunction or primary non-function (PNF), subjecting the recipient to increased morbidity and mortality risk. In recent years, evidence demonstrating the successful transplantation of marginal livers has grown and several strategies can be adopted to optimise these organs and improve post-transplant outcomes (14).

1.4 Features of a Marginal Liver

A liver is deemed to be marginal when there are certain donor and preservation characteristics which are known to increase the risk of post-transplant graft failure. Marginal livers have a lower tolerance of hypoxia, culminating in a more severe reperfusion injury (15) (Figure 1.2). Furthermore, the resilience of a marginal organ to recover from the physiological and metabolic traumas of brain death, organ procurement, preservation and implantation is diminished. As well as PNF, marginal livers are at increased risk of rejection and biliary complications including ischaemic cholangiopathy (16, 17).

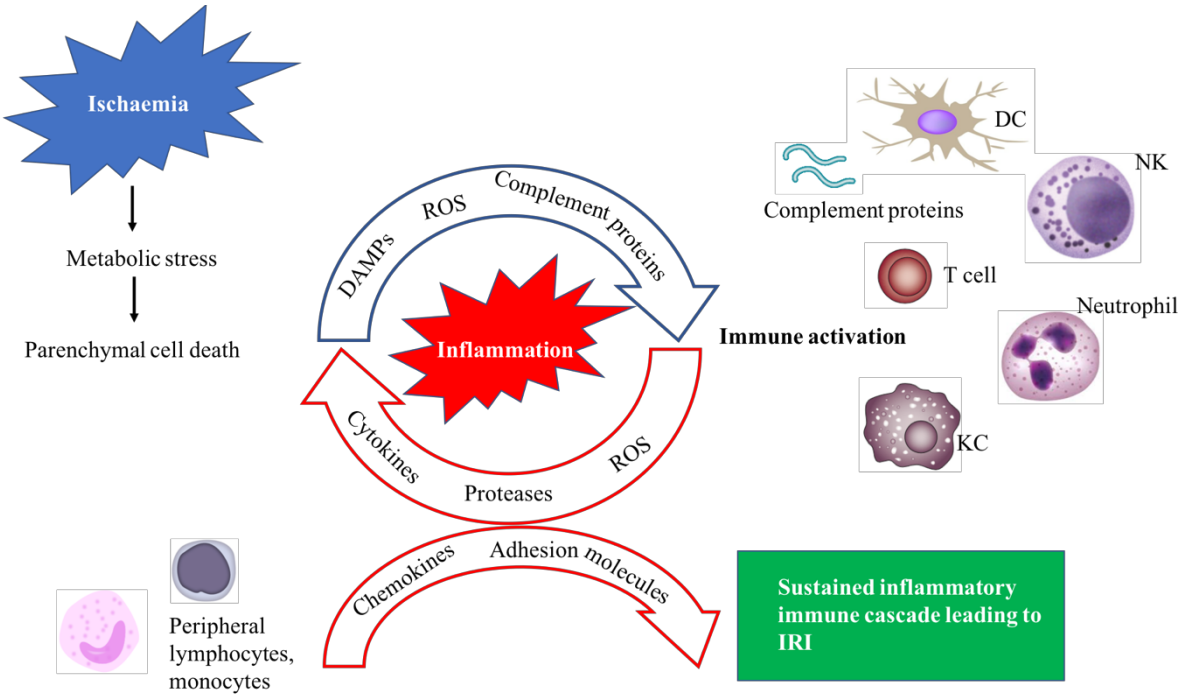


Figure 1.2. An overview of hepatic ischaemia/reperfusion injury (IRI). Ischaemic injury occurs as a result of glycogen consumption, lack of oxygen and ATP depletion. DAMPs are released following cell death, complement activation is induced by tissue injury, and mitochondrial ROS production is triggered by oxygenation. All of these factors contribute to liver immune activation after reperfusion. This involves multiple hepatic non-parenchymal cell types including Kupffer cells, dendritic cells, T cells, NK cells and neutrophils. The pro-inflammatory immune cascade activated by ischaemia and reperfusion is self-sustaining via the recruitment of peripheral immune cells from the circulation, ultimately responsible for liver reperfusion injury.
Abbreviations: DAMPs, danger-associated molecular patterns; DC, dendritic cell; KC, Kupffer cell; NK, natural killer cell; ROS, reactive oxygen species. Figure adapted from Zhai *et al.* (14).

Several population-based scoring systems have identified a number of factors which are independently associated with an increased risk of graft loss (Table 1.1). Although each factor in isolation increases the risk associated with the liver, the total risk is increased when high-risk characteristics are combined. It is also important to note that although prolonged cold ischaemia is a major risk factor for post-transplant graft survival, it is not included in the Euro-Transplant-derived or UK-derived risk scores because cold ischaemia time (CIT) is not known at the time the organ offer is made.

Table 1.1. Factors contributing to increased risk of graft loss according to 3 population-based scoring systems

Donor/preservation characteristic	**DRI (USA)** n = 20,023 *Feng S et al.* (18)	**ET-DRI (Europe)** n = 5,939 *Braat AE et al.* (19)	**DLI (UK)** n = 7,929 *Collett D et al.* (20)
Increasing donor age	✓	✓	✓
DCD graft	✓	✓	✓
Split graft	✓	✓	✓
Geographical allocation	✓	✓	×
Decreasing donor height	✓	×	✓
African-American race	✓	×	×
Cause of death (CVA)	✓	×	×
Cold ischaemia time	✓	×	×
Positive smoking history	×	×	✓
Elevated donor GGT	×	✓	×
Elevated donor bilirubin	×	×	✓

DCD – donor after circulatory death; CVA – cerebrovascular accident; DLI – donor liver index; DRI – donor risk index; ET – Eurotransplant; GGT – gamma glutamyltransferase

1.4.1 Donor Age

Although liver function does not appear to deteriorate with increasing age, the liver's ability to recover from the injuries associated with organ procurement and preservation is reduced

(21). The incidence of several liver diseases also increases with age and a reduction of liver volume and blood flow has been observed in older livers (22).

1.4.2 Donor after Circulatory Death (DCD)

In DCD donation, death is declared by circulatory (rather than neurological) criteria and there is an inevitable period of warm ischaemia before the liver is cooled by *in situ* perfusion. Despite an increased effort to transplant DCD livers, unfortunately, graft loss and recipient mortality have been shown to be almost twice as high as with livers from donors after brain-stem death (DBD) (23). During a DBD retrieval, although there are physiological changes as a result of brain death, there is minimal functional warm ischaemia time (FWIT) and oxygenation is maintained until the organ is perfused with cold preservation solution. During retrieval of a DCD organ, the FWIT is deemed to have started when the systolic blood pressure has a sustained fall below 50 mmHg and extends up to the onset of cold *in situ* perfusion (24). The duration of the FWIT is an important determinant of outcome and a recent large single centre study reported an increasing FWIT's association with recipient complications, specifically ischaemic cholangiopathy, graft failure and acute rejection (25). To this end, a FWIT of less than 20 minutes is advised (26-28). These criteria are arbitrary and the decision as to whether to proceed is multifactorial; as a result a number of livers are retrieved from DCDs that that are, on consideration of many factors, then declined.

The rates of DCD liver transplantation have greatly increased over the last 10 years in the UK and some other countries. However, as an overall percentage of transplanted livers, the percentage remains disproportionately low, because of poor utilisation (29). In the UK, 42% of the donor pool and 21% of transplants are made up of DCD livers (9). In the United States, DCD liver transplant rates are considerably lower than in the UK, with a modest increase in

recent years (Figure 1.3). In other regions DCD liver transplantation is limited mainly due to legal, cultural and logistical reasons (30).

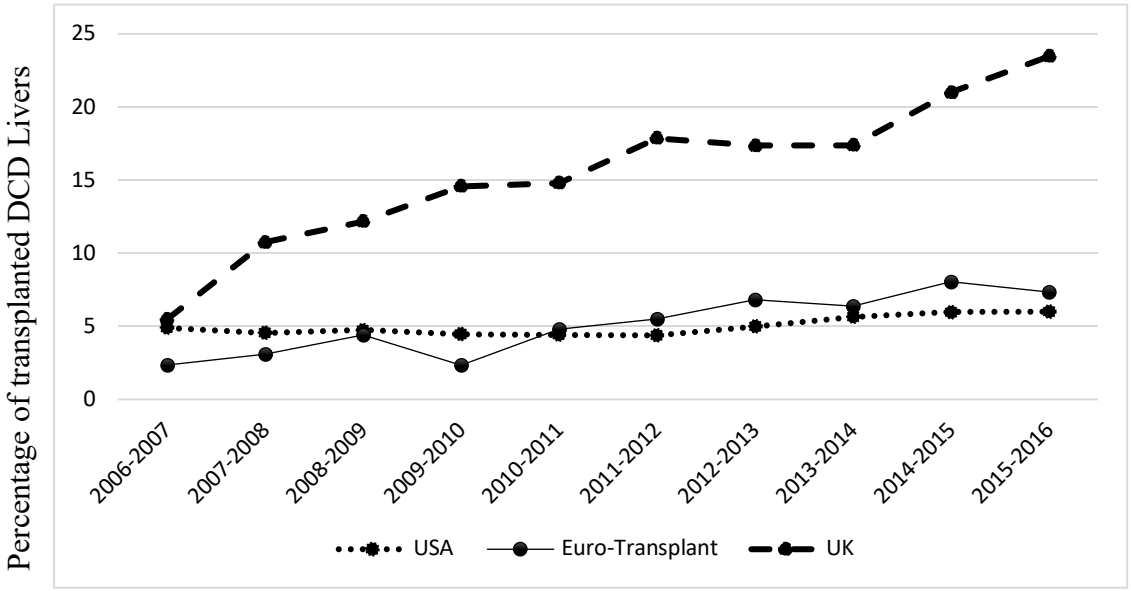


Figure 1.3. International DCD liver transplantation rates over the last 10 years

1.4.3 Split liver grafts

High quality donor livers can be split to provide grafts for two patients. Most commonly this involves one adult (right lobe) and one child (left lobe or left lateral segment). The use of a single liver for two adult patients has been associated with complications, particularly 'small for size syndrome' affecting the left lobe recipient (31). Liver splitting is a proven risk factor, and part of the donor risk index, and there is an increased risk of biliary complications (32). The CIT is often prolonged due to the time taken for the *ex* situ splitting procedure. Despite this, split grafts offer significant advantages to paediatric recipients, with a much-reduced time on the waiting list.

1.4.4 Steatotic livers

Steatotic livers derive poor outcomes when transplanted and a large number are, therefore, discarded. These grafts are considered in detail in section 1.6.

1.5 Liver Preservation

The aim of organ preservation in transplantation is to deliver a viable graft to a recipient which will exhibit primary function.

1.5.1 Static Cold Storage

Static cold storage (SCS) remains the standard of care preservation method in solid organ transplantation. University of Wisconsin (UW) solution is the most widely used fluid for the preservation of liver allografts from deceased donors, followed by histidine-tryptophan-ketoglutarate (HTK) solution (33). A systematic review by O'Callaghan *et al* demonstrated comparable post-transplant outcomes irrespective of the preservation solution used (34). The associated trauma of the organ retrieval process, through to transplantation of the liver, with the intervening preservation period, results in the physiological and metabolic phenomenon of ischaemia/reperfusion injury (IRI) (Figure 1.2). This remains the Achilles heel of solid organ transplantation and can lead to poor post-transplant outcomes including patient death and PNF as well as acute and chronic rejection (15). The first "hit" and ischaemic injury occurs during *in situ* cold perfusion where organs are flushed at ice-cold temperature with preservation solution. Cessation of blood flow to the liver prevents the supply of oxygen, nutrients and co-factors but does not stop metabolic activity completely. It is merely slowed to 1.5- to 2-fold for every 10^{o}C drop in temperature. Anaerobic metabolism ensues, resulting in the accumulation of metabolic waste products, including breakdown products of adenosine triphosphate (ATP) (35). Cell swelling and lysis occurs

due to ATP depletion which causes a loss of transcellular electrolyte gradients, influx of free calcium and subsequent activation of phospholipases (36) . The second “hit”, or reperfusion injury, results from the efflux of toxic molecules formed from metabolic products of ischaemia (37) and a profound inflammatory immune response that involves both direct and indirect cytotoxic mechanisms (15).

Despite the limitations of cold storage and the associated IRI, liver transplantation remains successful, with 1- and 5-year survival rates in the region of 85% and 75%, respectively (3). Whilst standard criteria donors typically possess sufficient physiological reserve to tolerate the injury associated with SCS, marginal organs may not and the role of cold storage for these grafts is therefore limited.

1.5.2 Normothermic Machine Perfusion

Preserving organs at normothermia is not a new concept. In 1935, Carell and Lindbergh developed a perfusion chamber for the normothermic preservation of organs and demonstrated their viability when perfused with oxygenated serum at normothermia for several days (38). However, due to its complexity, it was not widely adopted. Later, in the 1960s, several groups explored machine perfusion as a method of dynamic liver preservation but with complex logistical considerations and the advent of simple and effective static cold storage solutions (39), interest waned. Now, some 50 years later, the transplant community has seen a resurgence in enthusiasm for machine perfusion of organs. This is largely due to the need to expand the donor pool by successfully transplanting marginal organs.

NMP maintains the liver *ex situ* in a functioning state, providing it with oxygen and nutrients at 37ºC. Several NMP circuits have been described in detail (40-44); these function on the principle of physiological preservation which maintains cellular metabolism throughout the

preservation period. Principal components include: blood reservoir, pump(s) (some circuits comprise two pumps, for portal venous and hepatic arterial flow), an oxygenator, and a heat exchanger. NMP's superiority over SCS in terms of synthetic and metabolic liver function and post-transplant survival has been shown in large animal models (41, 42). However, the precise mechanism(s) underpinning the beneficial effects of NMP have not yet been fully elucidated. It is likely that perfusion helps maintain a healthy endothelium and replenish ATP. The importance of increasing hepatic ATP in human liver transplantation has previously been shown, with a direct correlation between high hepatic ATP content and good post-transplant outcomes (45). The role of NMP in ATP regeneration has been confirmed in animal experiments where rapid ATP recovery following initiation of NMP has been demonstrated (46-48) as well as mitochondrial ATPase activity (49). More recently, human studies have demonstrated histological evidence of glycogen repletion during NMP (50). Glycogen is essential to maintain hepatocellular integrity and function by supplying glucose for ATP generation. Once glycogen is consumed, ATP depletion ensues, leading to irreversible cell injury and necrosis (51). A difference in gene expression has also been shown between transplanted human NMP and SCS livers. Comparing gene expression between pre- and post-reperfusion biopsies, genes upregulated after NMP were mainly those involved in tissue regeneration, tissue growth/repair and those involved in control of inflammation (52). In contrast, upregulated genes in SCS were mainly those implicated in inflammation, apoptosis and activation of coagulation (52). Further studies are required to further explore NMP's mechanism of action which will undoubtedly allow clinicians and scientists to further exploit the technology to improve graft outcomes.

1.5.2.1 Clinical Experience of NMP

In recent years, clinical experience of NMP has grown and the body of evidence supporting the beneficial impact of NMP in liver transplantation has increased. The first NMP livers were transplanted as part of a phase one study undertaken by Ravikumar *et al* which demonstrated the safety and feasibility of NMP from retrieval to transplantation, including transportation (4). This was the first study to report post-transplant outcomes from a series of livers preserved by NMP. As well as safety and feasibility (the primary purpose) this trial demonstrated a significant biochemical benefit in the form of a halving of peak serum aspartate aminotransferase (AST) when compared to matched controls preserved via SCS (4). The peak serum AST in the first 7-days post-transplant is a validated surrogate marker for graft and patient survival (53-55). Two further pilot studies were subsequently performed by Selzner *et al* (56) and Bral *et al* (57) which demonstrated the safety and feasibility of the technology and compared post-transplant outcomes to cold-stored controls. Selzner and colleagues (56) demonstrated a non-significant reduction in peak serum AST in the NMP-preserved livers compared to retrospective SCS controls. In contrast, Bral and colleagues (57) observed a non-significant increase in the median peak serum AST in the NMP cohort. Neither of these studies was randomised or powered to demonstrate a difference in outcomes. Across these three studies, only one graft was lost during preservation, due to an occult twist in the portal vein which was obscured within the hepatic hilum, compromising perfusion of the liver (57). A multi-centre randomised controlled trial to compare the efficacy of NMP versus SCS in liver transplantation was recently conducted by the Consortium for Organ Preservation in Europe (COPE; www.cope-eu.org) (58). In this study by Nasralla *et al* (58), a total of 222 livers were transplanted (121 NMP, 101 SCS) with the primary endpoint of difference in peak serum AST between the two treatment arms. The study achieved its primary endpoint with a significant ($p < 0.0001$) 49.4% reduction in peak

serum AST in the NMP group (58). A notable finding was a reduced rate of liver discard in organs randomised to NMP; there was a significant difference between the two groups (32 SCS vs 16 NMP; p = 0.01) (59). Although no difference in patient or graft survival was seen between the groups, it was noted that a much larger trial would be required to test this outcome. Importantly, this trial demonstrated improved early graft function and reduced IRI in the context of significantly prolonged preservation times and fewer organ discards (58). These findings could have implications in increasing organ utilisation and addressing the donor organ shortage.

1.5.2.2 The Role of NMP in Increasing Organ Utilisation

At present, livers are discarded based on reported donor characteristics as well as the gross appearance of the organ (18-20). However, the lack of validated, objective predictors of function inevitably results in organs being turned down that would have functioned if transplanted. As well as reducing IRI and promoting liver regeneration (52), the ability to perform a viability assessment on the liver *ex situ* will undoubtedly increase the number of transplanted livers as surgeons can make an objective assessment of a liver's function during preservation, with the potential to predict post-transplant outcomes. Mergental *et al* reported the normothermic reconditioning of 6 livers which had been rejected by all UK liver transplant centres (50). Five of these livers were subsequently transplanted as they demonstrated *ex situ* function; specifically lactate clearance, bile production, acid/base homeostasis, stable flow dynamics as well as healthy graft appearance and consistency. At a median of 7 months (range 6-19 months) follow-up, all recipients remained well with functioning grafts (50). Watson *et al* (60) also published their experience of transplanting livers which had been declined by other UK liver transplant centres, following NMP and assessing organ quality and function. They observed that preserving livers at physiological

oxygen tensions resulted in a reduced incidence of post-reperfusion syndrome and suggested the importance of biliary pH in predicting post-transplant cholangiopathy (60). The same group subsequently built upon their initial experience and recently published their experience of perfusing 47 livers, of which 22 were transplanted (61). Based on their observations during perfusions and outcomes, they postulated that certain criteria should be fulfilled during perfusion of the liver in order to achieve successful transplantation with satisfactory outcomes and these are described in Table 1.2 (62). The Birmingham group has recently completed recruitment to a further clinical trial to assess more formally the benefit of NMP in liver grafts declined by other centres (63).

Table 1.2. The "Cambridge Criteria" of variables associated with successful transplantation of normothermic perfused livers.

Maximum bile pH > 7.5
Bile glucose concentration ≤ 3mmol/L or ≥ 10mmol/L less than perfusate glucose
Able to maintain perfusate pH > 7.2 without > 30mmol bicarbonate supplementation
Falling glucose beyond 2 h or perfusate glucose under 10mmol/L which, on challenge with 2.5g glucose, does subsequently fall
Peak lactate fall ≥ 4.4mmol/L/kg/h
Alanine aminotransferase (ALT) < 6000U/L at 2 h

1.5.2.3 Clinical adoption of NMP

As experience with this novel technology increases, new questions are being posed which will refine the way this technology is used and ultimately enhance its potential. NMP is logistically more challenging and costly than SCS, particularly when used for the whole preservation period, where dedicated personnel may be required to attend the donor hospital and different arrangements made for transportation. Indeed, Selzner *et al* acknowledged that

the use of NMP prolonged the organ retrieval process by 2 h because of back-bench preparation of the liver, cannulation, and connection to the device (56). The majority of NMP livers have been transplanted following perfusion for the entire preservation period from the donor to the recipient, including transportation (continuous NMP) (4, 56-58). The logistics of continuous NMP, whilst achievable, can be challenging, particularly in the context of organs transported by air. The clinical implementation of NMP may be facilitated by the use of NMP after a period of cold storage. This allows the transport of organs in an ice-box, thereby simplifying logistics and reducing costs (post-static cold storage normothermic machine perfusion, pSCS-NMP) (64). However, there is experimental evidence suggesting that rapid re-warming after a period of cooling can induce cellular injury (65). This scenario has been simulated in an experimental model by introducing a period of cold preservation prior to normothermic preservation (66). Porcine livers were subjected to 60 min of warm ischaemia and then assigned to either normothermic preservation for 24 h or cold preservation in UW solution for 4 h followed by 20 h NMP (66). All livers were then reperfused on a (freshly primed) normothermic perfusion circuit as a surrogate for transplantation. Livers that were subjected to pSCS-NMP had increased markers of hepatocellular injury, sinusoidal endothelial cell dysfunction and Kupffer Cell injury (66). The histology of livers that had been exposed to 4h of cold preservation before normothermia showed more necrosis and destruction of architecture (66). However, consideration is required as this study was an 'extreme' model: the pig liver is more sensitive to preservation injury and was subjected to a level of hypoxic injury that would not currently be accepted in clinical practice (i.e. 60 min). The situation in the clinical environment may be different in this regard and two clinical studies investigating NMP of discarded or high-risk human livers have demonstrated evidence that pSCS-NMP can be implemented without any apparent detriment to the graft (50, 60, 61).

1.6 Hepatic Steatosis and Liver Transplantation

The utilisation of steatotic livers is limited by a greatly increased susceptibility to IRI (Figure 1.2) and poor post-operative outcomes (67-78). As a result, an estimated 1000 steatotic livers are discarded each year in the United States (79) and in the UK, hepatic steatosis is the most common reason for non-utilisation, comprising 47% of organ discards in the last year (personal correspondence, NHSBT).

Hepatic steatosis is a condition where excess fat, in the form of triglycerides (TG), accumulates in the cytoplasm of hepatocytes. It can be caused by excessive alcohol intake (alcoholic liver disease, ALD) or a condition known as non-alcoholic fatty liver disease (NAFLD). NAFLD has now emerged globally as the most common cause of liver disease with a prevalence of 25-45% (80, 81). Obesity (BMI $\geq$ 30kg/m^2) is a major risk factor for NAFLD, with up to 90% of obese individuals being affected (82). The number of obese deceased organ donors in the UK is also increasing and in 2017, 25% of the donor population was made up of donors with a BMI $\geq$ 30kg/m^2 (83). Indeed, it is estimated that steatosis exists in 13-28% of deceased donor livers (84). It is notable that the majority of these organs exhibit near-normal biochemical function in the form of liver function tests prior to retrieval.

Changes in population phenotype mean that increasing numbers of steatotic livers in the donor pool are inevitable and in light of the donor organ shortage, methods to salvage these livers for transplantation are of great importance.

1.6.1 Classifying and quantifying hepatic steatosis

In the context of transplantation, the classification and quantification of hepatic steatosis is variable and subjective. The gold standard assessment involves histological analysis of a

haematoxylin and eosin (H&E) stained liver section where steatosis can be described as macrovesicular steatosis (MaS) or microvesicular steatosis (MiS). MaS describes large lipid droplets (LD) which displace the nucleus to the periphery of the cell, causing structural disruption (85). Often, MaS can be present with both large and small droplets that may be seen to coalesce (86). MaS can be further sub-divided into large droplet MaS (a single LD, larger than half of the cell, displacing the nucleus) or small-droplet MaS (LD is smaller than half of the cell and does not displace the nucleus) (87). MiS is characterised by multiple small LDs which create a foamy appearance and uniformly occupy the whole cell with a centrally-located nucleus. This often represents a separate clinical entity commonly characterised by an acute change as seen in encephalopathy and liver failure and the underlying disease processes are those of severe mitochondrial dysfunction (88, 89). True MiS comprises around 10% of hepatic steatosis (86) but is rarely seen in the context of transplantation as these livers would not be considered for donation due to the underlying aetiology. It is likely that the majority of MiS referred to in the transplantation literature is in fact small-droplet MaS. This makes robust conclusions and comparisons between studies difficult, creating more uncertainty between the types of steatosis which may be amenable to transplantation.

The quantification of steatosis remains an area of controversy. Traditionally, livers with MaS are divided into 3 groups according to the percentage of cells that are infiltrated by fat: mild (<30%), moderate (30-60%) and severe (>60%) (90). This can either be assessed by a histopathologist on liver biopsy as described above, or indeed by an experienced surgeon on macroscopic evaluation of the liver. Macroscopically, a severely steatotic liver is considered to have yellow parenchyma, rounded edges and a firm texture (91). Although histological assessment is the gold standard, it is not performed in all cases; a survey of surgeons in the

UK and USA concluded that biopsies were obtained in only around 50% of cases where the liver appeared to be macroscopically abnormal (92). Studies exploring concordance and correlation between histological and macroscopic evaluation display conflicting results. In a study by El Badry *et al,* 46 liver sections were scored by 4 different histopathologists and the quantification of steatosis was strongly observer-dependent and not reproducible (93). However, Hall *et al* explored 12 histopathologists' assessments of 20 liver biopsies and showed substantial agreement in the inter-observer concordance of steatosis scores (kappa = 0.64) (94). When assessing the surgeon's ability to predict steatosis, Yersiz *et al* showed that clinical assessment might be as reliable as other methods for predicting steatosis (87). This is contradicted by other studies which have demonstrated that disparity exists between surgeon's assessment and histological grade, resulting in livers being discarded on the basis of steatosis that could have been transplanted in up to one in six cases (95, 96).

The majority of histological analysis is performed on frozen-sections, as dictated by the time constraints when assessing a liver in the context of organ donation and transplantation. However, frozen-section specimens are notoriously of poor quality and it is often difficult to distinguish between fat vacuoles and artefactual spaces (87, 96, 97). Histopathological assessment is further limited by the fact that only a small quantity of tissue is taken (~1/50,000th relative to total liver size) (98) and biopsies are subject to sampling variability. It is also unclear whether steatosis in one part of the liver is representative of the whole organ (99).

The optimal method of quantifying hepatic steatosis should be practical, cost-effective, rapid and reproducible. Computational digital image analysis may provide a more accurate, un-biased and standardised approach. Studies have demonstrated good correlation between

histopathologists' assessment and image analysis software but histopathologists consistently overestimate compared to image analysis (93, 94, 100, 101). If image analysis was incorporated into standard practice, this would need to be considered in order to prevent severely steatotic grafts with a high risk of failure from being used. Although digital image analysis is useful in quantifying steatosis, it is unable to detect necrosis, fibrosis, malignancy and bile duct injury; all of which need to be assessed when considering a high-risk graft for transplantation.

1.6.2 Effect of steatosis on post-transplant outcomes

Concerns regarding the transplantation of steatotic livers are certainly justified. Based on the evidence in the literature, the risk is limited to MaS livers with several studies advocating the safe transplantation of livers with MiS even at high percentages of steatosis (67, 74, 76, 102-104). Spitzer *et al* performed a large-scale study of 5051 patients and demonstrated that MaS involving more than 30% of hepatocytes decreased graft survival post-transplant by 71% (RR 1.71, $P = 0.007$) (75). However, other groups have shown excellent results with McCormack *et al* publishing outcomes from 20 severely steatotic livers (>60%) and highlighting 95% 1-year patient survival (105); although only 2 livers displayed MaS >60% in this study. A systematic review conducted by Chu *et al* analysed 34 studies and concluded that livers with >30% MaS are associated with PNF and reduced graft survival (106). Quantifying the true risk of a steatotic graft from the literature is difficult as studies vary in the type of biopsy taken (core needle or wedge), tissue fixing and staining protocols used and the definition and quantification of steatosis itself varies greatly. These studies are also subject to considerable selection bias; often steatotic livers are only transplanted when the graft is otherwise considered to be low-risk (DBD and short CIT). Surgeons will not use

organs for which there is a valid concern that they will not function and the risks associated with these grafts are reflected in their poor utilisation.

1.6.3 Why are steatotic livers so high risk?

Steatotic livers are subject to severe IRI (107) (Figure 1.2). In human liver transplantation, highly elevated post-operative transaminase levels are associated with histological evidence of reperfusion injury (108, 109). There is good evidence to suggest that post-transplantation, recipients of steatotic livers have significantly higher transaminase levels and evidence of early graft dysfunction (77, 105, 110-117).

Experimental *in vitro* cellular and *in vivo* animal models suggest that the liver damage sustained during IRI is mainly initiated in the parenchymal cells and that excess lipids within the hepatocytes exacerbate this response (67, 68, 118). Berthiaume *et al* demonstrated this in a hepatocyte cell culture model of IRI, where the level of injury (as measure by liver enzyme release) correlated with the intracellular TG content (68). The same study also demonstrated that the injury could be reversed through the reduction of TG, supporting the hypothesis that steatosis is independently associated with IRI (68).

Several mechanisms have been suggested to explain the exacerbating effect of steatotic livers on IRI. All of these mechanisms are interlinked through complex cellular processes and mechanisms.

1.6.3.1 Lipid peroxidation

Human and animal studies have demonstrated that post-ischaemia and reperfusion, steatotic livers are subject to more lipid peroxidation (68-71). This describes the oxidative

degradation of lipids and defines the process by which free radicals scavenge electrons from lipids in cell membranes, resulting in cellular injury (119). This process is initiated by the reduction of hydrogen peroxide resulting in the formation of a hydroxyl radical which leads to the destruction of polyunsaturated fats (120). Lipid peroxidation has been used as an indirect measurement of oxidative damage induced by reactive oxygen species (ROS) (121) which remain critical to the generation of reperfusion injury (122).

1.6.3.2 Impaired sinusoidal blood flow

Animal models have shown that fat droplets within the hepatocytes impart structural injury and obstruction to the microcirculation causing narrowed and tortuous microvessels (123-125). In practice, this results in reduced hepatic and sinusoidal blood flow; Seifalian *et al* demonstrated diminished microcirculation in human steatotic compared to lean livers (126). The deleterious effects of impaired blood flow (and therefore oxygenation) are exacerbated during reperfusion of the graft, when an enhanced ischaemic injury facilitates the production of more ROS and Kupffer cell activation (127). Activated Kupffer cells produce endothelin-1 (ET-1), a potent vasoconstrictor in excess of the vasodilator nitric oxide, therefore compounding the issue (128).

1.6.3.3 Enhanced pro-inflammatory environment

Post-IRI, steatotic livers are subject to a more florid inflammatory response, characterised by the increased release of pro-inflammatory mediators TNF-α (71, 129) and neutrophil infiltration (130). Cytokine release causes endothelial dysfunction with the expression of adhesion molecules and activation, migration and accumulation of platelets and leukocytes (131, 132). Activated Kupffer cells, in addition to sustained ET-1 production, have been shown to possess lower phagocytic activity and overproduction of ROS, IL-6 and IL-1β in

steatotic compared to lean livers (133, 134). Persistent activation of inflammatory cells increase ROS and protease production, thereby aggravating hepatocellular injury (133).

1.6.3.4 Mitochondrial dysfunction

One of the most damaging consequences of IRI is related to the deterioration of mitochondrial function and consequent alterations in energy metabolism (135). Mitochondrial energy supply is fundamental for cellular viability and disruption to mitochondrial processes interrupts normal cellular bioenergetics, impairs cellular function and leads to cell death by necrosis or apoptosis (136). It is thought that mitochondrial dysfunction may be initiated through the production of ROS (137, 138). Mitochondrial proton adenosine triphosphate activity (ATPase activity), which is the key enzyme of ATP synthesis and oxidative phosphorylation in the mitochondrion (139, 140), was shown to decrease rapidly after 6 hours of cold storage in steatotic compared to lean livers (141). ATP depletion in steatotic livers results in failure of ATP-dependent Na/K membrane pumps leading to progressive cell swelling and necrosis (142). Indeed, it is likely that cellular necrosis (rather than apoptosis) is favoured in steatotic livers as the former is ATP-independent (79).

1.6.4 Strategies to reduce IRI in steatotic livers

Attempts to improve post-transplant outcomes in steatotic livers have included treatments to attenuate the IRI to which these grafts are particularly susceptible. These include pharmacological approaches, such as the addition of carvidelol to the preservation solution in a rat model of hepatic steatosis. In this experimental model, reduced hepatic death markers, vascular resistance and reactive oxygen species, as well as increased bile production and hepatic ATP levels were observed post reperfusion (70). Further separate

animal studies have examined the effects of glutathione (GSH)-ester (69), tacrolimus (143) and N-acetylcysteine (130) on hepatocellular injury post ischaemia/reperfusion. They observed reduced lipid peroxidation, liver injury markers and microcirculatory injury post reperfusion, respectively. Despite this, levels of injury remained higher than those of steatotic livers not subjected to ischaemia/reperfusion or indeed, lean livers (69, 70, 143).

Rather than identifying methods to reduce IRI, targeting the primary initiating event, namely hepatic steatosis, may yield improved transplantation outcomes. By eliminating the root cause of the problem, the associated complications may be avoided. Liver de-fatting has been successfully implemented in the setting of human living donation where donors were treated with a combination of a protein-rich diet, exercise and fibrate drugs for 2-8 weeks which significantly reduced hepatic macrovesicular steatosis and facilitated successful transplantation (144). Experimental studies have also demonstrated that when fatty hepatocytes or livers are cleared of intracellular lipids, they recover the normal response to IRI similar to that of their lean counterparts (68, 145). These studies describe de-fatting strategies that are effective when implemented over a period of days or weeks. However, in the setting of deceased organ donation, this is unsuitable. For the de-fatting approach to be effective on procured liver grafts, it is necessary for the kinetics of liver de-fatting to be accelerated so that it can be implemented over a period of hours.

1.8 Hepatic Steatosis and NMP

The key to transplanting steatotic livers may lie in the preservation method. NMP provides the potential to reduce IRI by avoiding the deleterious effects of cooling. It also provides a platform to treat the liver *ex situ* in an attempt to remove the fat during preservation which

may further improve outcomes. When considering that steatotic livers often display normal biochemical function in the donor and if successfully transplanted, the fat within the liver can dissipate within days to a few weeks (146, 147), it is not unreasonable to hypothesize that these grafts can be salvaged or even enhanced during preservation. In order to better understand how liver "de-fatting" may occur in an *ex situ* environment, it is important to consider the processes involved in hepatic lipid metabolism.

1.8.1 Hepatic lipid metabolism

The accumulation of fat in the liver most likely occurs due to an imbalance between fatty acid (FA) synthesis, input, and disposal (148). The liver derives fat from 3 main sources: 1) uptake of circulating plasma non-esterified fatty acids (NEFA) that originate from the lipolysis of subcutaneous and visceral adipose tissue; 2) diet; or, 3) endogenous synthesis from non-lipid precursors (*de novo* lipogenesis, DNL) (149-151). FAs are removed from the liver either by secretion in very low density lipoproteins (VLDL) as triglyceride (TG) or through oxidation (the tricarboxylic acid (TCA) cycle and ketogenesis). If not removed, FAs are esterified and stored in the liver, predominantly as TG (151). Hepatic fatty acid metabolism is a rapidly changing, dynamic process which in health, is under tight hormonal regulation and dependent on metabolic and nutritional state. An overview of hepatic lipid metabolism is shown in Figure 1.4.

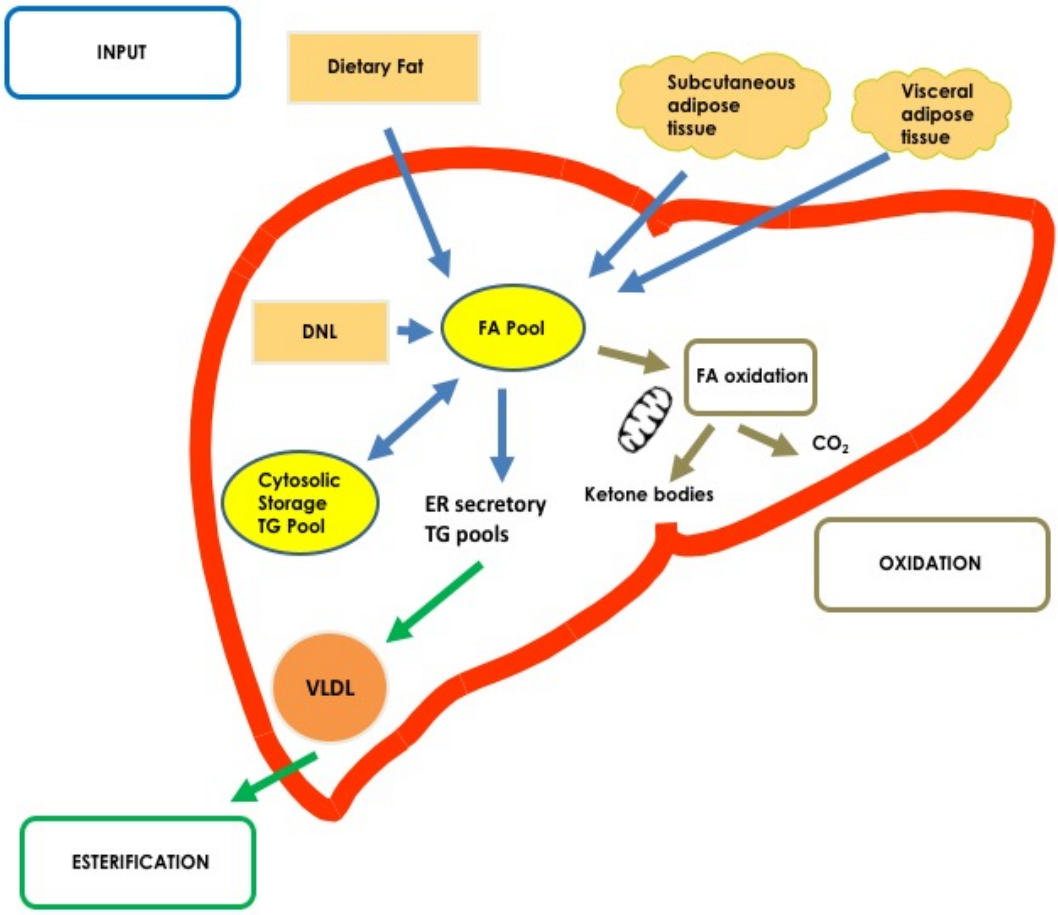


Figure 1.4. Overview of hepatic lipid metabolism. FAs derived from diet or the lipolysis of subcutaneous and visceral adipose tissue enter the liver as NEFA. Here, they mix with FA already stored in the liver (from the TG pool) and from those synthesised via DNL. From the FA pool, FAs are preferentially partitioned towards the oxidative pathway where the acetyl CoA produced can enter the Krebs' Cycle to produce ATP and CO_2 or ketogenic pathway, producing 3-hydroxybutyrate (3-OHB). TG is hydrolysed from the storage pool to release FAs that are re-esterified to TG and utilised in the production of VLDL particles and secreted (150). Abbreviations: DNL, *de novo* lipogenesis; ER, endoplasmic reticulum; FA, fatty acid; VLDL, very low density lipoprotein.

1.8.1.2 Lipid synthesis and esterification

FAs taken up by the liver are esterified with glycerol-3-phosphate to predominantly form TG along with other lipid species including phospholipids and cholesterol esters (150). Further contributing to the FA pool are FA that are synthesized by de novo lipogenesis (DNL) from non-lipid precursors such as glucose and amino acids (152) (Figure 1.5). This DNL pathway is stimulated by insulin and malonyl-CoA, an intermediate in the DNL

pathway, is a key co-ordinator of FA oxidation through its ability to inhibit carnitine-palmitoyl transferase-1 (CPT-1) therefore limiting FA entry into the mitochondrion for oxidation. The esterification of FA (in the form of fatty acyl-CoA) with glycerol-3-phosphate is also stimulated by insulin. As insulin is the hormone of the "fed" state, the liver tends towards the storage of FAs as TG, rather than partitioning fatty acyl CoAs into oxidation pathways. Under these conditions, hepatic energy requirements are largely met through the oxidation of amino acids (153). The stored TG, acts as a substrate for the hepatic secretion of fat into the bloodstream, in the form of VLDL. When secreted, VLDL particles contain TG, cholesterol ester, apolipoprotein B100 (ApoB) and small amounts of apolipoprotein E and C. Each VLDL particle contains just one molecule of ApoB, which stays with the particle throughout its lifetime. VLDL particles have a surface coat of phospholipids and unesterified cholesterol (154).

1.8.1.1 Fatty acid oxidation

The liver predominantly oxidises FAs by mitochondrial β-oxidation as a means of energy production to support its numerous metabolic activities. FAs shift from glycerolipid synthesis into the oxidation pathway when fatty acyl-CoA enters the mitochondrion. As the mitochondrial membrane is impermeable to fatty acyl-CoAs, the acyl group is transferred to the small molecule, carnitine. This process is catalysed by the enzyme CPT-1. At the cellular level, the activity of this enzyme is controlled by malonyl-CoA, which provides an important link between carbohydrate and fat metabolism and is a potent intermediate in the DNL pathway (Figure 1.5) (152). Under conditions of carbohydrate excess, malonyl-CoA concentrations are high and fat oxidation is therefore suppressed via CPT1 inhibition. This complements the suppression of FA release from adipose tissue under the regulation of insulin. Consequently, in conditions favouring carbohydrate excess, the fate of FAs in the

liver is shifted away from oxidation towards esterification (150). Within the mitochondria, the acetyl CoA generated can then enter the Krebs' cycle for oxidation to CO_2 or undergo ketogenesis. During ketogenesis, two molecules of acetyl-CoA are combined within the mitochondrion to form acetoacetic acid, from which the other major ketone body, 3-hydroxybutyrate (3-OHB), is produced and secreted into systemic circulation; 3-OHB is often used as a surrogate marker of hepatic FA oxidation (155).

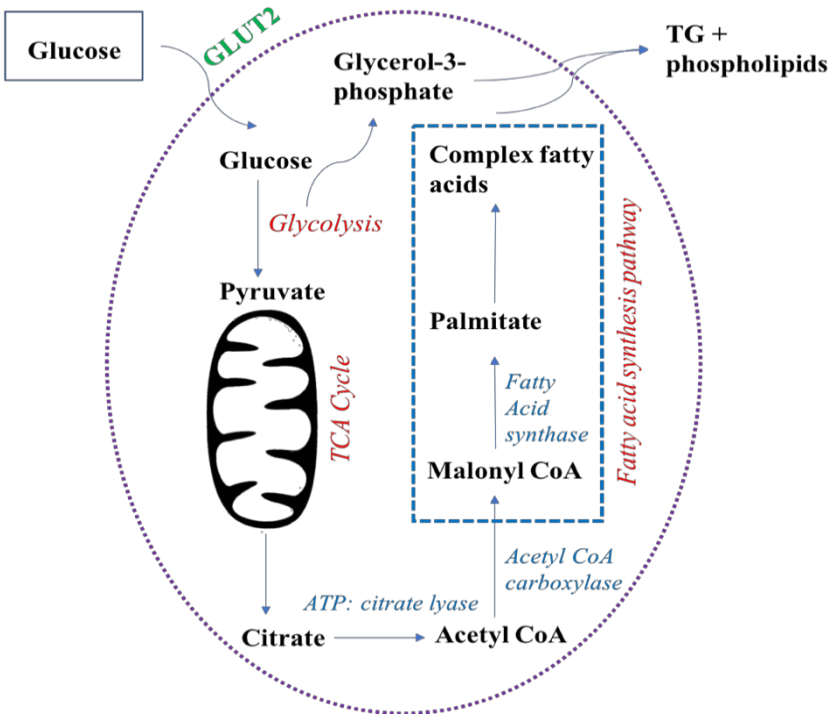


Figure 1.5. Schematic over view of hepatic *de novo* lipogenesis (DNL) whereby fatty acids are synthesised from non-lipid precursors. It is effectively a pathway for the disposal of surplus carbohydrate and is stimulated by conditions where carbohydrates are in excess. Briefly, glucose is taken up by the glucose transporter, enters the glycolytic pathway and produces pyruvate. Pyruvate is converted to acetyl CoA (feeding the Krebs' cycle) in the mitochondrion but it cannot cross the inner mitochondrial membrane so is converted to citrate. ATP: citrate lyase converts the citrate back to acetyl CoA which is then acted upon by acetyl CoA carboxylase (the first committed step in the DNL pathway), producing malonyl CoA which is then utilised as a substrate for the production of palmitic acid (16:0) by fatty acid synthase (FAS). FAS produces mainly palmitic acid using 8 molecules of acetyl CoA. Mammals can then modify palmitic acid to produce other fatty acids. Since these can be synthesised, they are non-essential fatty acids. Each of the enzyme reactions in the DNL pathway are stimulated by insulin. Figure adapted from Ameer *et al* (152).

1.8.2 NMP and liver "de-fatting": exploring the evidence

Few studies have explored the structural and functional effects of machine perfusion on steatotic livers. Jamieson *et al* explored the effects of NMP on steatotic porcine livers (156). This study demonstrated that during NMP, steatotic livers maintained perfusate homeostasis, bile and Factor V production and displayed similar perfusion haemodynamics to lean control livers. Notably, the steatotic livers displayed higher perfusate glucose and urea levels (156). In this study, the authors observed a reduction in steatosis from 28% to 15% over the course of the 48 h perfusion and also observed a decrease in lipid droplet size (156). Nagrath *et al* tested the effect of several de-fatting agents (Table 1.3) on normothermically perfused fatty livers isolated from Zucker rats (157). They observed a 65% reduction in hepatic triglyceride content after only 3 h of perfusion. A 30% decrease in hepatic triglyceride was seen when control perfusate with no de-fatting agents were used (157).

Table 1.3. De-fatting agents used by Nagrath *et al* (157) with their proposed mechanism of action in the context of hepatic lipid metabolism.

De-fatting Agent	**Function**
Pregnane X receptor ligand hypericin (HPC)	Increased peroxisomal β-oxidation of very long chain fatty acids (158).
Peroxisome proliferator-activated receptor α (PPARα)	PPARα activation increases mitochondrial fatty acid oxidation. As natural ligand for PPARα is thought to be a fatty acid, this may be seen as a way of increasing the oxidation of fatty acids when fatty acid supply is high (159).
Visfatin	This is an insulin-mimetic adipokine whose role in de-fatting is not properly established (160).
Scorparone	This constitutive androstane receptor ligand upregulates PPAR (161).
Forskolin	Glucagon mimetic cAMP activator which results in increased lipolysis of lipid droplets and fatty acid oxidation (162)

It is worth noting that Liu *et al* explored the de-fatting potential of machine perfusion at sub-normothermic temperatures (20ºC) (163). However, no significant changes in steatosis were observed and it was concluded that normothermia is required to ensure the liver's optimal metabolic activity (163). This finding was corroborated in a murine model, and findings suggested that machine perfusion's de-fatting potential was temperature-dependent, favouring normothermia (71). These preliminary data in animal models have highlighted NMP's potential to enhance the preservation of steatotic livers and support the hypothesis that TG metabolism can be altered during preservation, such that hepatic steatosis can be reduced which may result in the successful transplantation of these organs.

Although the ability to reduce hepatic TG content appears to be possible in animal models (71, 156, 157), where steatosis has been artificially induced for experimental purposes, it is unclear whether these findings are applicable to steatotic human livers; the limited available data would suggest not. Liu *et al* perfused 10 discarded livers with variable degrees of baseline steatosis for 24 h (164) and demonstrated a significant increase in perfusate TG levels over the duration of the perfusion but did not observe any reduction in steatosis (164). Banan *et al* have reported results from two human livers which were preserved normothermically with the addition of de-fatting agents, namely l-carnitine and exendin-4 (165). L-carnitine increases the rate of fatty acid transport to the mitochondria and has been shown to be important in β-oxidation of fatty acids from the mitochondrial membrane (166) and exendin-4 has been shown to reduce oxidative stress in steatotic models (167). However, in the two treated livers, only one showed a minimal reduction in the degree of macrovesicular steatosis (10%) after 8h NMP (165).

1.9 Overview and Aims

The success of liver transplantation is limited by a shortage of available donor organs. NMP has the potential to transform liver transplantation by minimising the risk of graft injury during preservation and improving organ utilisation through the use of higher-risk organs (such as those with steatosis) without compromising outcomes.

The aims of this thesis are:

- Facilitate the clinical adoption of NMP by investigating the safety and feasibility of cold storage prior to NMP by conducting a clinical trial (Chapter 3).
- Compare post-transplant outcomes from steatotic livers undergoing NMP and cold storage and explore the histological and biochemical changes in steatotic NMP livers as well as NMP's impact on IRI (Chapter 4).
- Explore the structural and functional effects of NMP, with or without de-fatting adjuncts, on human steatotic livers. An attempt will be made to understand how key pathways in liver fat metabolism could be exploited *ex situ* to successfully improve liver function and reduce the degree of steatosis (Chapter 5).

Chapter 2

General Methods

The methods described in this chapter are commonly used within this thesis. Methods specific to individual chapters are found in the methods section of those chapters.

2.1 Normothermic Machine Perfusion (NMP)

All normothermic liver perfusions described in this thesis were conducted using the OrganOx *metra* (1st Generation) (OrganOx Ltd, Oxford, UK) NMP device (Figure 2.1) (4, 56-58). This is a commercially available device, CE-marked for the normothermic preservation of donor livers for up to 24 h (4, 56-58).

Figure 2.1. The OrganOx *metra* (1st Generation) liver NMP device

The device comprises a centrifugal pump, heater/oxygen concentrator, blood (perfusate) reservoir and blood gas analyser (168). The device perfuses the liver via a closed system –

with cannulation of the hepatic artery, portal vein and inferior vena cava (IVC) (169). Arterial flow is delivered continuously (as opposed to pulsatile) with automated pressure control (60 to 75mmHg) (169). A schematic representation of the device is shown in Figure 2.2.

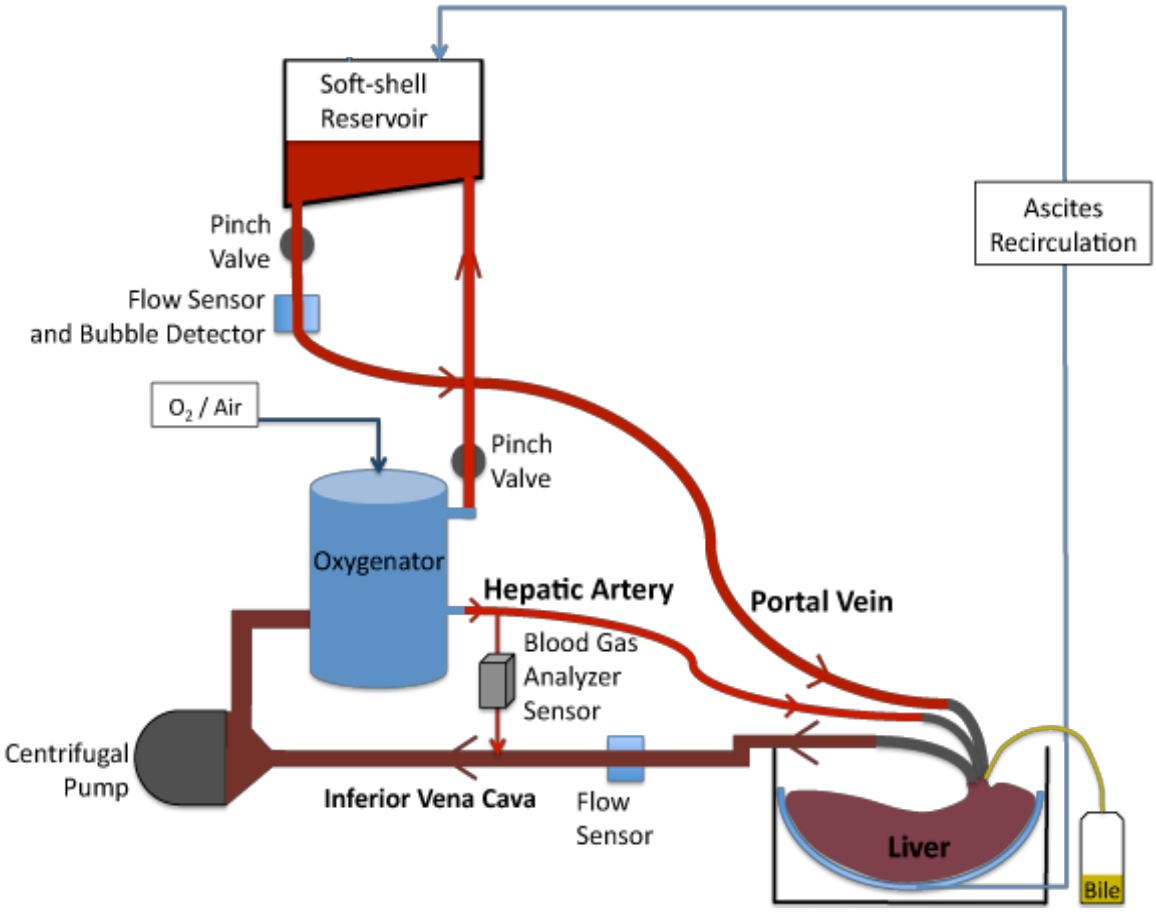


Figure 2.2. Schematic overview of OrganOx metra. Perfusate leaves the liver via the IVC, passing through a flow sensor, before reaching the centrifugal pump. Perfusate is then pumped to the heater and oxygen concentrator (from air), warming the perfusate to 37°C and oxygenating to achieve a pO_2 between 12-18kPa. Perfusate is then delivered directly to the liver through the hepatic artery via a low flow, high pressure system or to the portal reservoir. The pinch valve on this line is used to regulate the arterial pressure. A shunt from the arterial to IVC line passes through a blood gas analysis sensor which provides real-time pO_2, pCO_2 and pH. From the reservoir, perfusate passes through the portal flow sensor to the portal vein and into the liver via a high flow, low pressure system. The portal pressure is fixed, dictated by the height of the fluid level in the reservoir. The portal pinch valve provides a safety mechanism to prevent air from entering the liver. If the portal reservoir empties (e.g. excess bleeding from the liver), 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 re-fill. Any bleeding form the 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 chamber in the liver bowl, passing through a sensor to record output in ml/h.
Figure adapted from Ravikumar *et al* (4).

The perfusate comprises three units of donor/recipient-matched or group O packed red cells as an oxygen carrier and 500 ml of Gelaspan (B Braun, Sheffield, UK), a colloid added to normalise the haematocrit and osmolarity. For clinical perfusions (Chapters 3 and 4), blood was sourced from the donor or recipient hospitals. For research perfusions (Chapter 5), red cells were sourced from the blood bank in Oxford, either as recently expired red cells or purchased as non-clinical issue surplus (Filton, Bristol, UK). Boluses of 10% calcium gluconate (B Braun), 10,000 IU unfractionated heparin sodium (Wockhardt UK Ltd, Wrexham, UK) and 750mg of cefuroxime (Flynn Pharma Ltd, Dublin, Ireland) were added to the reservoir during priming of the device in order to correct the binding of citrate to calcium, prevent thrombosis in the circuit and protect from bacterial overgrowth, respectively. Once the device reached 37ºC, a bolus of 30ml 8.4% sodium bicarbonate (B Braun) was administered to normalise the perfusate pH prior to connection of the liver.

During perfusion, four continuous infusions were administered:

1. 25,000 IU unfractionated heparin sodium (Wockhardt UK Ltd) to prevent thrombosis during perfusion.
2. 200 units insulin (Actrapid) (Novo Nordisk, West Sussex, UK) to assist with perfusate glucose control.
3. 4.5g sodium taurocholate (bile salts) (OrganOx Ltd) to compensate for loss of exogenous bile salts.
4. 0.5mg epoprostonol sodium (Flolan®) (Glaxo, Middlesex, UK) to optimise micro-perfusion.

All solutions were made up to 30ml with 0.9% sodium chloride (B Braun) and administered via an automated pump at a rate of 1ml/h. In addition, 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.

Following device set-up, it was primed to allow the perfusate to reach optimal operating conditions: temperature (37°C), pO_2 (12-18 kPa), pCO_2 (4-6 kPa) and pH (7.25 – 7.35). During this time, the liver was prepared and cannulated as described below.

During perfusion, real-time perfusion parameters are displayed on the graphical user interface (GUI) on the *metra* (Figure 2.3). These data are recorded and stored on the device and can be downloaded for further analysis.

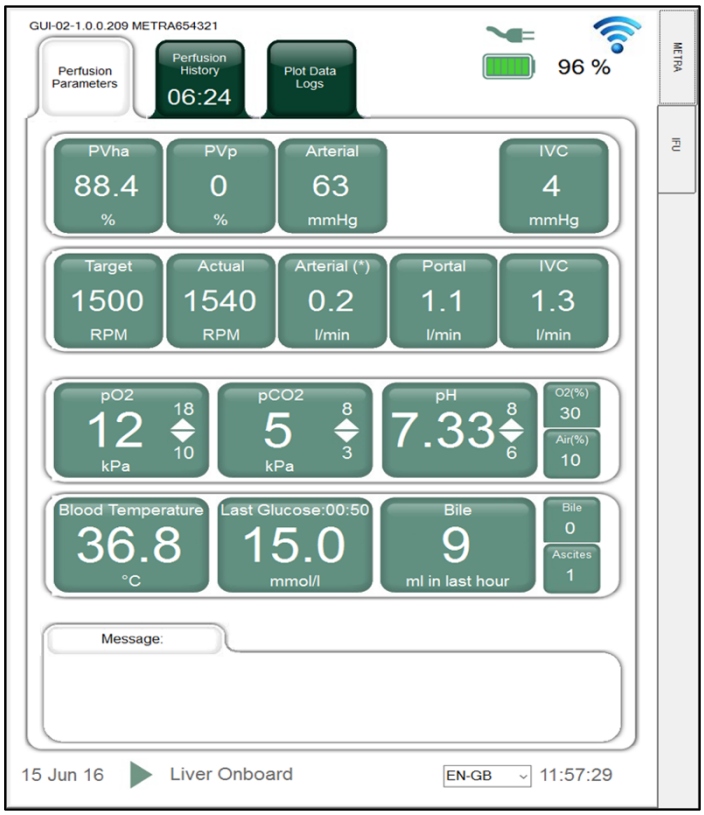


Figure 2.3. OrganOx *metra* GUI. Here, real-time pressure and flow dynamics are displayed. The arterial flow rate is not directly measured, rather it is derived as the difference between portal and IVC flow. Blood gas and pH values are provided and refreshed every 10 seconds. Glucose values are inputted manually every 4 h and this regulates the TPN infusion. Bile production is also displayed and updated every hour.

2.2 Liver Preparation and Cannulation

During deceased donor multi-organ retrieval (170), all 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 (without cold storage) and placed on the *metra* (Chapter 4) or cold stored in UW solution and transported on ice to the respective centre (Chapters 3 and 5).

Livers were prepared as described by Makowka *et al* (171). Briefly, the infrahepatic IVC was dissected free from surrounding tissue and the adrenal vessel ligated. Diaphragmatic tissue was dissected from the posterior aspect of the liver. The remnants of diaphragm were dissected from the suprahepatic IVC and the orifices of the phrenic veins oversewn with 4:0 prolene sutures (Ethicon, Johnson & Johnson Medical Devices, Wokingham, UK). The suprahepatic IVC was oversewn with prolene (Ethicon). Dissection of the portal vein was performed to demonstrate the bifurcation and any small branches, ligated. Dissection of the common hepatic artery and coeliac trunk was undertaken to the level of the gastroduodenal artery (GDA). Meticulous haemostasis was carried out through the ligation of any small branches and lymphatic tissue. Any aberrant vessels were carefully preserved and re-constructed prior to cannulation of the liver. The gallbladder was closed and minimal bile duct dissection performed. After the placement of a purse-string suture at the infrahepatic IVC using 2.0 prolene (Ethicon), a 28 Fr bespoke cannula (Sorin, Gloucester, UK) was inserted and secured in place. 20 Fr and 10 Fr cannulae (Sorin) were secured in the portal vein and coeliac trunk, respectively, with 2.0 vicryl ties (Ethicon). The common bile duct was cannulated with a 12-18 Fr T-tube (Summit Medical, Cheltenham, UK) and secured with 2.0 vicryl (Ethicon). Following cannulation, the liver was primed with 0.5-1 L of Gelaspan (B Braun), initially through the portal and then arterial cannula. Care was taken to

remove all pockets of air from the vessels and cannulae. The liver was then connected to the *metra* and perfusion commenced. The prepared and cannulated liver is shown in Figure 2.4.

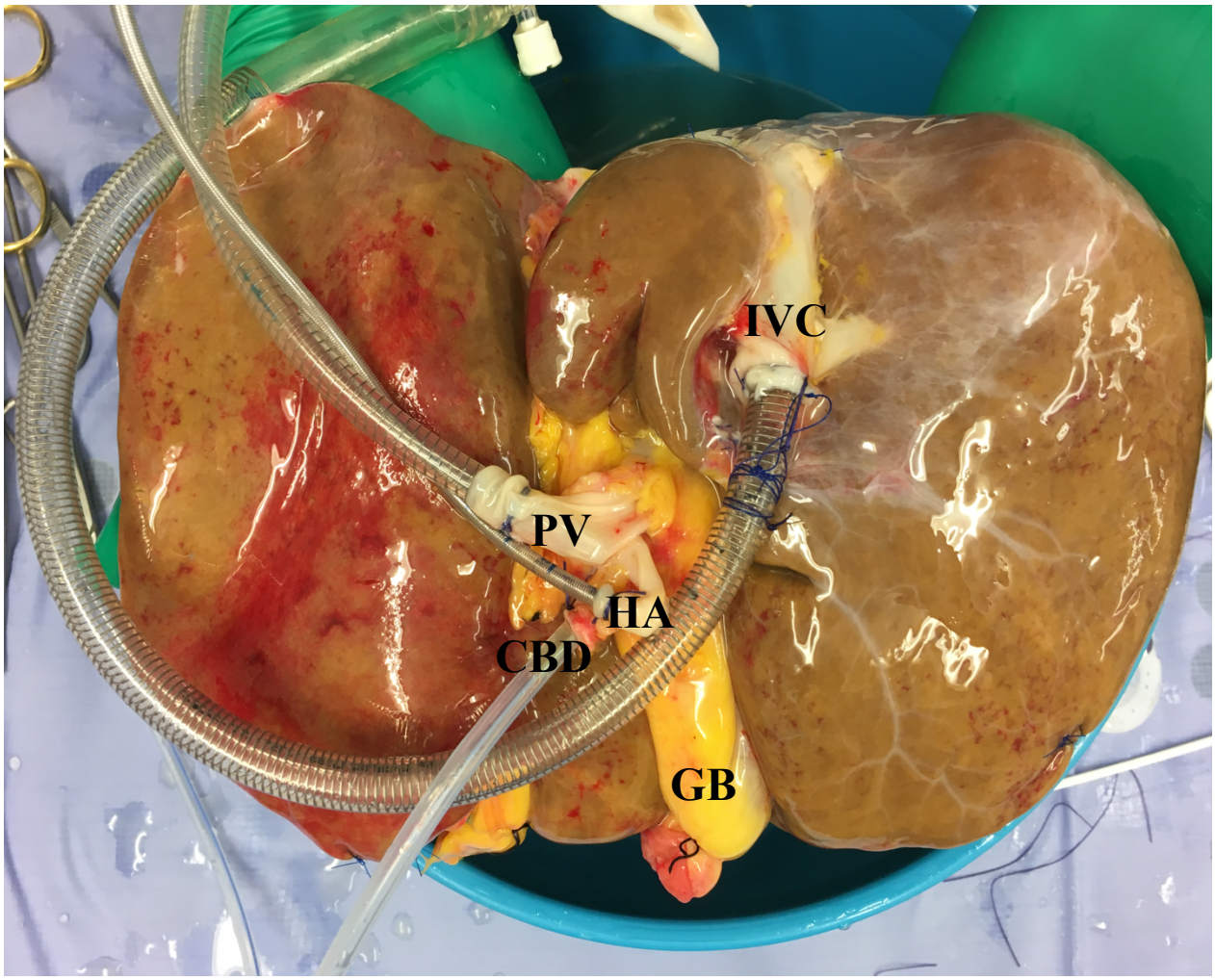


Figure 2.4. Image of cannulated liver, ready for the commencement of NMP. The inferior vena cava (IVC), portal vein (PV), hepatic artery (HA) and common bile duct (CBD) are all cannulated. The gallbladder (GB) is also present and has been closed.

2.3 Liver Tissue Preparation and Staining for Light Microscopy

2.3.1 Tissue Acquisition

All liver biopsies described throughout this thesis were obtained with a Biopince™ Full Core Biopsy Instrument (Argon Medical Devices, Texas, USA) using a 33mm throw length (yielding a 29mm specimen length). Biopsy specimens were subsequently fixed in 10%

formalin and stored at 4°C or snap-frozen in liquid nitrogen and stored at -80°C for future processing and analysis.

2.3.2 Paraffin-embedding of formalin-fixed tissue

Formalin-fixed biopsies were placed in cassettes, washed in phosphate buffered saline (PBS) (Sigma-Aldrich, Dorset, UK), before being automatically processed through 70% to 100% methanol (VWR International Ltd, Leighton Buzzard, UK), xylene (Fisher Scientific UK Ltd, Loughborough, UK) and paraffin (VWR International Ltd) using a Leica TP1020 automatic tissue processor (Leica Biosystems, Newcastle, UK) to fix, dehydrate and infiltrate the tissue. Tissue was then embedded in blocks using melted paraffin and allowed to cool.

2.3.3 Tissue Sectioning

Formalin-fixed paraffin-embedded (FFPE) specimens were sectioned at 4μm thickness using a microtome (Leica Biosystems). All sections were cut onto superfrost plus microslides (VWR International Ltd).

2.3.4 Haematoxylin & Eosin (H&E) Staining

2.3.4.1 Principle

H&E is an all-purpose stain used to identify basic tissue structures. It involves electrostatic attraction between oppositely charged ions; one of which is fixed in the tissue, the second of which is in the dye. Haematoxylin in complex with aluminium salts, is catanionic and acts as a basic dye. It is positively charged and can react with negatively charged, basophilic cell components, such as nucleic acids in the nucleus (172). These stain blue as a result. Eosin is anionic and acts as an acidic dye. It is negatively charged and can react with

positively charged, acidophilic components in the tissue, such as amino groups in proteins in the cytoplasm (172). These stain pink as a result.

2.3.4.2 Method

Slides were processed through 3x 5 min washes of xylene (Fisher Scientific UK Ltd) to de-wax the specimens. Sections were then de-hydrated in 3x 5 min washes of 100% methanol (VWR International Ltd) and 1x 5 min wash of 70% methanol (VWR International Ltd). After rinsing the slides in water, they were placed in haematoxylin (Sigma-Aldrich) for 5 min. Following a further 2 min rinse in water and 1min rinse in acid alcohol, slides were placed in eosin (Sigma-Aldrich) for 5 min. Sections were once again dehydrated in 3x 3 min washes of 70% to 100% methanol (VWR International Ltd) and cleared in 3x 3 min washes of xylene. The H&E-stained sections were mounted under a cover slip with distyrene plasticizer xylene (DPX) (Sigma-Aldrich), ready for light-microscopy analysis.

2.3.5 Periodic acid-Schiff (PAS) Staining

2.3.5.1 Principle

This staining method is used in the detection of polysaccharides – macromolecules composed of monosaccharide units joined by covalent bonds (173). The main polysaccharide identified is glycogen (173). The first reaction in the stain involves periodic acid acting as an oxidising agent to oxidise the carbon-to-carbon bonds between two adjacent hydroxyl groups (174). This produces Schiff-reactive aldehyde groups. In the second reaction, the tissue reacts with Schiff reagent (174). This comprises a mixture of basic fuchsin, hydrochloric acid and sodium metabisulphite. The basic fuchsin in the mixture reacts with newly formed aldehyde groups in the tissue to produce a bright magenta colour. The intensity of the colour is proportional to the concentration of hydroxyl groups originally

present in the monosaccharide units (174). Haematoxylin is used as a counter-stain to visualise other tissue elements.

2.3.5.2 Method

Slides were process through xylene (Fisher Scientific UK Ltd) and methanol (VWR International Ltd) as described above (section 2.3.4.1). Slides were then immersed in periodic acid solution (VWR International Ltd) for 10 min and rinsed in water before immersion in Schiff solution (VWR International Ltd) for 30 min. After rinsing in warm tap water, slides were placed in haematoxylin (Sigma-Aldrich) for 5 min. Slides were then dehydrated and cleared and cover slips mounted as described above (section 2.3.4.1).

2.4 Image Analysis for Glycogen Quantification

Serial images across each PAS-stained section were obtained using a Canon EOS 700D digital camera during light microscopy (Olympus BH-2, Southend-on-Sea, UK) at 10x magnification and open-source image analysis software (ImageJ, https://imagej.net/Welcome) (175, 176) was used to quantify the amount of positive stain in each image. The total area of the section was calculated (Figure 2.5A) and the image converted to grey-scale (Figure 2.5B). A threshold for positive stain was established and the same threshold was used for each image analysed (Figure 2.5C). The remaining positive-threshold area was calculated and the remaining positive-stain area compared to the total area of the tissue was interpreted as the amount of glycogen in the tissue (Figure 2.5D). The amount of glycogen in the tissue was calculated as a percentage.

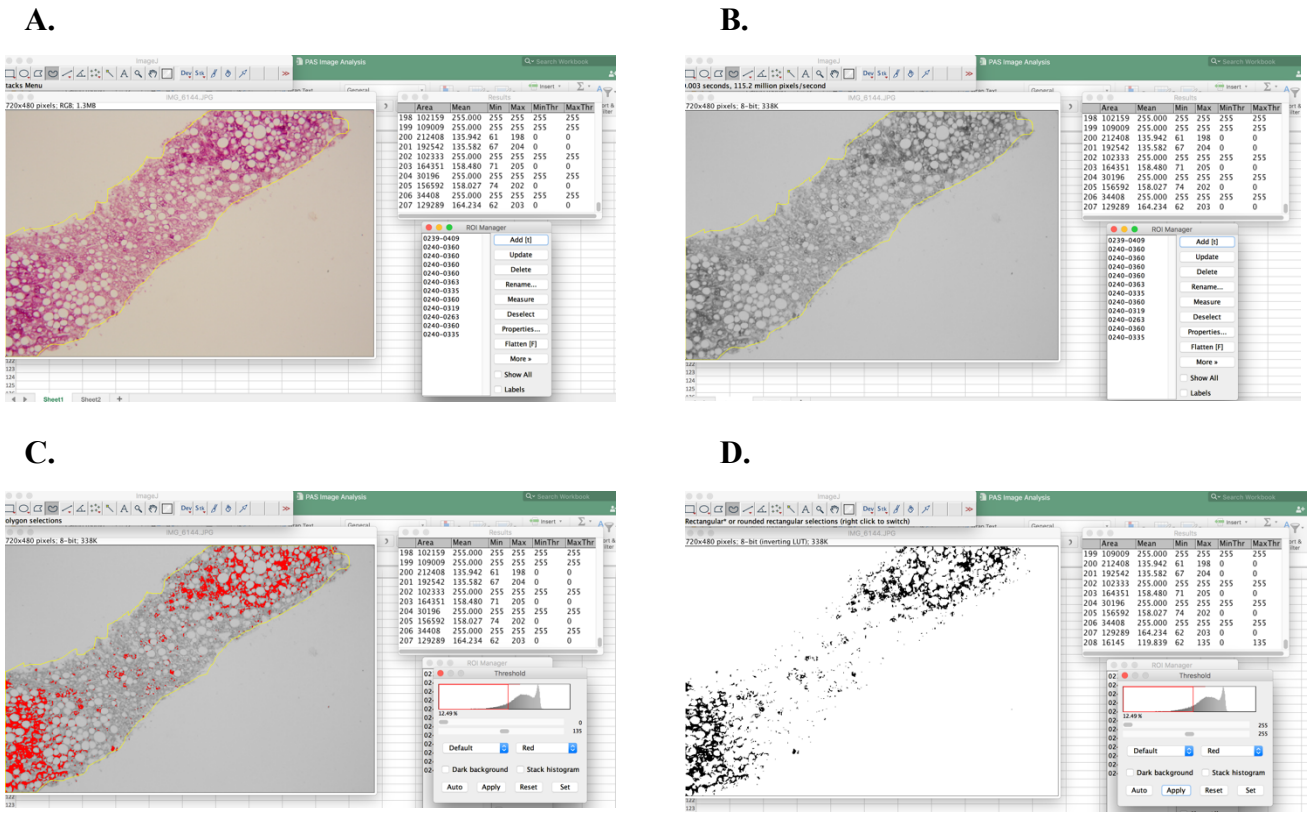


Figure 2.5A-D. Image analysis process for Periodic Acid Schiff (PAS)-stained sections. (**A**) Total tissue area calculated; (**B**) Image converted to grey-scale; (**C**) Positive stain threshold set; and (**D**) Amount of positive stain compared to tissue area calculated.

2.5 Perfusate Sampling and Analysis

During all perfusions described in this thesis, perfusate sampling was performed from the arterial sampling port on the *metra*, located on the shunt between the hepatic artery and IVC. Perfusate samples were either tested immediately after collection on clinical ABL90 Flex or ABL800 Flex Analysers (Radiometer, Crawley, UK) for: pO_2, pCO_2, pH, haemoglobin (Hb), haematocrit (Hct), sodium (Na), potassium (K), calcium (Ca), lactate and glucose or processed and stored at -80ºC for future analysis. Prior to storage, perfusate was centrifuged at 3500rpm for 15 min at room temperature. Supernatant was aspirated and pipetted in 500µL aliquots and initially stored on dry ice and then at -80ºC.

2.5.1 Perfusate Biochemistry

Perfusate 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
- Haemolysis Index

2.5.2 Triglyceride (TG) Measurement

2.5.2.1 Principle

The TG assay kit (Randox Laboratories Ltd, County Antrim, UK) is used for the determination of TG concentrations in serum or plasma. The reaction starts from the enzymatic hydrolysis of TG by lipase to glycerol and free fatty acids. The glycerol formed is then phosphorylated by glycerol kinase to glycerol-3-phosphate. Glycerol-3-phosphate is oxidised by glycerol phosphate oxidase producing dihydroxyacetone phosphate and hydrogen peroxide (H_2O_2). Peroxidase catalyses the redox-coupled reaction of H_2O_2 with 4-aminoantipyrine (4-AAP) and N-Ethyl-N-(3-sulfoproryl)-m-anisidine (ESPA), leading to production of a purple colour (Figure 2.6). The absorbance is measured at 540nm.

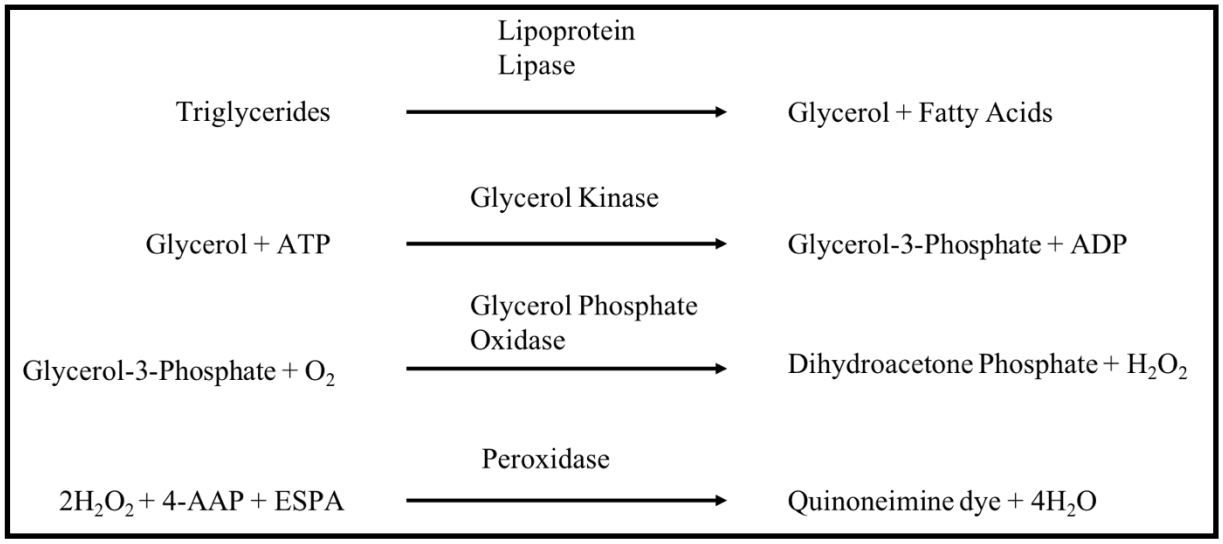


Figure 2.6. Series of reactions for the determination of TG concentrations (Randox Laboratories Ltd).

2.5.2.2 Method

Perfusate was thawed and vortexed. TG concentration was then determined on an iLAB 650 Clinical Chemistry analyser (Instrumentation Laboratory, Milano, Italy) using the TG assay kit (Randox Laboratories Ltd). Prior to running samples, the iLAB was calibrated using a range of 50, 100, 200 and 1000μM of glycerol standards.

2.5.3 3-Hydroxybutyrate (3-OHB) Measurement

2.5.3.1 Principle

The D-3-OHB assay kit (Randox Laboratories Ltd) is used for the determination of 3-OHB in serum and plasma. It is a kinetic enzymatic method based on the oxidation of 3-OHB to acetoacetate by the enzyme 3-Hydroxybutyrate dehydrogenase (3-HBDH). Subsequent to this oxidation, the cofactor NAD+ is reduced to NADH and the associate change in absorbance can be directly correlated with the 3-OHB concentration. The absorbance is measured at 505nm.

2.5.3.2 Method

Perfusate was thawed and vortexed. 3-OHB concentration was then determined on an iLAB 650 Clinical Chemistry analyser (Instrumentation Laboratory, Milano, Italy) using the D-3-OHB assay kit (Randox Laboratories Ltd). Prior to running samples, the iLAB was calibrated using 3 different concentrations (62.5, 125 and 500μmol/L) of the calibration standard supplied in the kit.

2.5.4 Apolipoprotein B (ApoB) Measurement

2.5.4.1 Principle

The ApoB assay kit (Randox Laboratories Ltd) is used for the determination of ApoB concentration in serum and plasma. This method is based on the reaction of a sample containing human ApoB and a specific antiserum to form an insoluble complex which can be measured turbidimetrically at 340 nm. By constructing a standard curve from the absorbances of standards, the concentration of ApoB can be determined.

2.5.4.2 Method

Perfusate was thawed and vortexed. A standard curve was created through 5 serial dilutions of the lyophilised calibrator with normal saline. ApoB concentration was then determined on an iLAB 650 Clinical Chemistry analyser (Instrumentation Laboratory, Milano, Italy) using the ApoB assay kit (Randox Laboratories Ltd).

2.5.5 Total Cholesterol (TC) Measurement

2.5.5.1 Principle

The TC assay kit (Randox Laboratories Ltd) is used for the determination of TC concentration in serum and plasma. Cholesterol is determined by enzymatic hydrolysis and

oxidation. The indicator is a quinoneimine formed from H_2O_2 and 4-AAP in the presence of peroxidase and phenol.

2.5.5.2 Method

Perfusate was thawed and vortexed. TC concentration was then determined on an iLAB 650 Clinical Chemistry analyser (Instrumentation Laboratory, Milano, Italy) using the TC assay kit (Randox Laboratories Ltd). Calibration was performed using the quality control serum available with the kit.

2.6 Statistical Methods

All analyses in this thesis were performed using GraphPad Prism version 7 for Mac OS X, GraphPad Software, La Jolla California USA. For continuous variables, a D'Agostino-Pearson normality test was performed to establish data distribution. A Student's *t*-test was used to analyse parametric, continuous variables and the Mann-Whitney U test used for non-parametric continuous variables. A Fisher's exact test used for categorical variables. One-way ANOVA was used to compare more than two groups. Survival analyses were performed using the log-rank test. Pearson r test was used to test for correlation. Continuous variables are presented as mean ± standard deviation (SD) or median with range. A p value <0.05 was considered statistically significant.

Chapter 3

The effect of cold storage prior to normothermic machine perfusion: facilitating clinical adoption of a novel technology

3.1 Introduction

Pilot studies have demonstrated the safety and feasibility of NMP in the clinical setting (4, 56, 57). Recently, a multicentre phase III randomised-controlled trial demonstrated the superiority of NMP compared to SCS in terms of biochemical markers of early graft injury, reduced IRI and improved organ utilisation (58). In this, the only randomised trial of NMP to date, perfusion was implemented for the entire preservation period, from retrieval to transplantation, including transportation (continuous NMP). Clinical adoption of NMP may be facilitated by the use of NMP after a period of SCS, thereby simplifying logistics and reducing costs. However, there is experimental evidence suggesting that rapid re-warming after a period of cooling can induce cellular injury (65). To date, no formal comparison has been made in human liver transplantation between continuous and post SCS-NMP (pSCS-NMP). It is unclear whether any deleterious effects of SCS on the graft can be mitigated by NMP, resulting in comparable outcomes to grafts where cold ischaemia has been minimised.

The aims of this chapter are:

- To assess the safety and feasibility of pSCS-NMP.
- To compare perfusion parameters between pSCS-NMP and continuous NMP livers.
- To compare clinical outcomes between pSCS-NMP, continuous NMP and SCS livers.

3.2 Methods

3.2.1 Study Design and Ethical Approval

In this prospective clinical trial, livers were recruited by three UK transplant centres until 30 recipients were transplanted after the intervention. Outcomes were compared to the livers transplanted as part of the recently-completed phase III randomised controlled trial comparing continuous NMP with SCS (58). The current trial was approved by the London-Dulwich research ethics committee (16/LO/2196) and registered (NCT03176433).

3.2.2 Eligibility and Consent

Whole livers from DBD and DCD (Maastricht category III (177)) aged at least 16 years were eligible. Recipients were eligible provided they were at least 18 years old and listed for a liver-only transplant, excluding those with fulminant liver failure, due to the poor prognosis of this group regardless of organ quality.

The study protocol stipulated that livers should be cold-stored for a minimum of 3 h and maximum of 8 h before being placed on the NMP circuit. These time limits were established in order to ensure the intervention was tested consistently and to reflect the UK practice of cold ischaemia times (29).

3.2.3 Post-SCS-NMP (study) group

Livers were retrieved and transported in UW solution at 4^{o}C according to standard practice. The device and liver were prepared and perfusion commenced as described in Chapter 2, sections 2.1 and 2.2. On arrival of the organ at the transplanting centre a standard liver back-table was performed (17).

The minimum NMP duration was set at 4 h, the time taken for ATP repletion in animal DCD models (48), and to provide time for optimal functional assessment. Livers could be perfused for a maximum of 24 h, as per the regulatory approval of the device, governed by local operating room logistics. Duration of perfusion was at the discretion of the operating surgeon. Blood gas analysis, sampled from an outlet via the hepatic artery line, was performed after 15 min and one hour from the beginning of perfusion and then at least 4 hourly until the end of perfusion. A decision was made to transplant the liver based on its objective function during NMP. Functional parameters, including: lactate clearance, glucose metabolism, pH maintenance, bile production, bile pH, perfusate transaminase levels and flow rates, were used to establish liver quality and function as has been previously described (50, 60, 61). The final decision to transplant the liver was made by the transplanting surgeon.

3.2.4 Control Groups

Livers that were transplanted by the UK centres of the phase III randomised controlled trial comparing the efficacy of continuous NMP (n = 104) with SCS (n = 182) in liver transplantation (ISRCTN 39731134) (58) were used as the control groups. We decided to include the whole UK cohort within this previous trial (rather than matching), in order to minimise any potential selection bias. To confirm the comparability of the groups, donors were compared for donor type, age, sex, BMI, and DLI (20) . Recipients were compared for age, sex, BMI and MELD score.

In the SCS control group the organ retrieval, preservation and transplantation was performed according to conventional standard practice with the liver flushed and stored in UW solution at 4^{o}C.

In the continuous NMP control group, the donor liver was connected to the OrganOx *metra*® normothermic device at the donor hospital after a standard deceased donor organ retrieval. It remained on the device for the duration of preservation until the transplanting surgeon was ready to implant the liver into the recipient.

3.2.5 Study End-Points

The primary endpoint was 30-day graft survival, in order to test the safety and feasibility of the pSCS-NMP approach.

Secondary end-points included:

- Peak serum AST in the first 7 days post-transplant. This is a validated surrogate marker, predictive of PNF as well as graft and patient survival (53, 54). It is also associated with histological evidence of moderate to severe reperfusion injury (108, 109).
- Early allograft dysfunction (EAD) (178): any one of: (i) bilirubin > 172 μmol/l on day 7 post-transplant; (ii) INR > 1.6 on day 7 post-transplant; (iii) peak-AST > 2000 IU/L during the first 7 days.
- Model for Early Allograft Dysfunction (MEAF) score (179): a validated continuous scoring model graded 0-10 comprising the highest serum ALT and INR in the first 3 post-operative days and bilirubin value on day 3 following liver-transplantation. The MEAF score has been proposed as a superior predictor of transplant survival than EAD (180).
- PNF: irreversible graft dysfunction requiring emergency liver replacement during the first 10 days after liver transplantation, in the absence of technical or immunological causes.

- Post-reperfusion syndrome (PRS): a decrease in mean arterial pressure (MAP) of more than 30% from the baseline value for more than one minute during the first five minutes after reperfusion (181, 182).
- Need for renal replacement therapy (RRT).
- Duration of initial post-operative critical care and hospital stays.
- Adverse event rates and severity, graded according to the Clavien-Dindo classification (183).
- Biliary complications: bile leak, anastomotic and non-anastomotic strictures.
- Graft histology
- 6-month graft and patient survival

3.2.6 Perfusate Biochemistry

Perfusate sampled at 15 min, 1 h and at the end of preservation was collected, processed, stored and analysed as described in Chapter 2, section 2.5 for 20 pSCS-NMP livers. Analytes assessed at each time point included: ALT, AST, GGT, bilirubin, ALP, LDH and haemolysis index. Changes in analytes were recorded over time and the correlation at each time point with peak AST was assessed.

3.2.7 Post-SCS NMP Liver Histology

For the 30 transplanted pSCS-NMP livers, core biopsies were obtained at the end of SCS and at the end of NMP and fixed in formalin (Chapter 2, section 2.3.1). Sequential biopsies in individual livers all came from the same part of the liver. Sections were stained with H&E (Chapter 2, section 2.3.4) and PAS (Chapter 2, section 2.3.5). Each section was scored by a consultant histopathologist, blinded to the time at which the biopsy was taken. In order to

establish potential mechanisms and to investigate any progression during NMP, H&E sections were scored for small and large droplet macrovesicular steatosis (none/mild/moderate/severe), necrosis, fibrosis, sinusoidal leukocytosis, sinusoidal dilatation and PAS sections scored for glycogen depletion (none/focal/scattered/confluent).

3.3 Results

Between 4th May and 18th July 2017, 30 liver transplants were performed using donor organs subjected to pSCS-NMP during preservation. In total, 51 livers were recruited to the study with 21 being excluded for the following reasons: non-proceeding DCD (n = 12); suboptimal appearance at retrieval (n = 4); poor function during NMP (n = 3); change to super-urgent recipient (n = 1); leak from NMP disposable circuit during priming before commencement of NMP resulting in liver remaining cold-stored (n = 1).

3.3.1 Donor and Recipient Demographics

For the 30 pSCS-NMP the indications for transplant were: primary sclerosing cholangitis (n = 6), non-alcoholic steatohepatitis (n = 6), hepatocellular carcinoma (n = 5), alcoholic liver disease (n = 5), hepatitis C cirrhosis (n = 4), autoimmune hepatitis (n = 2), primary biliary cirrhosis (n = 1) and polycystic liver disease (n = 1).

Baseline donor and recipient demographics of the study (pSCS-NMP) and control groups (continuous NMP or SCS) were similar (Table 3.1). However, the DLI score of the pSCS-NMP and continuous NMP groups was higher than that in the SCS arm (1.35 vs. 1.18), placing these donor livers in the highest-risk quartile (20).

Table 3.1. Donor and recipient demographics

		pSCS-NMP (n=30)	Continuous NMP (n=104)	SCS (n=82)	p value
Donor type	DBD (%)	22 (73)	73 (70)	66 (80)	0.27
	DCD (%)	8 (27)	31 (30)	16 (20)	
Donor age		56 (17 - 78)	55 (17 - 83)	55 (20 - 88)	0.9
Donor sex	Male (%)	18 (60)	58 (56)	44 (54)	0.83
	Female (%)	12 (40)	46 (44)	38 (46)	
Donor BMI (kg/m^2)		26 (17 - 46)	26 (16 - 47)	26 (17 - 43)	0.8
DLI		1.35 (0.6 - 3.15)	1.35 (0.76 - 4.13)	1.18 (0.63 - 3.64)	0.18
Recipient age		58 (25 - 73)	56 (20 - 73)	55 (22 - 71)	0.8
Recipient sex	Male (%)	22 (73)	74 (71)	59 (72)	0.97
	Female (%)	8 (27)	30 (29)	23 (28)	
Recipient BMI (kg/m^2)		26.9 (19.9 - 43.4)	26.2 (18.0 - 51.0)	27.0 (19.6 - 43.0)	0.99
MELD score		14 (7 - 24)	13 (6 - 33)	14 (6 - 28)	0.92

Data presented as median (range).

3.3.2 Preservation Duration

The total preservation time (mean) of livers in the pSCS-NMP group was 14 h 25 min ± 4 h 37 min; this included 6 h 1 min ± 1 h 21 min of SCS before 8 h 24 min ± 4 h 4 min of NMP. The longest total preservation time was 25 h 39 min. The total preservation time in the study group (pSCS-NMP) was significantly longer than either of the continuous NMP (14 h 25 min ± 4 h 37 min vs. 12 h 3 min ± 4h 11 min ($p = 0.008$)) and SCS (14 h 25 min ± 4 h 37 min vs. 7 h 37 min ± 2 h 24 min ($p < 0.0001$)) control groups.

3.3.3 NMP Parameters

3.3.3.1 Flow Dynamics

The mean arterial pressure during NMP in the study group was 67 ± 3 mmHg which resulted in a mean arterial flow rate of 0.44 ± 0.15 L/min. Mean arterial flow in the continuous NMP control group was significantly lower at 0.29 ± 0.12 L/min ($p < 0.0001$) despite no difference in arterial pressure. Similar mean portal flow rates were seen in the pSCS-NMP and continuous NMP control group (1.08 ± 0.12 L/min vs 1.1 ± 0.12 L/min, $p = 0.6$, respectively).

3.3.3.2 Acid-Base Homeostasis

Following the initial bolus(es) of 8% sodium bicarbonate (10 – 40 ml in the first 2 h of perfusion, according to pH), the pH of all transplanted livers increased from 6.99 ± 0.11 at 15 min to 7.33 ± 0.06 at the end of preservation. A mean pH of 7.32 ± 0.06 was achieved by 4 h which remained stable and unsupported from this time until the end of perfusion. Mean perfusate pH levels at 15 min, 60 min, 240 min and end of perfusion were similar to those seen in livers in the continuous NMP control group (n=69) (Figure 3.1).

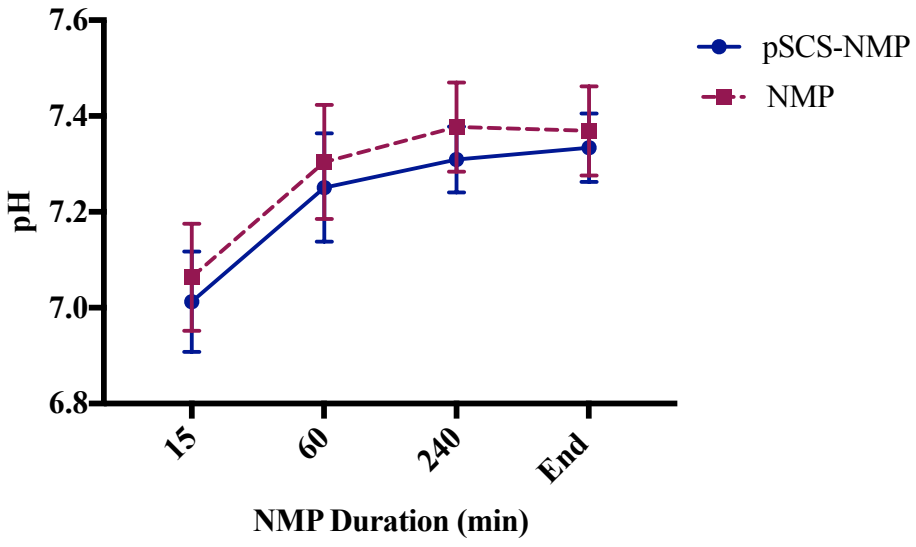


Figure 3.1. Comparison of perfusate pH during NMP between pSCS-NMP study group and continuous NMP control group. Data presented as mean ± SD.

3.3.3.3 Lactate Clearance

All transplanted livers showed evidence of lactate clearance from a starting median level of 7.3 mmol/L (1.9 - 10.1 mmol/L) to 0.8 mmol/l (0.2 - 2.2 mmol/L) by the end of preservation ($p < 0.0001$) (Figure 3.2). The perfusate lactate level was significantly ($p < 0.0001$) higher at 15 min in the continuous NMP control group compared to the pSCS-NMP group (Figure 3.2). However, by 60 min, lactate levels had decreased to 1.3 mmol/L (0.5 - 7.2 mmol/L) in the pSCS-NMP group and 1.3 mmol/L (0.3 - 12.5 mmol/L) in the continuous NMP group ($p = 0.87$) and remained comparable in the groups until the end of perfusion (Figure 3.2).

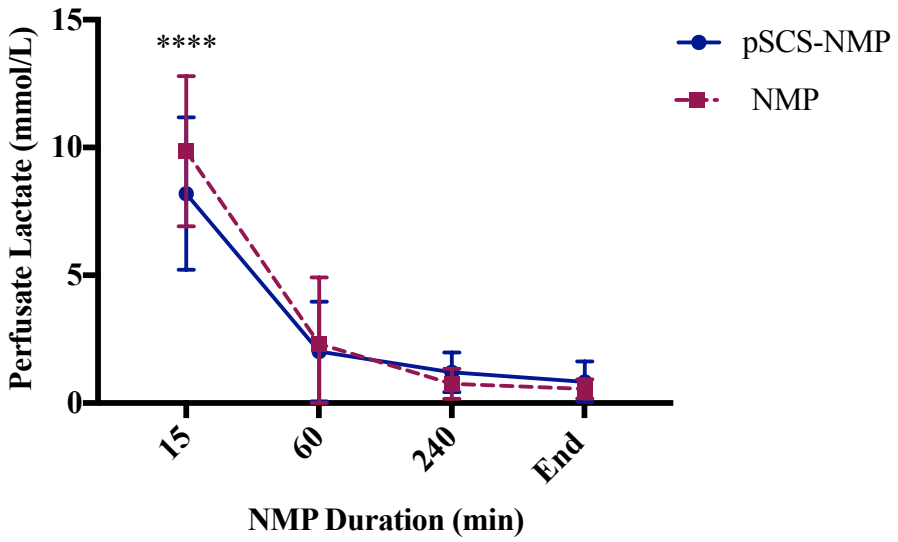


Figure 3.2. Comparison of perfusate lactate concentrations during NMP between pSCS-NMP study group and continuous NMP control group. Data presented as mean ± SD. **** p value <0.0001.

3.3.3.4 Glucose Metabolism

Glucose was metabolised by all transplanted livers as evidenced by a significant ($p < 0.0001$) decrease from 24.6 ± 7.1 mmol/L at 15 min to 10.8 ± 3.9 mmol/L at the end of perfusion (Figure 3.3). When comparing glucose metabolism at each time point between the pSCS-

NMP group and continuous NMP control group, no significant differences were observed over time (Figure 3.3).

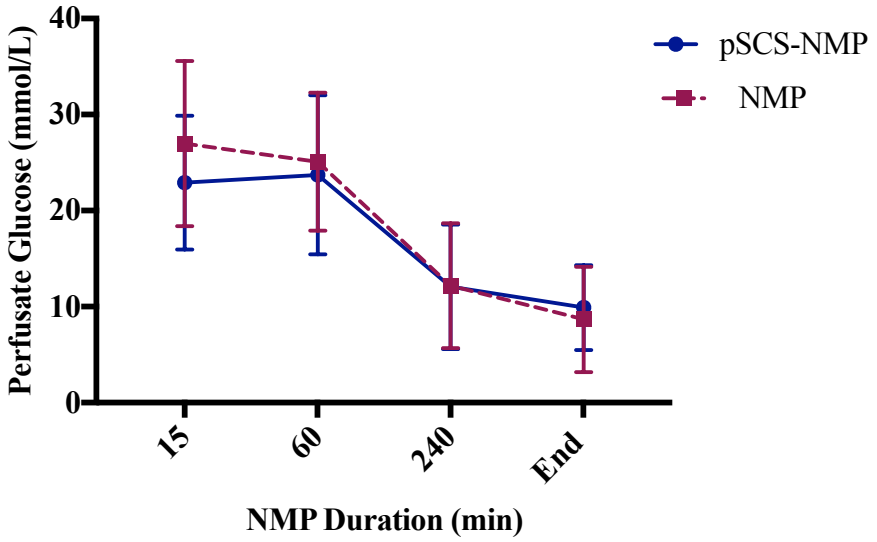


Figure 3.3. Comparison of perfusate glucose concentration during NMP between pSCS-NMP study group and continuous NMP control group. Data presented as mean ± SD.

3.3.3.5 Bile Production

Bile production was comparable in the NMP study group and the continuous NMP control group (10.5 ml/h (0 – 50 ml/h) vs 9 ml/h (0 – 42 ml/h) (p = 0.32), respectively). Four transplanted livers in the pSCS-NMP group produced no bile throughout normothermic preservation; this was in line with experience in the NMP control group.

3.3.4 Clinical Outcomes

3.3.4.1 Graft Survival (primary outcome)

Thirty-day graft survival was 93% in the pSCS-NMP group. The two graft losses were due to acute hepatic artery thrombosis (HAT); both patients were successfully re-transplanted. The donor and preservation data for all graft losses are provided below (Table 3.2). Thirty-

day graft survival was 98% in the continuous NMP control group (p = 0.22) and 99% in the SCS control group (p = 0.17).

There were no cases of PNF and median duration of initial inpatient stay was 13 (7 - 31) days. By reference, both continuous NMP and SCS control groups had a median duration of initial inpatient stay of 15 days.

Table 3.2. Demographics for graft losses.

	Donor type	Donor age	DLI	CIT	NMP duration	Mean arterial flow (ml/min)	Final perfusate lactate (mmol/L)	Bile production (ml/h)	Aetiology of graft loss
Liver 1*	DCD	26	1.41	7h 30min	8h 31min	490	0.9	0	HAT
Liver 2*	DBD	51	1.17	5h 11min	9h 23min	50	0.6	8	HAT
Liver 3	DBD	52	0.92	6h 36min	5h 31min	460	0.6	0	Recurrent hepatitis C infection
Liver 4	DBD	70	1.24	5h 5min	4h 57min	520	1.8	28	Portal hypertension due to AV fistula

* Graft loss within 30 days post-transplantation
Abbreviations – AV, arterio-venous; CIT, cold ischemia time; DBD, donor after brain-stem death; DCD, donor after circulatory death; DLI, donor liver index; HAT, hepatic artery thrombosis; NMP, normothermic machine perfusion

3.3.4.2 Adverse Events

Seven patients (23%) developed complications of grade IIIb severity (Clavien-Dindo classification (183)) or above. These included surgical intervention (re-transplantation (n =

2), bleeding (n = 1), corrective vascular intervention (n = 1) and sepsis control (n = 1)) and RRT alone (n=2).

The percentage of complications ≥ IIIb severity was 24% in the continuous NMP control group (p > 0.99) and 22% in the SCS control group (p > 0.99).

3.3.4.3 Early Biochemical Function

The median peak serum AST in the first 7 days postoperatively was 485U/L (92-8669U/L) in the pSCS-NMP group, which was similar to the level of 465U/L (68-8822U/L) in the continuous NMP control group (p=0.89), and significantly less than that of the SCS control group (1138U/L (105-11001U/L, p=0.003) (Figure 3.4A).

Four of the 30 pSCS-NMP livers (13%) displayed EAD; this is similar to what was observed in the continuous NMP cohort (11%) (p=0.74) and contrasts with the 33% incidence seen in the SCS group (p=0.06) (Figure 3.4B).

For the MEAF score calculation, data were missing for 5 (17%) pSCS-NMP livers, 26 (26%) continuous NMP livers and 21 (27%) SCS livers. Median scores were similar between the pSCS-NMP (3.00 (0.74 - 8.85)) and continuous NMP (3.55 (1.03 - 9.49)) groups (p = 0.22) and significantly reduced compared to the SCS control group (4.94 (1.07 - 9.42); p = 0.002) (Figure 3.4C).

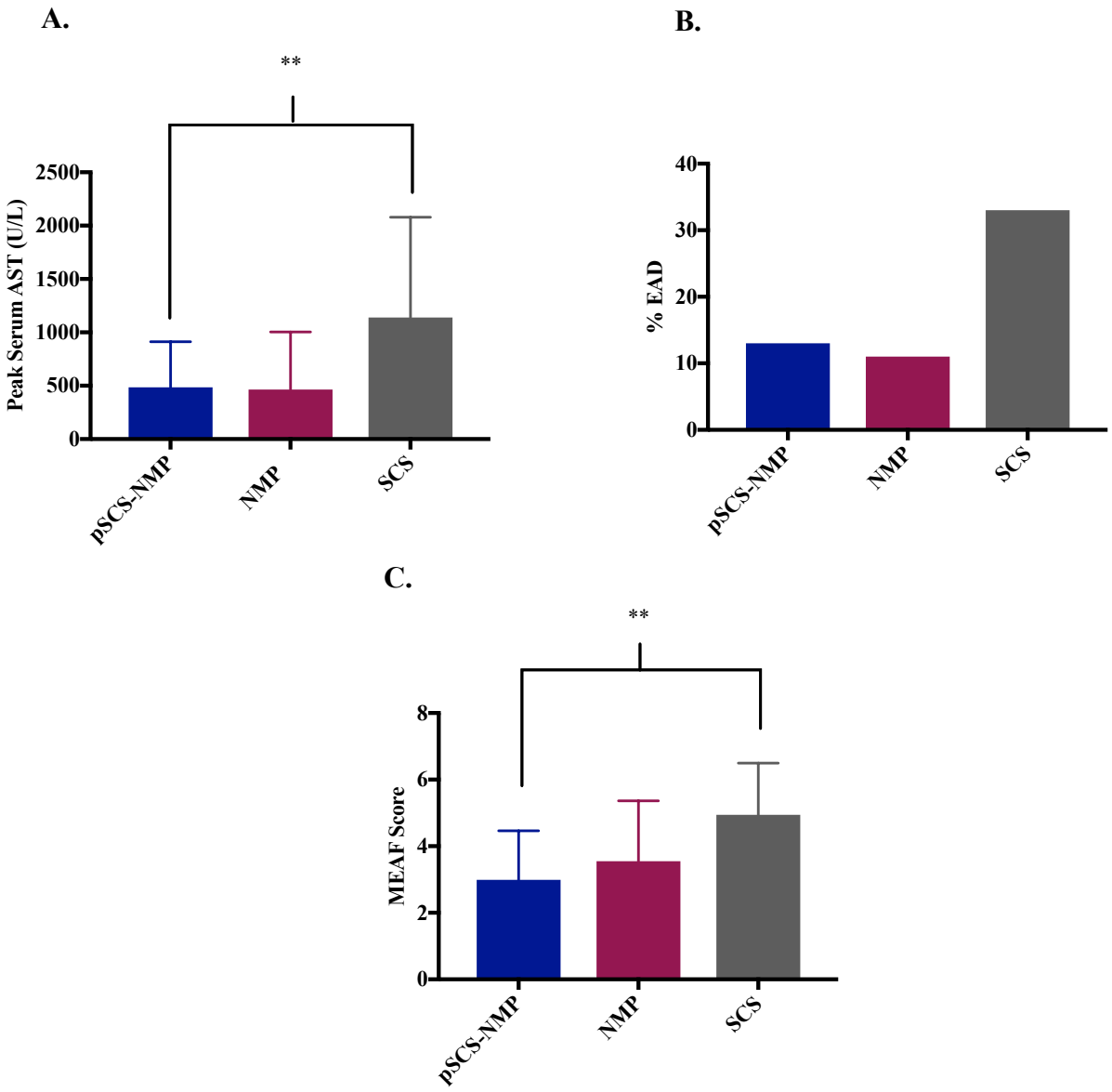


Figure 3.4A-C. Comparison of early biochemical outcomes in terms of **(A)** peak serum AST, **(B)** EAD and, **(C)** MEAF score between pSCS-NMP study group and continuous NMP and SCS control groups. Data presented as median with IQR **(A)**, absolute percentage **(B)** and mean ± SD **(C)**. ** p value < 0.01.

3.3.4.4 Physiological Response to Preservation Injury

Three patients (10%) in the pSCS-NMP group experienced post-reperfusion syndrome (PRS) immediately following portal reperfusion of the liver. This is similar (11%) to what had been experienced in the continuous NMP control group and a significant reduction compared to the SCS group (34%) ($p = 0.02$).

Five patients (17%) required RRT in the first 30-days post-transplantation. Although this is numerically lower than in the control groups (26% NMP and 24% SCS) these differences do not reach statistical significance (p = 0.34 and p = 0.45, respectively).

Median duration of initial critical care stay was 3.5 (range 1 - 20) days. This was similar to both control groups, with median durations of 4 (1 - 23) NMP (p = 0.95) and 4 (1 - 58) SCS (p = 0.28).

3.3.4.5 Biliary Complications

At 6-months follow-up in the pSCS-NMP study group, two patients experienced biliary complications; one with a biliary anastomotic leak and the other, an anastomotic stricture successfully managed by endoscopic retrograde cholangiopancreatography and stent insertion. No cases of ischaemic cholangiopathy were observed in the pSCS-NMP study group. This is similar to the continuous NMP and SCS controls with one case of clinically apparent ischaemic cholangiopathy in each group.

6-month Patient and Graft Survival

There were no significant differences in patient and graft survival in the three groups. Six-month graft survival in the pSCS-NMP group was 87% compared to 94% in the continuous NMP control group (p=0.16) and 95% in the SCS control group (p=0.11). Six-month patient survival was 90% in the pSCS-NMP group compared to 95% in the continuous NMP control group (p=0.31) and 98% in the SCS control group (p=0.09). Donor, preservation details and causes of all graft losses were previously shown (Table 3.2).

3.3.5 Perfusate Analysis

During perfusion, circulating levels of ALT remained fairly stable, with a slight increase from 1065 ± 223 U/L to 1461 ± 289 U/L ($p = 0.28$) (Figure 3.5A) and a similar pattern was observed with AST (increase from 1172 ± 217 U/L to 1532 ± 277 U/L, $p = 0.31$) (Figure 3.5B). LDH levels also remained stable, with only a slight increase seen from the beginning (2522 ± 448 U/L) until the end (3070 ± 364 U/L) ($p = 0.35$) (Figure 3.5C). Significant increases in GGT (7 ± 1 U/L to 79 ± 16 U/L, $p < 0.0001$) (Figure 3.5D), ALP (9 ± 1 U/L to 50 ± 10 U/L, $p = 0.0002$) (Figure 3.5E) and bilirubin (2 ± 0 μmol/L to 6 ± 2 μmol/L) ($p = 0.002$) (Figure 3.5F) were observed. There was no evidence of active haemolysis during the perfusion (Figure 3.5G).

There was no correlation between any of the analytes tested during perfusion and peak serum AST in the recipient (Figure 3.6A-G).

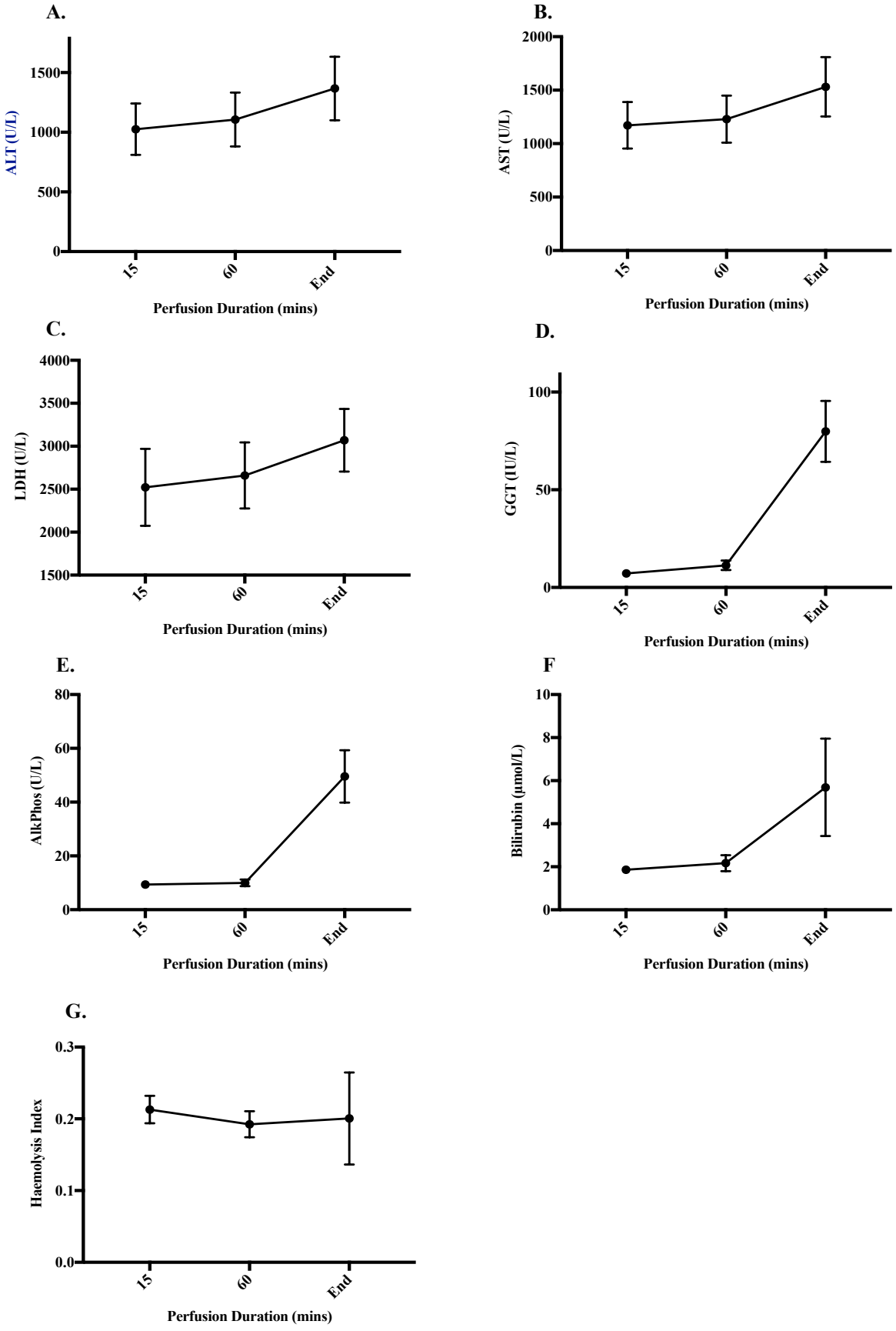


Figure 3.5A-G. Levels of perfusate markers from pSCS-NMP livers over time including: ALT **(6A)**, AST **(6B)**, LDH **(6C)**, GGT **(6D)**, ALP **(6E)**, bilirubin **(6F)** and haemolysis of red cells during the perfusion **(6G)**. Data presented as mean ± SD.

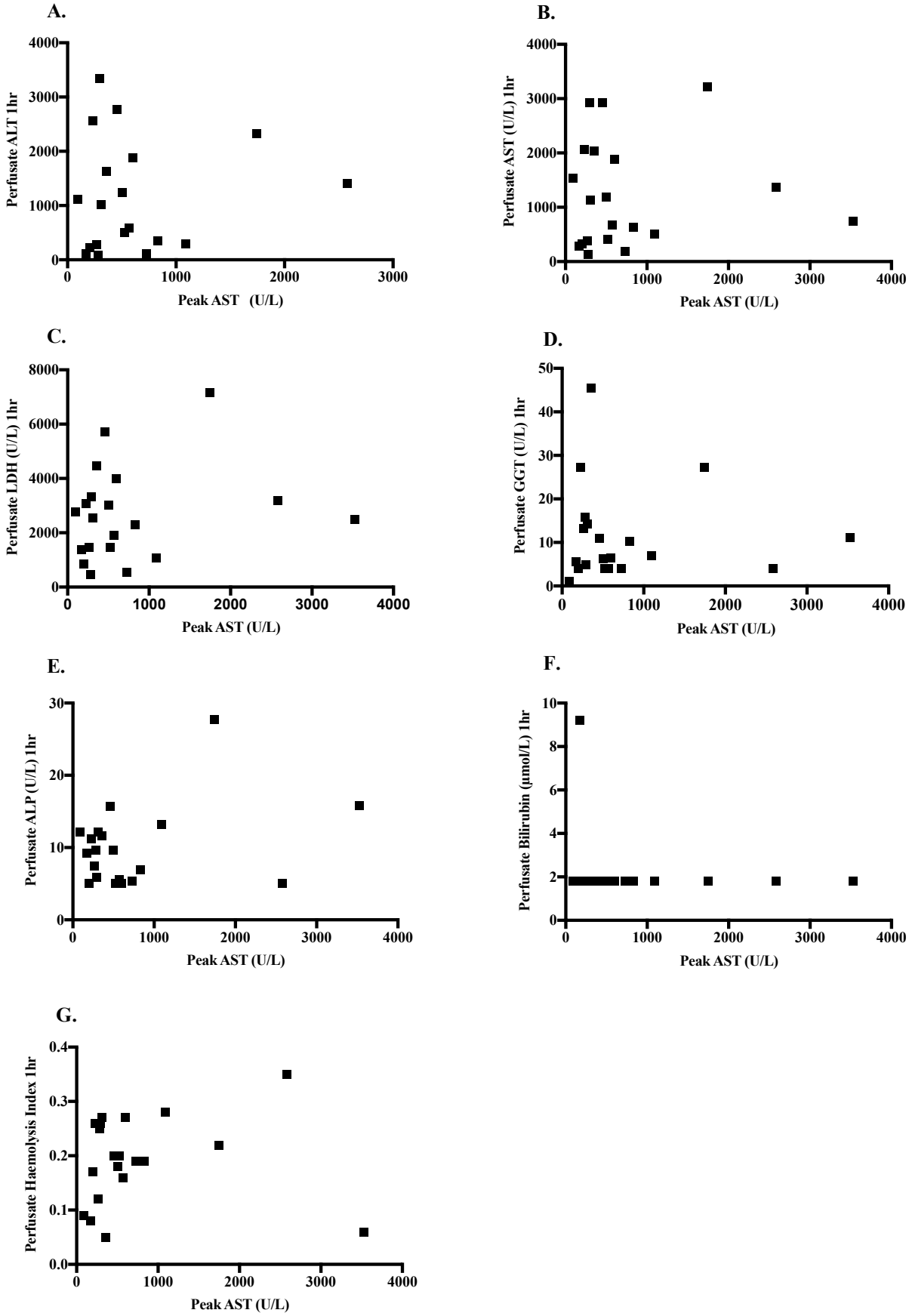


Figure 3.6A-G. Correlations between peak serum AST in the recipient and perfusate markers from pSCS-NMP livers at 1 h including ALT (r = 0.1, p = 0.68) **(A)**, AST (r = 0.04, p = 0.88) **(B)**, LDH (r = 0.2, p = 0.4) **(C)**, GGT (r = -0.02, p = 0.93) **(D)**, ALP (r = 0.32, p = 0.17) **(E)**, bilirubin (r = -0.16, p = 0.5) **(F)**, haemolysis index (r = 0.06, p = 0.82) **(G)**.

3.3.6 Histology

Histological assessment of the pSCS-NMP livers did not show any evidence of deterioration following NMP in terms of necrosis or fibrosis. Following the initial period of SCS, sinusoidal leukocytosis was observed in 50% of livers. This decreased to 17% of livers after NMP ($p = 0.03$) (Figures 3.7A-B). In 56% of livers, replenishment of glycogen stores was observed by at least one category (none, focal, scattered, confluent) after NMP (Figure 3.7C-D). Six livers displayed moderate/severe macrovesicular steatosis (predominantly large droplets) and the degree of steatosis did not appear to change following NMP.

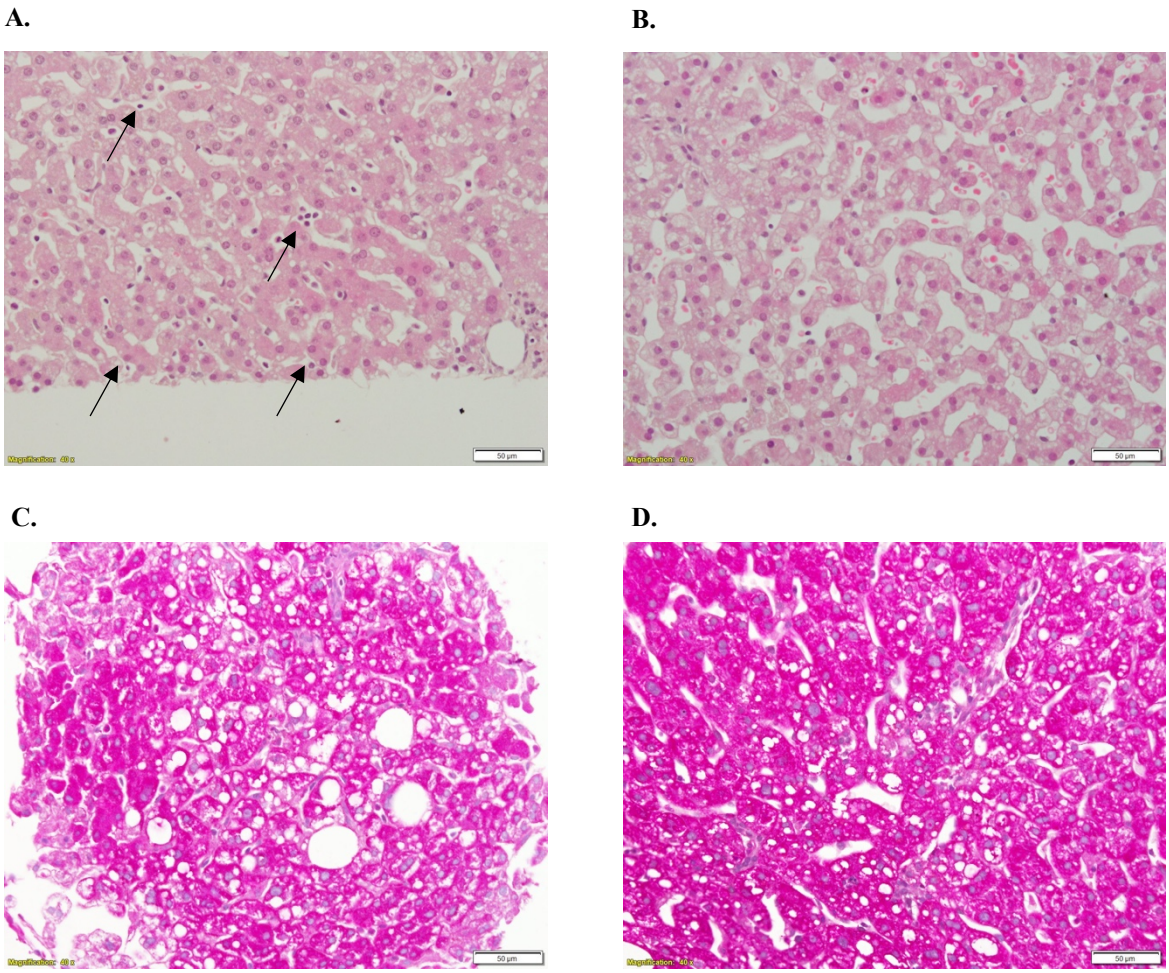


Figure 3.7A-D. Liver histology. H&E stained liver section demonstrating extensive sinusoidal leukocytosis after 6 h 42 min of SCS **(A)** with resolution after 11 h 58 min NMP **(B)**. PAS stained liver section demonstrating scattered glycogen depletion after 6 h 9 min of SCS **(C)** and complete replenishment of glycogen stores after 8 h 33 min of NMP **(D)**.

3.3.7 Livers Declined after NMP

Three livers in the pSCS-NMP group were discarded following a period of NMP due to concerns over their function on the device. The demographics of these livers and the justification for discard are shown below (Table 3.3).

Table 3.3. Donor and preservation characteristics of livers discarded after NMP

	Type	Age	DLI	CIT	Reason for Discard
Discard 1	DCD	70	2.5	7 h 34 min	• Climbing glucose • No bile production • Perfusate ALT 9268 U/L at 1 h
Discard 2	DBD	34	1.00	3 h 6 min	• Climbing glucose • No bile production • Lactate 4.1 mmol/L at 4 h • Persistent acidosis • Severe macrovesicular steatosis (80%)
Discard 3	DCD	59	1.74	7 h 12 min	• Lactate 4.2 mmol/L at 4 h • Bile pH 7.43 • Bile glucose 14 mmol/L

Abbreviations – ALT, alanine aminotransferase; CIT – cold ischaemia time; DBD, donor after brain-stem death; DCD, donor after circulatory death; DLI, donor liver index

3.4 Discussion

In this group of livers, the use of NMP following SCS (pSCS-NMP) with either NMP alone or SCS alone were compared. I have demonstrated comparable perfusion parameters and postoperative liver function between pSCS-NMP and continuous NMP livers and confirmed the previously reported benefit of NMP compared to SCS (4, 58) in terms of early graft injury, can be obtained using either modality.

Applying NMP after SCS has clear logistical and cost benefits. Removing the need to transport the device to and from a donor hospital undoubtedly reduces costs, whilst avoiding any prolongation of the donor procedure. Indeed, Selzner *et al* acknowledged that initiating NMP at the donor hospital, prolonged the procedure by 2 h (56). Although continuous NMP has been carried out in around 300 cases globally, and there have been no reported graft losses as a direct consequence of transportation (4, 56-58), transporting the liver on the device does add an inherent risk; any problem on the device is likely to be much more difficult to resolve if it were to occur in transit. Also, the simpler logistics are likely to facilitate the introduction of this technology into standard clinical practice.

There is an ongoing pressure to increase utilization of higher-risk deceased donor organs; this is driven by increasing numbers of waiting list deaths (10, 184). There is an expanding body of evidence that NMP is of benefit, not only by reducing preservation associated reperfusion injury (58), but also allowing surgeons to assess the viability of an organ before transplantation (50, 61). It is not clear whether the benefit of NMP is restricted to higher-risk organs – certainly the livers in this study had a median DLI within the top quartile of

UK donor livers, implying a 3-fold increase in the risk of graft loss compared to low-risk livers (20). It may not be possible to assess the benefit in low-risk organs until more experience has been gained in this context. Perhaps more importantly, livers in the upper quartile of donor risk have a 34% utilisation rate (20) and it is plausible that this technology, by removing the uncertainty surrounding these organs, may give surgeons the confidence to improve this rate of utilisation. This aligns with the observed increase in organ utilisation in the normothermic arm of the recently published randomised study of Nasralla *et al* (58).

The graft losses in this study do not appear to be attributable to the preservation method. However, it is notable that the hepatic artery flow was low in one of these livers which developed arterial thrombosis. In the case of sub-optmal hepatic artery flow, a doppler ultrasound scan during perfusion may be indicated to confirm vascular integrity and global organ perfusion. I observed a significantly higher arterial flow rate in the pSCS-NMP livers than in the continuous NMP control group during preservation (0.44 ± 0.15L/min vs. 0.29 ± 0.12L/min, respectively; $p < 0.0001$). The reasons for this are unclear, although a previous study observed initial vasoconstriction after cold flush followed by vasodilatation during cold storage in a pig renal artery which appeared to be attributable to intracellular sources of calcium (185). If this phenomenon also occurred in the hepatic artery, this might explain the observation, although this remains unproven.

The perfusate lactate level after 15min in the continuous NMP group (10.1mmol/L (1.2-16.0mmol/L)) was significantly higher than that of the pSCS-NMP group (7.3mmol/L (1.9-10.1mmol/L)) ($p<0.0001$). It has previously been shown that livers do not reach 4^{o}C until they have been in the ice box for around 90mins (186). It is therefore likely that the continuous NMP livers have more active anaerobic metabolism during the back-table phase,

as this takes place before complete cooling. Increased (anaerobic) metabolic activity may manifest as an increased lactate level at the beginning of perfusion. There is no evidence that this affects the liver's functional ability to clear the lactate or post-transplant outcome.

In this study, perfusate transaminase and LDH levels remained fairly stable, with no significant increase observed. This would suggest that there is no on-going liver injury during preservation and that the initial efflux of enzymes is most likely due to injury sustained during the retrieval process. Livers which had high or increasing transaminases were not transplanted as this may imply a serious initial insult or on-going injury. In this regard, my findings are consistent with Watson *et al* who observed a similar pattern of enzyme release (61). However, in the present study, there was no correlation between any perfusate markers and peak AST in the recipient. This is in contrast to Watson *et al* who identified a significant, positive correlation between perfusate and peak recipient transaminases (61). Furthermore, our group have previously demonstrated significantly higher perfusate ALT and LDH levels in livers with significant preservation injury (peak serum AST > 1000 U/L) (187).

In the present study, three recruited livers were not transplanted because of poor function, namely lactate clearance, pH stability and bile production during NMP. Although we cannot be sure that these livers would have experienced a poor post-transplant outcome, NMP appears to be able to discriminate between livers in terms of metabolic and synthetic function and concerns regarding a poorly functioning organ *ex situ* would appear to be logical. In each of these cases, the decision not to transplant the liver was made by the operating surgeon based on their own experiences and current evidence (61).

Contrary to established clinical practice, in this study there is no evidence that the prolonged preservation is detrimental to the graft. Histological analysis showed no new injury after NMP and there was a reduction in the clinical phenomenon of preservation injury after transplantation, as well as a reduction in sinusoidal leukocytosis after NMP, suggesting a reduced inflammatory stimulus.

The logistic benefits of NMP could be considerable. By facilitating prolonged preservation, centres would able to accept organs simultaneously or indeed plan elective operating more effectively. In the UK, from 2013-2016, a total of 163 livers were declined for logistical reasons alone (29). During this study, a shift from out-of-hours operating with 22 (73%) transplants being performed between 8am and 8pm was observed; the start time was often delayed, ensuring the staff were well-rested.

This study is not without limitations. The number of recruited livers is small, and therefore it is not adequately powered to detect differences between the pSCS-NMP and continuous NMP cohorts. The small sample size also precludes subgroup analyses, and it is possible that continuous NMP (as opposed to pSCS-NMP) may still be beneficial in the highest risk livers, in particular those with severe steatosis, where the adverse effects of cooling are well documented (75, 79). Use of a retrospective comparison with historical control groups is a recognised limitation, although use of the same inclusion criteria and comparison with the entire UK cohort from the previous RCT does help limit the risk of selection bias.

In summary, I have demonstrated that NMP after a period of SCS is both feasible and safe, demonstrating similar perfusion parameters and early clinical outcomes to continuous NMP and maintaining the benefits of NMP seen over SCS alone. This may have significant

logistical and cost benefits that are likely to facilitate the uptake of NMP in routine clinical practice.

Chapter 4

Investigating the clinical outcomes from steatotic livers preserved via normothermic machine perfusion

4.1 Introduction

NMP provides a potential platform to facilitate the transplantation of steatotic livers with improved outcomes. This could be achieved by avoiding the deleterious effects of cooling sustained during SCS and subsequently reducing IRI, to which steatotic livers are particularly susceptible (79). Experimental evidence from murine and porcine models suggest that NMP can decrease the fat content of the liver during a prolonged perfusion over several days (156), or over the course of a few hours in conjunction with de-fatting agents (157). However, to date, there have been no reports on the post-transplant outcomes of human steatotic livers preserved via NMP in transplantation. As steatotic livers form an increasing proportion of organ discards, enabling their safe and reliable transplantation would help bridge the gap between liver supply and demand, potentially making a significant contribution toward reducing waiting list deaths.

The hypothesis of this chapter is that clinical outcomes from steatotic livers preserved using NMP will be improved via a combination of enhanced preservation through the avoidance of cooling, a reduction in IRI and de-fatting.

Therefore, the aims of this chapter are:

1. To report and compare clinical outcomes in patients transplanted using steatotic livers preserved via NMP or SCS;

2. To explore changes in hepatocyte structure and function of human steatotic livers undergoing NMP; and,
3. To establish the impact of NMP on IRI.

4.2 Methods

As part of a phase III, multicentre RCT investigating the efficacy of NMP compared to SCS in liver transplantation (ISRCTN 39731134) (58), a biological sample biobank was established. The biobank was managed by the Consortium for Organ Preservation in Europe (COPE, www.cope-eu.org), and an application was approved by the management board (ref. Liver-13-OX-151104) to access liver biopsies, NMP perfusate and recipient blood samples that were collected as part of the trial protocol (Table 4.1).

Table 4.1. Samples obtained for the COPE Biobank

Sample	**Time points**
Liver core biopsy (3 samples per liver)	Taken pre- and post- preservation (NMP or SCS) and post-reperfusion.
Perfusate (3 samples per liver)	Collected at 15 and 60 min after initiation of NMP and at the end of NMP (which ranged between 4 – 24 h).
Recipient blood (2 samples per recipient)	Collected prior to transplantation (on induction of anaesthesia) and 60 min following reperfusion.

Samples were identified and selected from the COPE liver database based on the following criteria:

1) Moderate and severely steatotic livers (as assessed by lead multi-organ retrieval surgeon), which were successfully transplanted with a full complement of samples available in both NMP and SCS arms; and,
2) Lean controls (no steatosis as assessed by lead multi-organ retrieval surgeon) for both NMP and SCS groups.

All livers were matched for: donor type (DBD or DCD), donor age, DLI (20), recipient age

and recipient MELD score.

As the macroscopic appearance of the liver does not necessarily correspond to the histological degree of steatosis (188), all liver biopsies were stained with H&E (Chapter 2, section 2.3.4) and scored for total macrovesicular steatosis by a consultant histopathologist. Samples for further analysis were subsequently selected based on their histological steatosis score. This ensured that all selected livers objectively scored positively for steatosis. Livers with >10% total macrovesicular steatosis were included in the steatotic liver groups and those with <5% in the lean liver groups. The number of livers included for final analysis are shown in Table 4.2.

Table 4.2. Liver phenotype and preservation method.

Liver phenotype	Preservation Method	Number
Steatotic	NMP	18
Steatotic	SCS	15
Lean	NMP	15
Lean	SCS	10

4.2.1 Clinical Outcomes

Using the COPE clinical trial database, clinical data including donor and recipient demographics, preservation data and post-transplant outcomes were determined. This information was then reported for patients undergoing liver transplantation with steatotic livers preserved via NMP and SCS and compared with lean controls for each group.

Outcomes reported included:

- Peak serum AST in the first 7 days post- transplant.
- EAD (178), defined by any one of:
    - Bilirubin > 171 μmol/L on day 7 post-transplant
    - INR > 1.6 on day 7 post-transplant

- Peak AST > 2000 U/L within the first 7 days post-transplant

- PNF, defined as irreversible graft dysfunction requiring emergency liver replacement during the first 10 days after liver transplantation, in the absence of technical or immunological causes.
- Evidence of post-reperfusion syndrome as defined by a decrease in mean arterial pressure (MAP) of more than 30% from the baseline value for more than one minute during the first five minutes after reperfusion (181, 182).
- Need for RRT.
- Critical care stay duration.
- Duration of initial inpatient stay.
- 1-year graft and patient survival.

4.2.2 Changes in hepatocyte structure and function

In order to establish whether the degree of steatosis can be reduced during NMP, all H&E stained liver sections were scored for total macrovesicular steatosis before NMP, at the end of NMP and post-reperfusion. A consultant histopathologist who was blinded to both the preservation method and the timing of the biopsy scored all of the sections. A comparison was made between the amount of macrovesicular steatosis at each time point for the steatotic NMP and SCS livers as well as matched lean livers in each preservation group.

In order to gain an initial understanding and explore intrahepatic metabolism during NMP, perfusate collected at 15 and 60 min from initiation of NMP and at the end of NMP (range 4 - 24 h) was analysed for: TG, total cholesterol, 3-OHB, urea and ALT. These perfusate markers were compared during NMP between steatotic and lean livers. All analyses were

performed using an ILab 600 or 650 chemical analyser (Instrumentation Laboratory, Bedford, USA) (Chapter 2, section 2.5).

4.2.3 IRI

Several histological methods were adopted in an attempt to quantify and compare the extent of IRI between the groups:

4.2.3.1 Neutrophil infiltration

The extent of neutrophil infiltration post-transplantation was assessed by a blinded consultant histopathologist in each of the steatotic and lean liver groups for both NMP and SCS.

4.2.3.2 Glycogen quantification

As glycogen depletion post-reperfusion has also been implicated in IRI, whereby glycogen consumption has resulted as a consequence of the ischaemic injury, glycogen was assessed in a sub-set of livers. Livers with the highest levels of macrovesicular steatosis were selected from the NMP and SCS groups (n = 5 in each group). Pre-preservation, post-preservation and post-reperfusion sections were stained with PAS (Chapter 2, section 2.3.5) and glycogen was quantified as described in Chapter 2, section 2.4.

4.2.3.3 Lipid Peroxidation

4-hydroxynonenal (HNE) is a reactive aldehyde and an end-product of lipid peroxidation, produced by ROS (189). Post-reperfusion sections were stained with anti-4HNE antibody (abcam, ab46545). Briefly, sections were de-waxed and de-hydrated as previously described (Chapter 2, section 2.3.4.2). Antigen retrieval was performed by warming the slides in an

EDTA-TRIS buffer solution. Endogenous peroxidase activity was then blocked by immersing the slides in hydrogen peroxide (VWR International Ltd) and methanol (VWR International Ltd) for 30 min. After washing in TRIS-buffer saline, the non-specific binding of antisera were blocked by adding non-immune swine serum (Fisher Scientific UK Ltd) (1 in 20 dilution with TRIS-buffer) for 20 min. The anti-4HNE antibody (abcam, ab46545) was diluted in TRIS-buffer at a concentration of 1 in 200 and 100 μL was added to each section and left overnight at 4ºC. After washing in TRIS-buffer saline, the secondary antibody was applied (α rabbit horseradish peroxidase (Fisher Scientific UK Ltd)) diluted 1 in 100 with TRIS-buffer. Tyramide amplification was then performed prior to re-washing in TRIS-buffer saline and cover-slip placement. A confocal microscope was used to detect green fluorescence (positive for 4HNE) and sequential images were obtained at 10x magnification across each section.

4.2.3.4 Systemic inflammation

Pre- and post-reperfusion recipient plasma samples were analysed for key cytokines which have previously been implicated in hepatic IRI (190). A bespoke human magnetic luminex® assay kit (R&D Systems) containing the analytes: CXCl8/IL-8, IL-10, IL-2, TNF-α, IFN-γ, IL-13, IL-4, IL-1β, IL-17A and IL-6 was used. The nature and function of each of these are listed in Table 4.3. The quantity of each cytokine identified in the plasma was compared pre- and post-reperfusion for each of the steatotic NMP (n=14), steatotic SCS (n=11), lean NMP (n=6) and lean SCS groups (n=7).

Table 4.3. Analytes investigated in the plasma of liver transplant recipients pre- and post-reperfusion along with their main functions.

Analyte	Function
CXCL8/IL-8	Induces chemotaxis in target cells, primarily neutrophils and causes them to migrate toward the site of infection. Induces phagocytosis. IL-8 is also known to be a potent promoter of angiogenesis.
IL-10	Anti-inflammatory cytokine.
IL-2	Regulates activity of white blood cells
TNF-α	Cell signalling protein involved in systemic inflammation and makes up acute phase reaction.
IFN-γ	Important activator of macrophages and inducer of Class II major histocompatibility complex (MHC) molecule expression.
IL-13	Central mediator of the physiologic changes induced by allergic inflammation in many tissues.
IL-4	Stimulation of activated B-cell and T-cell proliferation and differentiation of B cells into plasma cells. It is a key regulator in humoral and adaptive immunity.
IL-1β	Produced by activated macrophages and is important mediator of the inflammatory response. Is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis.
IL-17A	Pro-inflammatory cytokine produced by activated T cells.
IL-6	Important mediator of fever and of the acute phase response.

4.3 Results

4.3.1 Donor and Recipient Demographics

All livers included in the study were from DBD donors and were well matched for other donor and recipient characteristics with no significant differences between groups (Tables 4.4 and 4.5).

Table 4.4. Donor demographics.

		Steatotic NMP	Steatotic SCS	Lean NMP	Lean SCS	p value
Age (years)		54 ± 12.47	51 ± 9.69	59 ± 17.79	55 ± 18.89	0.51
Sex	Male (%)	67	53	33	50	0.42
	Female (%)	33	47	67	50	
BMI (kg/m^2)		31 ± 8.07	30 ± 5.56	26 ± 6.87	27 ± 4.25	0.16
DLI		1.34 ± 0.43	1.14 ± 0.24	1.21 ± 0.26	1.15 ± 0.16	0.27

Data presented as mean ± SD.

Table 4.5. Liver recipient characteristics.

		Steatotic NMP	Steatotic SCS	Lean NMP	Lean SCS	p value
Age (years)		58 ± 11.22	56 ± 10.46	51 ± 11.36	56 ± 9.44	0.33
Sex	Male (%)	89	73	60	70	0.30
	Female (%)	11	27	40	30	
BMI (kg/m^2)		31 ± 5.72	30 ± 5.94	27 ± 8.07	27 ± 3.91	0.15
MELD		16 ± 5.52	13 ± 6.26	15 ± 5.09	14 ± 6.04	0.62

Data presented as mean ± SD.

4.3.2 Steatosis Scores

The median steatosis percentage in the steatotic NMP group was 40% (10 – 80%) and in the steatotic SCS group was 20% (10-90%) (p = 0.69). The distribution of the degree of steatosis for all the groups are shown in Figure 4.1.

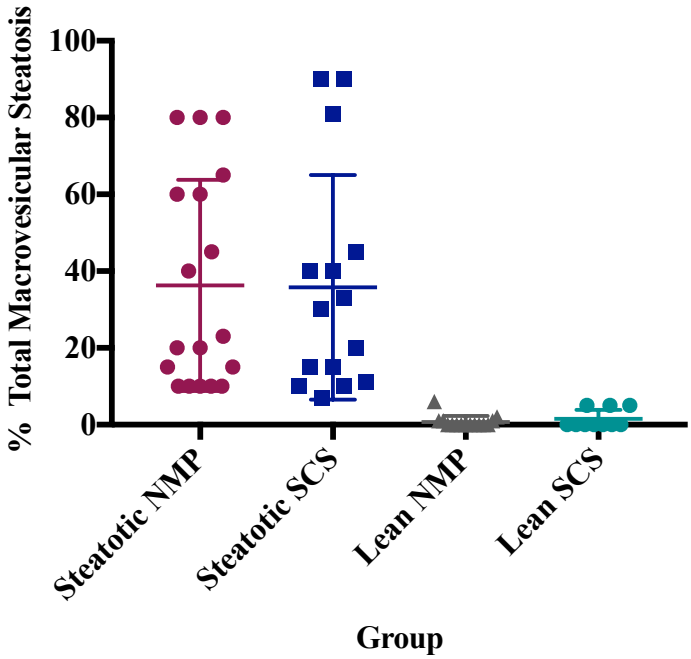


Figure 4.1. Scatter dot plot representing total macrovesicular percentage score for each group. The mean ± SD are shown/presented within each group.

4.3.3 Clinical Outcomes

There was a significant reduction in median peak serum AST between steatotic NMP (822 U/L (103 - 5101 U/L)) and steatotic SCS livers (2316 U/L (281 - 7245 U/L)) (p = 0.03)

(Figure 4.2A). The median peak serum AST from a steatotic NMP liver was comparable to a lean SCS liver (822 U/L (103 - 5101 U/L) vs. 811 U/L (131 - 2823 U/L), respectively; p = 0.98) (Figure 4.2B) but a significant reduction in peak serum AST was observed between lean and steatotic NMP livers (320 U/L (88 - 1493 U/L) vs. 822 U/L (103 - 5101 U/L), respectively; p = 0.02) (Figure 4.2C).

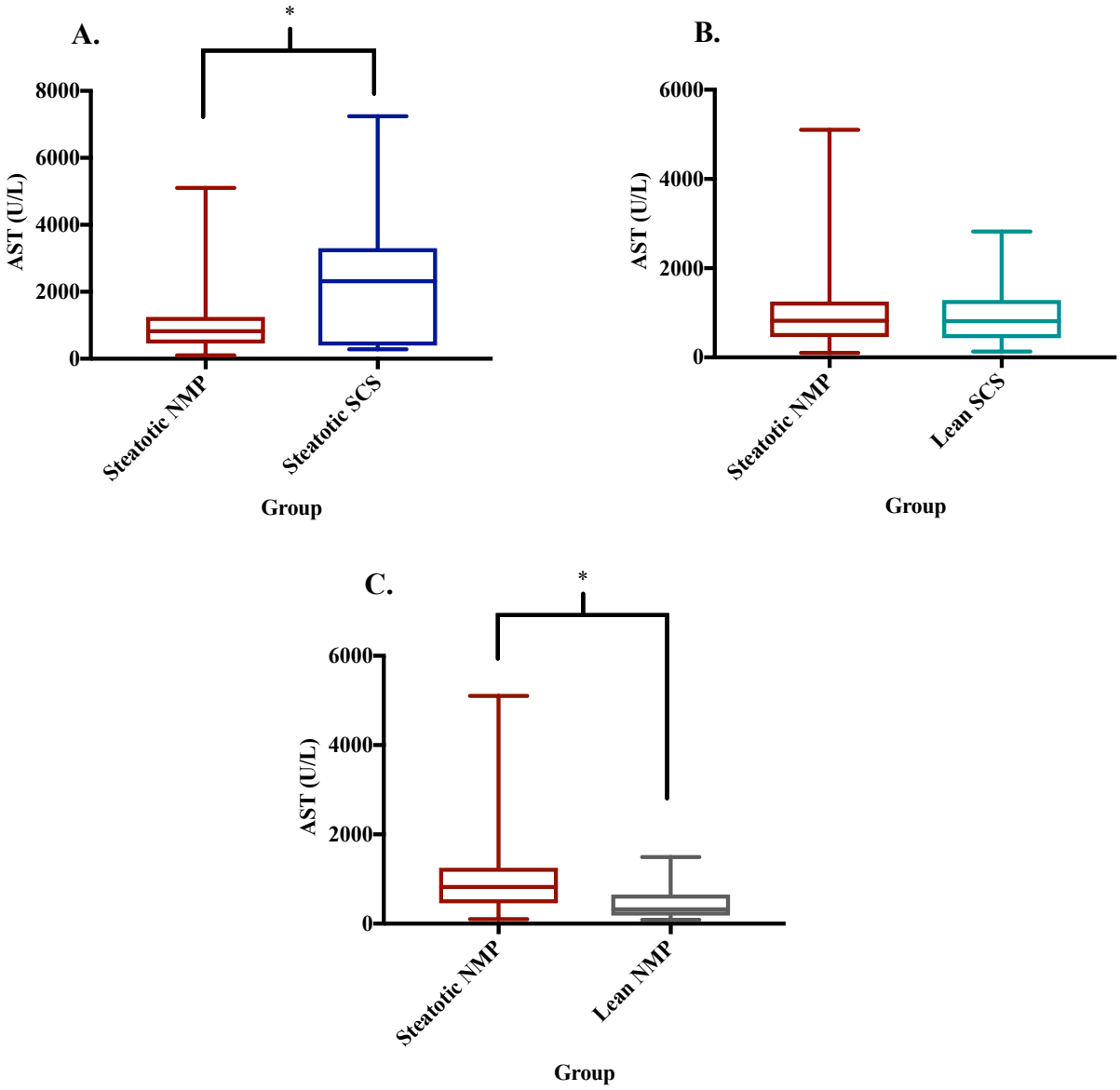


Figure 4.2A-C. Box and whisker plots displaying a significant reduction in peak serum AST between steatotic NMP and SCS livers **(A)**, comparable peak serum AST in steatotic NMP and lean SCS livers **(B)** and a significant reduction in peak serum AST between lean and steatotic NMP livers **(C)**. Data presented as median with range. *p < 0.05

A significant reduction in EAD was observed in the steatotic NMP group compared to the steatotic SCS group (11% vs. 60%, p = 0.008) and there was also a non-significant reduction in the incidence of post-reperfusion syndrome between the two groups (17% vs. 33%, p = 0.42). No difference in the need for RRT or duration of critical care or hospital stay was observed between the two steatotic liver groups (Table 4.6). However, there was a significant reduction in median critical care duration between steatotic and lean NMP livers (4.5 days (1 - 22 days) vs. 3 days (1 - 12 days), p = 0.04). The results for the investigated post-transplant outcomes and the comparison between groups are shown in Table 4.6.

Table 4.6. Comparison of post-transplant outcomes between donor recipient groups.

Outcome	**Steatotic NMP**	**Steatotic SCS**	**Lean NMP**	**Lean SCS**
EAD (%)	11	60**	7	10
PRS (%)	17	33	20	7
RRT (%)	39	40	13	20
Duration of critical care stay (days)	4.5 (1-22)	7 (2-30)	3* (1-6)	3 (1-12)
Duration of initial inpatient stay (days)	19 (7-70)	15 (7-63)	14 (6-97)	15.5 (8-35)

Data presented as percentages or median and range. **p < 0.01 steatotic NMP vs steatotic SCS; *p < 0.05 steatotic NMP vs lean NMP
Abbreviations – EAD, early allograft dysfunction; PRS, post-reperfusion syndrome; RRT, renal replacement therapy

Only one liver in the study population developed PNF. This was a steatotic liver preserved via NMP. Although it appeared healthy and homogeneously perfused, it failed to produce bile and the perfusate remained persistently acidotic with a lactate concentration > 4 mmol/L for the duration of NMP. All other grafts in the population were functioning and patients were alive at 1-year post-transplant as summarised in Table 4.7.

Table 4.7. One year graft and patient survival for all study groups.

	Steatotic NMP	Steatotic SCS	Lean NMP	Lean SCS
Graft Survival (%)	94	100	100	100
Patient Survival (%)	94	100	100	100

Data presented as a percentage**.**

4.3.4 Histological Changes in Steatosis

Neither NMP or SCS had an impact on the total amount of macrovesicular steatosis during preservation and post-reperfusion with a similar mean steatosis percentage throughout (Figure 4.3A-B).

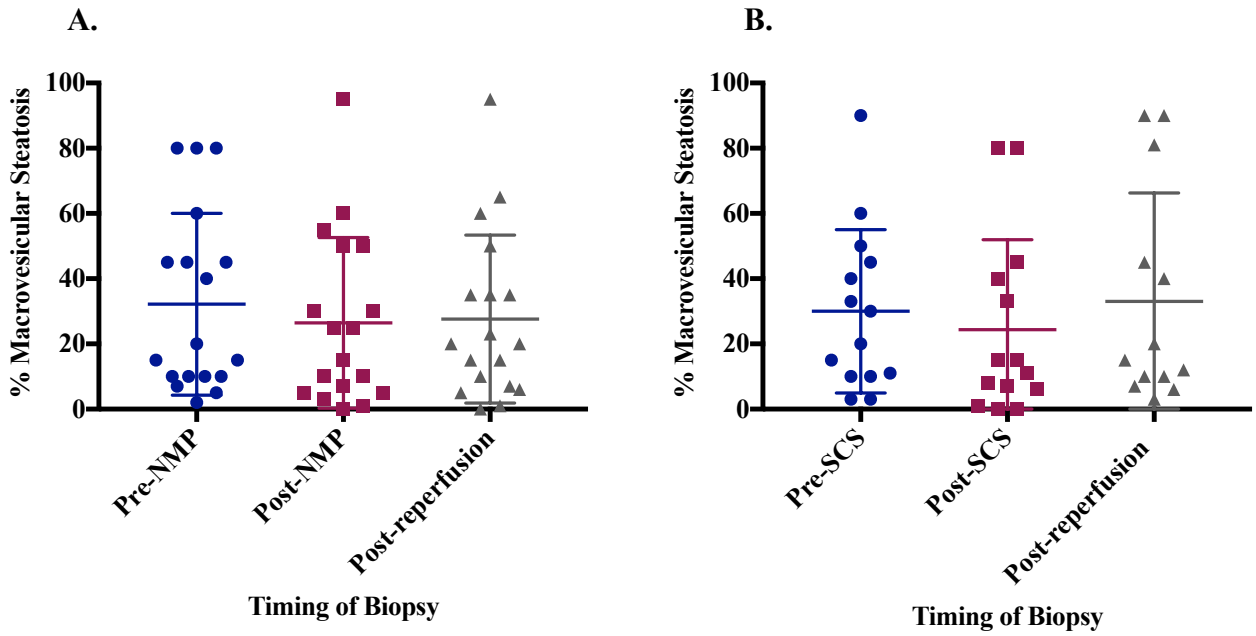


Figure 4.3A-B. Scatter-plot demonstrating percentage of total macrovesicular steatosis throughout preservation and post-reperfusion in steatotic NMP livers (**A**) and steatotic SCS livers (**B**). The mean ± SD is presented for each group.

4.3.5 NMP Perfusate Lipid Metabolites

Perfusate TG levels were higher at all time points in steatotic compared to lean livers; this difference was significant at 60 min into the perfusion (552 ± 260 µmol/L vs. 354 ± 213

µmol/L, respectively; p = 0.02, Figure 4.4A). Perfusate 3-OHB concentrations were significantly higher in steatotic than lean livers at 15 min (p = 0.008), 60min (p = 0.003) and at the end of perfusion (p = 0.007) (Figure 4.4B). Perfusate ALT levels were also higher in the steatotic compared to lean livers and this difference was statistically significant at the end of NMP (p = 0.04) (Figure 4.4C). The levels of circulating total cholesterol and urea in the perfusate were comparable between steatotic and lean livers (data not shown).

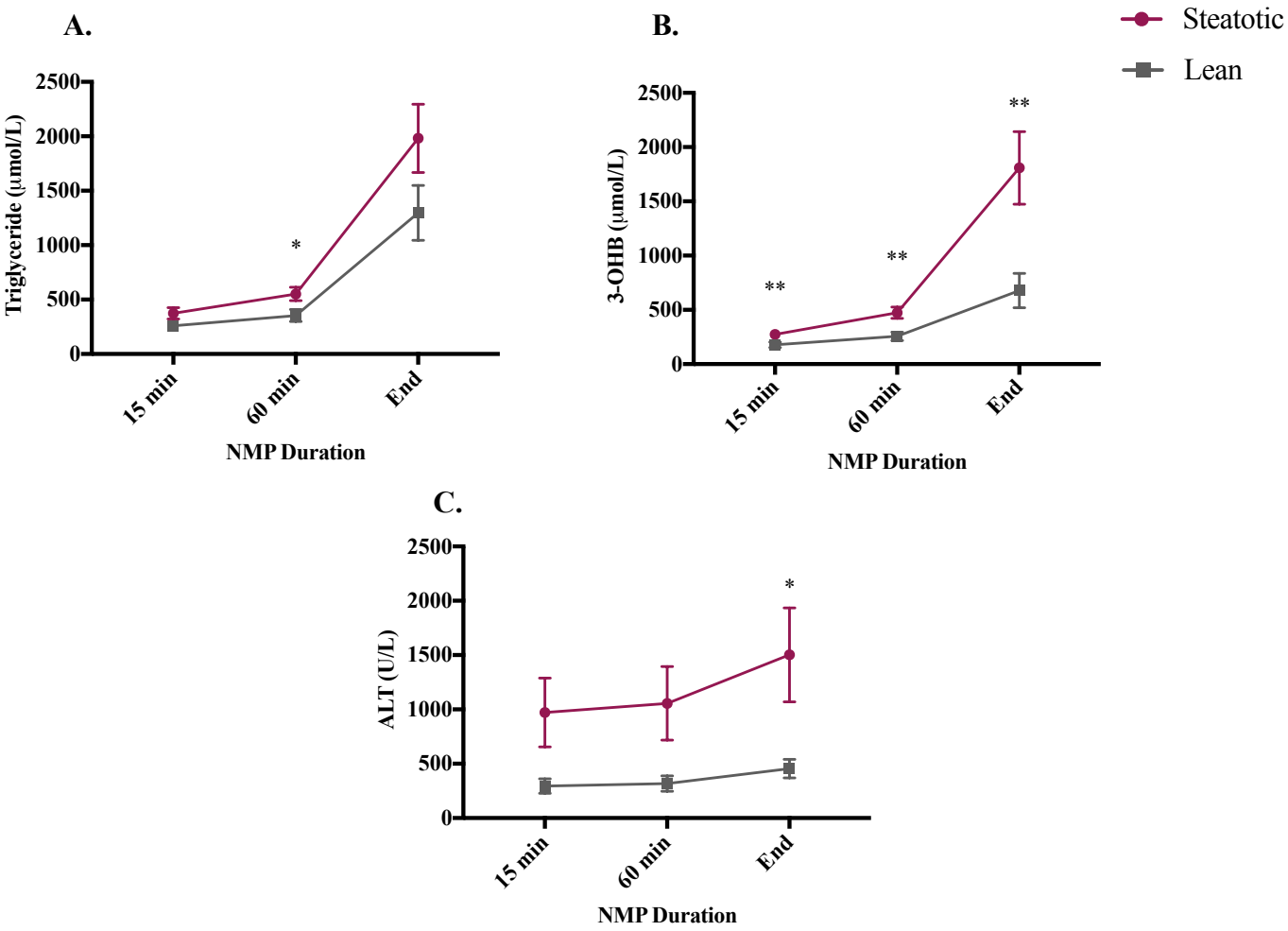


Figure 4.4A-C. Comparison of circulating TG **(A)**, 3-OHB **(B)** and ALT **(C)** in the perfusate during NMP between steatotic and lean livers. Data presented as mean ± SD. * $p < 0.05$, ** $p < 0.01$

A significant, positive correlation was observed between the percentage of macrovesicular steatosis in the liver and the perfusate TG ($r = 0.48$, $p = 0.005$), 3-OHB ($r = 0.46$ $p = 0.008$) and ALT ($r = 0.43$, $p = 0.01$) levels at 60mins (Figure 4.5A-C).

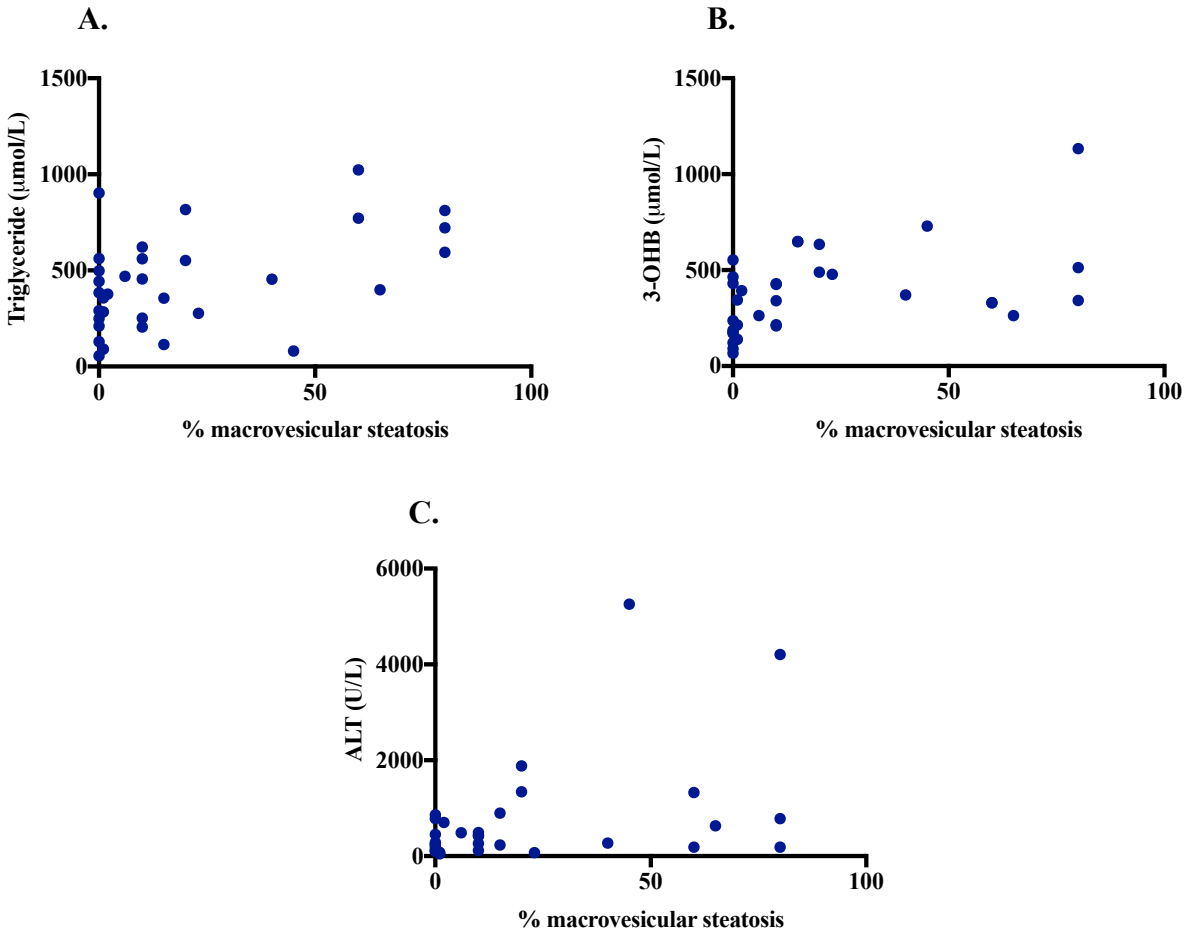


Figure 4.5A-C. Associations between % macrovesicular steatosis and perfusate TG at 60 min **(A)**; perfusate 3-OHB at 60 min **(B)**; and, perfusate ALT at 60 min **(C).**

4.3.6 IRI

Moderate/severe neutrophil infiltration was observed in the post-reperfusion biopsies in 29% of steatotic NMP livers. This is comparable to the steatotic SCS livers, where 36% were assessed as having moderate/severe neutrophil infiltration post-reperfusion ($p > 0.99$). The degree of neutrophil infiltration was lower in the lean NMP liver group with only 7%

demonstrating moderate/severe neutrophil infiltration although this difference was not significantly lower than in the steatotic NMP livers ($p = 0.18$).

In the sub-set of livers in which glycogen depletion was quantitatively assessed ($n = 5$ for each group) a median increase in hepatic glycogen content was observed in steatotic NMP livers (from 43% (33-70%) to 66% (22-69%)), compared to depletion in the steatotic SCS livers (from 56% (31-63%) to 42% (16-57%) throughout the course of preservation and reperfusion (Figure 4.6A). However, the change between groups did not reach statistical significance ($p = 0.77$). Lean NMP livers maintained their glycogen content throughout preservation and reperfusion (from 78% (68-79%) to 76% (66-77%)), whereas lean SCS livers tended ($p = 0.07$) to deplete their glycogen to a greater extent over the same time-course (65% (49-83%) to 42% (15-64%)) (Figure 4.6B). There was no significant difference between the change in glycogen content between steatotic and lean NMP livers or steatotic and lean SCS livers. Representative images of serial sections highlighting changes in glycogen storage during preservation in each group are shown in Figure 4.7.

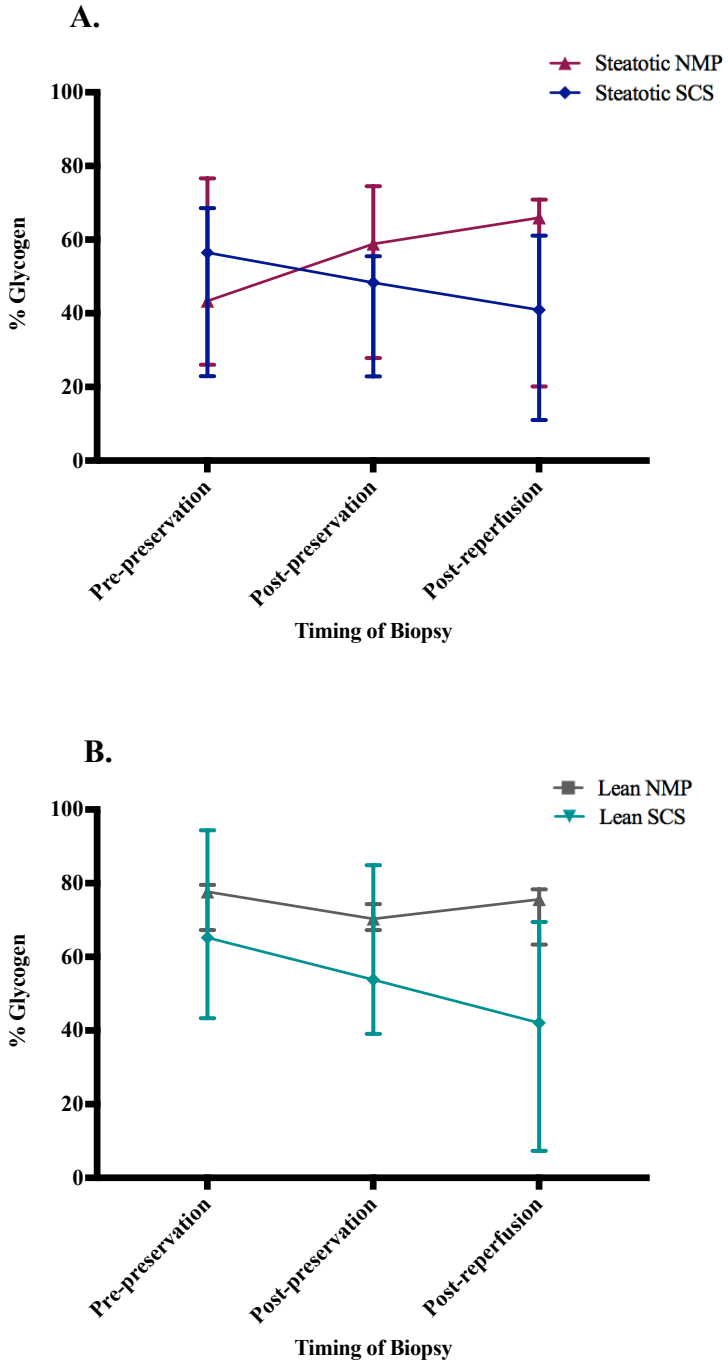


Figure 4.6A-B. Glycogen repletion throughout preservation and after reperfusion in steatotic NMP livers compared to depletion in steatotic SCS livers **(A)** and glycogen store maintenance compared to depletion in the lean NMP and lean SCS livers **(B)**. Data presented as median (range).

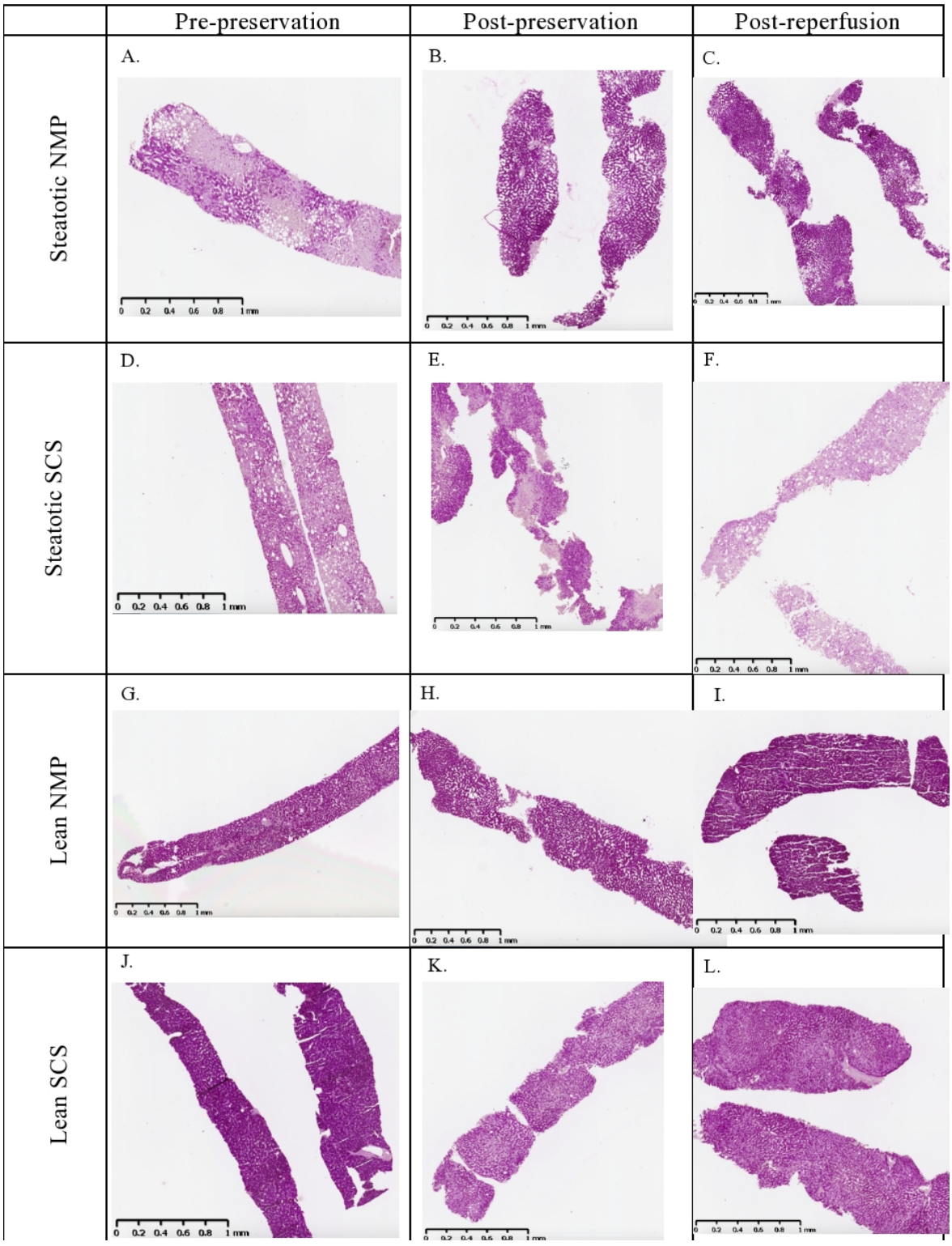
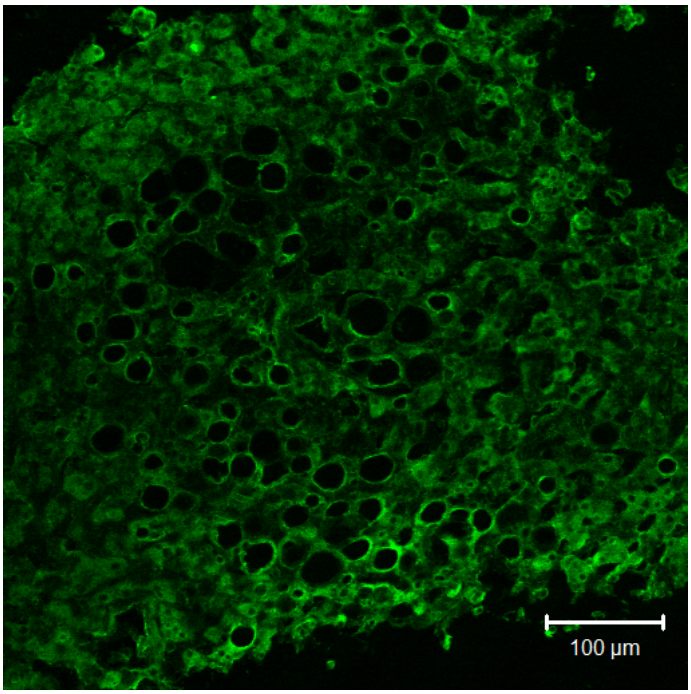


Figure 4.7A-L. Representative images of PAS-stained liver sections demonstrating glycogen repletion **(A-C)** and glycogen maintenance **(G-I)** during preservation and post-reperfusion in steatotic and lean NMP livers and glycogen depletion during preservation and post-reperfusion, most predominantly in steatotic SCS **(D-F)** and also in lean SCS **(J-L)** livers. A darker purple stain is positive for glycogen.

There also appeared to be a reduction in lipid peroxidation in NMP compared to SCS livers, as evidenced by a reduction in positive 4-HNE staining post-reperfusion (Figure 4.8).

A.



B.

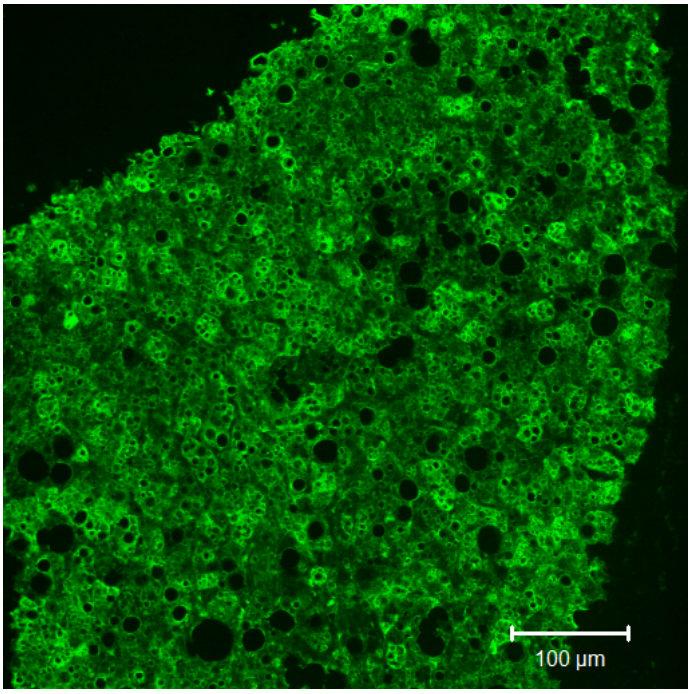


Figure 4.8A-B. Post-reperfusion 4-HNE stained sections showing more severe lipid peroxidation in a cold-stored steatotic liver **(B)** compared to a normothermic steatotic liver **(A)** as characterised by greater 4-HNE immunofluorescent uptake.

4.3.7 Cytokine Analysis

Two cytokines (IL-17A and IL1β) were below the level of detection in all samples and so further analysis could not be performed. Of the remaining 8, a significant increase in recipient plasma levels of IL-8, IL-10 and IL-6 was seen post reperfusion across all 4 groups (Table 4.8). However, a significant increase post-reperfusion of IL-2 and TNFα was only seen in the steatotic SCS livers (Table 4.8). When comparing post-reperfusion plasma in steatotic NMP and SCS livers, the circulating levels of IL-10, IL-2, TNFα, IFN-γ and IL-4 were significantly higher in the steatotic SCS livers (Figure 4.9).

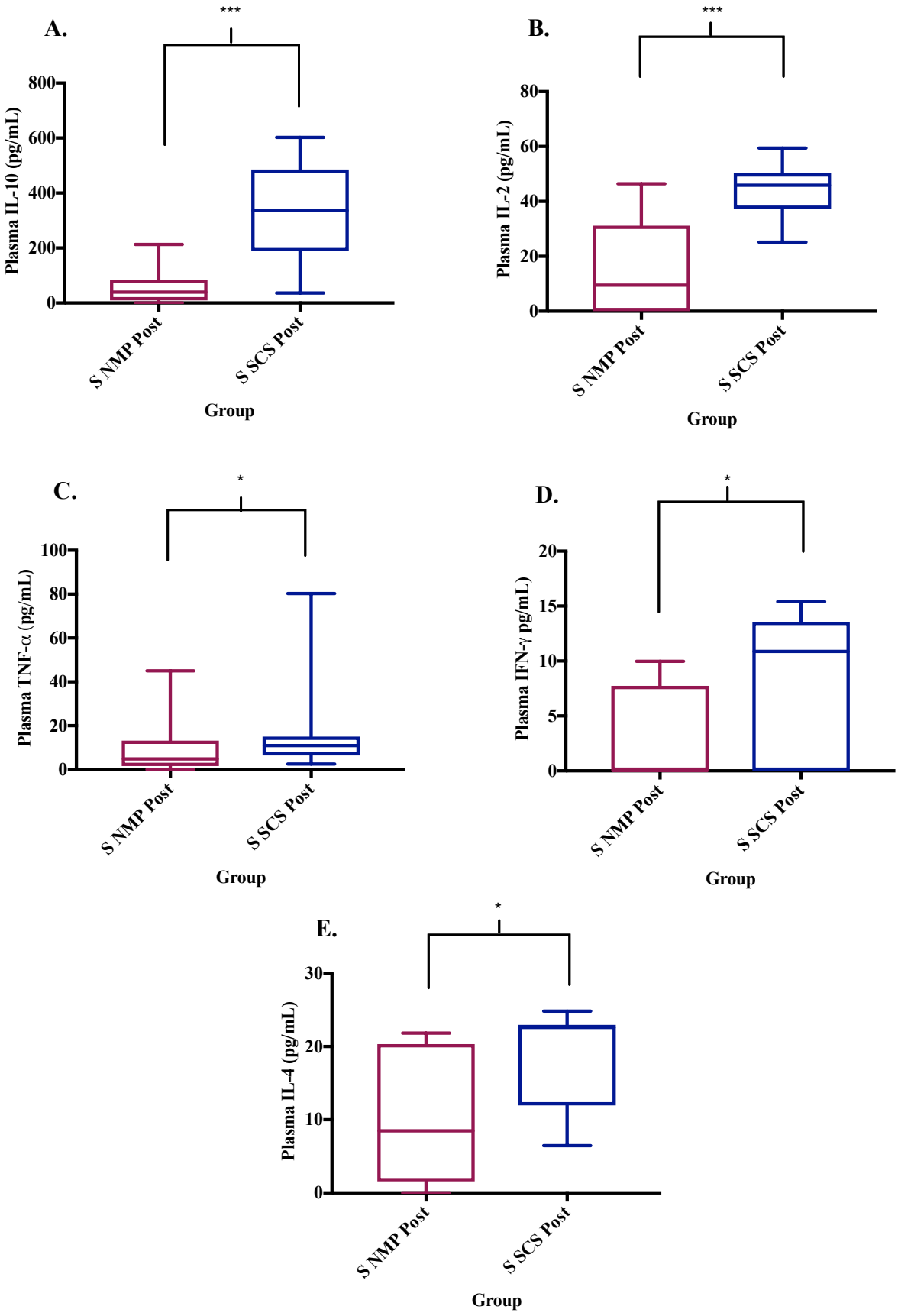


Figure 4.9A-E. Box and whisker plots demonstrating a significant reduction in IL-10 **(A)**, IL-2 **(B)**, TNF-α **(C)**, IFNγ **(D)** and IL-4 **(E)** between steatotic NMP and SCS livers. Data presented as median with range. * p <0.05, ***p <0.001 between group comparison.

Table 4.8. Pre- and post-reperfusion plasma cytokine levels for each liver sub-group.

Cytokine	Steatotic NMP		Steatotic SCS		Lean NMP		Lean SCS	
	Pre	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**
IL-8 (pg/mL)	7.0 (0 -18.6)	42.3*** (0 - 1464)	10.1 (0 - 79.7)	133.6*** (31.2 - 1024)	10.1 (4.1 - 65.7)	72.0* (7.8 - 297)	5.0 (0 - 97.1)	36.5 (17.3 - 159)
IL-10 (pg/mL)	0 (0 - 2.9)	39.5*** (0 - 213.0)	0 (0 - 2.1)	336.6*** (36.6 - 602.3)	0.1 (0 - 11.6)	61.9* (2.7 – 204.0)	0 (0 - 2.1)	245.4*** (150.9 - 533.6)
IL-2 (pg/mL)	0 (0 - 29.3)	9.5 (0 - 46.4)	26.7 (0 - 33.6)	45.9** (25.2 - 59.4)	0 (0 - 43.6)	0 (0 - 44.6)	0 (0 - 25.9)	0 (0 - 46.7)
TNF-α (pg/mL)	3.7 (0 - 13.3)	4.9 (0 - 45.1)	3.5 (0 - 10.2)	10.9** (2.5 - 80.3)	4.8 (2.8 - 13.8)	6.6 (3.3 - 18.9)	3.0 (0 - 5.7)	2.6 (0.9 - 7.9)
IFN-γ (pg/mL)	3.0 (0 - 11.8)	0 (0 – 10.0)	5.6 (0 - 14.5)	10.9 (0 - 15.4)	0 (0 - 15.8)	0 (0 - 10.9)	0 (0 - 0.9)	0 (0 - 11.8)
IL-13 (pg/mL)	247.7 (213.8 - 293.8)	279.0* (279.0 - 412.9)	263.3 (176.0 – 279.0)	308.2** (279.0 – 362.6)	263.3 (247.7 – 279.0)	289.2 (269.2 – 301.4)	238.7 (213.8 - 263.6)	293.6 (279.0 – 308.2)
IL-4 (pg/mL)	15.2 (0 - 21.8)	11.3 (0 - 35.1)	20.3 (7.2 - 22.6)	22.6 (6.5 - 24.8)	11.4 (4.1 - 23.4)	10.2 (2.1 - 25.5)	13.0 (7.8 - 21.1)	2.1 (0 - 21.8)
IL-6 (pg/mL)	11.0 (2.5 - 142.4)	352.1*** (50.2 - 4683)	11.4 (2.7 - 91.4)	552.3*** (274.2 - 1623)	9.5 (0.7 - 581.5)	177.8† (62.6 - 446.3)	7.5 (1.4 - 141.3)	308.4** (109.5 - 729.1)

Data presented as median and range. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ †$p = 0.06$ pre vs post measurements for the respective groups.

4.4 Discussion

In this chapter, through the analysis of biological samples obtained as part of a RCT, I have investigated a potential benefit of NMP in steatotic liver transplantation. From the data presented, NMP appears to be effective in attenuating IRI in steatotic livers compared to their cold stored counterparts. This is evidenced by a significant reduction in clinically apparent graft injury post-transplantation in terms of peak serum AST and EAD as well as histological changes such as replenishment of glycogen stores and less lipid peroxidation. Furthermore, patients transplanted with steatotic livers preserved via NMP had a reduced systemic inflammatory response compared to SCS livers, with significantly reduced circulating levels of IL-10, IL-2, TNF-α, IFN-γ and IL-4, post-reperfusion. However, when compared to lean NMP livers, recipients receiving steatotic NMP livers had a significantly higher peak serum AST with a significantly prolonged critical care stay. This suggests that there is potential to further optimise steatotic grafts during NMP in order to achieve outcomes comparable to their lean counterparts.

When comparing steatotic and lean livers during NMP, there are clear differences in perfusate metabolites, namely TG and 3-OHB. A steatotic liver mobilises more TG (which is incorporated into very low density lipoproteins, VLDL) and the increase in 3-OHB suggests greater fatty acid oxidation through ketone body production. The difference observed in the perfusate most likely represents mobilisation of the increased fatty acid pool within the steatotic liver providing fatty acyl CoA as substrate to esterification and oxidation pathways. Liu *et al* also observed a progressive increase of TG in the perfusate when steatotic human livers were perfused for 24 h (164) but with no control group, they were unable to establish whether this effect was greater in steatotic livers. In the present study, there was a significantly higher perfusate ALT concentration in the steatotic compared to

the lean NMP livers. This supports the notion that steatotic livers are subject to a greater liver injury which is initiated in the parenchymal hepatocytes (67, 68, 118).

In contrast with the previous studies conducted by Jamieson *et al* (156) and Nagrath *et al* (157), the degree of macrovesicular steatosis did not reduce following NMP. In these porcine and murine experiments, a 50% reduction in hepatic steatosis observed over 48 h of NMP alone (156), or 3 h of NMP with de-fatting agents (157). In the current study, using histological assessment, we did not observe a reduction in steatosis in the livers following NMP. This may be due to the fact that livers were preserved for between 4 – 24 h (as dictated by the clinical trial protocol), with no standardised preservation duration. It is, therefore, difficult to make a comparison based on the variable time-course of perfusion. Furthermore, it is unclear whether the findings from artificially-induced hepatic steatosis in an animal model are representative of human disease. In the study of discarded human steatotic livers by Liu *et al*, no reduction of hepatic steatosis was seen over a 24 h period of NMP. Banan *et al* have reported results from two human livers which were preserved normothermically with the addition of de-fatting agents, namely l-carnitine and exendin-4 (165). L-carnitine increases the rate of fatty acid transport to the mitochondria and has been shown to be important in mitochondrial β-oxidation of fatty acids (166) and exendin-4 has been shown to reduce oxidative stress in steatotic models (167). However, in the two treated livers, only one showed a (minimal) reduction in the degree of macrovesicular steatosis after 8 h NMP.

In the current study I observed an increase in the histological hepatic glycogen content during preservation which is maintained post-reperfusion in both steatotic and lean normothermic livers. The converse is true for both steatotic and lean SCS livers where glycogen depletion is observed throughout preservation and post-reperfusion. This

observation may provide an explanation for the improved early post-operative graft function and attenuated reperfusion injury in the normothermic livers. During cold storage, the hypoxic conditions activate glycolysis and the energy derived from this process is critical for cellular integrity and survival (191). Cell death may, therefore, ensue once hepatic glycolysis is inhibited due to the accumulation of end products or a lack of substrate (glycogen) (192). Cherid *et al* evaluated 62 liver biopsies and observed 48% peri-portal and 78% glycogen depletion during cold ischaemia; with an even greater increase in glycogen depletion observed after reperfusion (193). Glycogen depletion is important when considering IRI and post-transplant outcomes as glycogen is important in maintaining hepatocellular integrity and function by supplying glucose for ATP generation. Once glycogen is consumed, ATP depletion ensues, leading to irreversible cell injury and necrosis (51). Several studies have demonstrated the importance of *ex vivo* hepatocellular ATP and post-transplant graft function with a strong correlation between high ATP content and good transplant outcomes (45, 194-196). The observed changes between the groups in glycogen storage during preservation in this study, may provide an explanation to support the reduction in reperfusion injury in the NMP livers.

Human and animal studies have demonstrated that post-ischaemia and reperfusion, steatotic livers are subject to more lipid peroxidation (68-71). This process is initiated by the reduction of hydrogen peroxide resulting in the formation of a hydroxyl radical which leads to the destruction of polyunsaturated fats (120). Lipid peroxidation has been used as an indirect measurement of oxidative damage induced by ROS (121) which remain critical to the generation of reperfusion injury (122). In this study, I observed a reduction in lipid peroxidation in steatotic livers post-reperfusion compared to their lean counterparts, as evidence by a immunohistochemical reduction of 4-HNE (an end product of lipid

peroxidation (189)). This may further explain why the steatotic grafts are subject to less IRI and improved post-transplant function.

The liver is a prominent cytokine target due to its key role in the acute phase response (197) and hepatocytes are highly susceptible to cytokine activity in acute and chronic pathophysiological conditions (197). Experimental models have demonstrated that upon reperfusion of a steatotic liver, there is an increased release of the pro-inflammatory mediator TNF-α (198, 199). TNF-α can facilitate the sinusoidal transmigration of inflammatory cells (200, 201) and induce the expression of CXCL8, a neutrophil activator (202). In this study, a significant increase in systemic TNF-α post-reperfusion was only seen in the steatotic cold-stored livers, post-reperfusion levels significantly higher in the steatotic SCS compared to steatotic NMP livers. A recent study by Sosa *et al* characterised the systemic cytokine signatures in patients undergoing liver transplantation and compared these to histological and clinical evidence of IRI (190). They observed significantly elevated post-operative plasma levels of IL-4, TNF-α, Il-13, IL-10 and IL-2 in patients with IRI (190). In this study I identified a significant increase in TNF-α, IL-10, IL-2 and IL-4 in the steatotic SCS compared to NMP livers, further supporting the hypothesis that NMP attenuates IRI in liver transplantation.

The study detailed in this chapter is not without its limitations. The sample size is small and it is therefore difficult to draw robust conclusions, particularly when there is such a broad range of macrovesicular steatosis in both NMP and SCS groups (10-90%). However, to my knowledge this is the only series highlighting clinical outcomes from steatotic human livers preserved via NMP with well-matched controls. Despite the wide variability in the degree of steatosis, these were indeed similar between the groups and therefore outcome

comparisons and interpretations are valid. Furthermore, important differences in liver fat metabolism were observed, even at these relatively low levels, between the steatotic and lean NMP liver groups. The variable duration of preservation duration in the NMP livers makes conclusions about any de-fatting potential difficult. In order to properly test this hypothesis, livers would all need to be preserved for the same duration and it is likely that a prolonged preservation (up to 48 h) may be required.

In conclusion, this study demonstrates that NMP has the potential to improve early post-operative graft function by reducing IRI as characterised clinically, histologically and by the systemic inflammatory cytokine signature. However, outcomes from steatotic livers preserved via NMP are not as positive (in terms of the markers of reperfusion injury) as in lean livers undergoing NMP. The differences in lipid metabolism shown in this study highlight the potential to intervene on these livers in order to manipulate the metabolic pathways which may be responsible for de-fatting.

Chapter 5

Exploring the structural and functional effects of normothermic machine perfusion and de-fatting agents on human steatotic livers

5.1 Introduction

In Chapter 4, I demonstrated that NMP enhances the preservation of steatotic livers with improved post-transplant outcomes compared to cold stored steatotic livers. However, over the preservation time (between 4 – 24 h), no reduction in liver fat was observed. Steatotic livers have complex metabolic demands and the presence of fat may still impact on post-transplant outcomes. This is suggested by the more severe preservation injury observed in the steatotic compared to lean NMP livers in Chapter 4. When considering the transplantation of livers with more severe steatosis (than those discussed in Chapter 4), it may be necessary, during preservation, to reduce the amount of fat within the liver in order to further improve outcomes. The ability to reduce the amount of intrahepatic fat has been demonstrated in animal models with or without the addition of specific de-fatting agents (156, 157). However, limited data from human studies have not been successful in demonstrating a reduction of intrahepatic fat during NMP (164, 165).

The hypothesis of this chapter is that the amount of liver fat can be reduced during prolonged NMP through the addition of de-fatting interventions.

The aims of this chapter are:

- To investigate the impact of de-fatting agents on *ex situ* liver function.

- To explore the effects of de-fatting interventions on liver fat content.
- To understand the potential mechanisms pertaining to any alteration in lipid metabolism and structure during NMP.

5.2 Methods

Between September 2016 and August 2018, 21 steatotic livers declined for transplantation by all UK centres and offered for research were included in this experimental study. Authorisation for research use of the liver was obtained by the specialist nurse in organ donation in accordance with NHSBT guidelines. The study was approved by the North East – Tyne and Weir South research ethics committee (16/NE/0248) and by the NHSBT Research, Innovation and Novel Technologies Advisory Group (study reference: liver 60).

5.2.1 Liver Procurement

All livers recruited to this study were retrieved as part of a multi-organ deceased donor organ retrieval (170). All livers were flushed *in situ* with UW solution, packed on ice and transported to the Oxford Transplant Centre.

5.2.2 Perfusion Protocols

The OrganOx *metra* NMP device was primed and livers were prepared and cannulated as described in Chapter 2, sections 2.1 and 2.2, respectively. Livers were sequentially assigned to one of 3 groups and perfused for up to 48 h with frequent perfusate and biopsy sampling (Table 5.1).

Table 5.1. Sampling schedule during NMP. Samples were collected, processed and stored as described in Chapter 2, sections 2.3.1 and 2.5.

		Time (h)											
Sample Obtained		0	0.5	1	4	8	12	18	24	30	36	42	48
	Blood Gas		x	x	x	x	x	x	x	x	x	x	x
	Perfusate	x	x	x	x		x		x		x		x
	Core Biopsy	x					x		x		x		x

5.2.2.1 Group 1 – NMP Alone

Livers assigned to this group were perfused using the standard NMP set-up as described throughout this thesis. No interventions were assigned to this group.

5.2.2.2 Group 2 – NMP + Lipid Apheresis Filtration

In this group, livers were perfused with the addition of a lipoprotein apheresis (DALI® 500) filter (Fresenius Medical Care (UK) Ltd, Huthwaite, UK). In clinical practice, this haemofiltration system is used for patients with severe hyperlipidaemia, refractory to maximal medical therapy (203). The filter consists of a matrix of polyacrylate beads, effective for the adsorption of cholesterol, lipoprotein (a) and triglycerides (203). The adsorption of lipoproteins occurs by polyacrylate ligands covalently binding to the polyacrylamide surface. Polyacrylate, consists of polyanions, with negatively charged carboxylate groups (204). The polyanions interact selectively with the cationic groups in the lipoproteins, and due to this electrochemical interaction, the lipoproteins are immobilized on the beads (203). Figure 5.1 demonstrates and details the incorporation of the filter into the NMP circuit. Prior to filtration, the filter was primed with 2 L of Gelaspan (B Braun, Sheffield, UK). Besides lipoproteins, the filter adsorbs the positively charged ions calcium

and magnesium. Priming therefore saturates the adsorber with these cations, preventing hypocalcaemia and hypomagnesaemia (205). In this context, priming also prevented excessive volume loss from the circuit upon commencement of filtration.

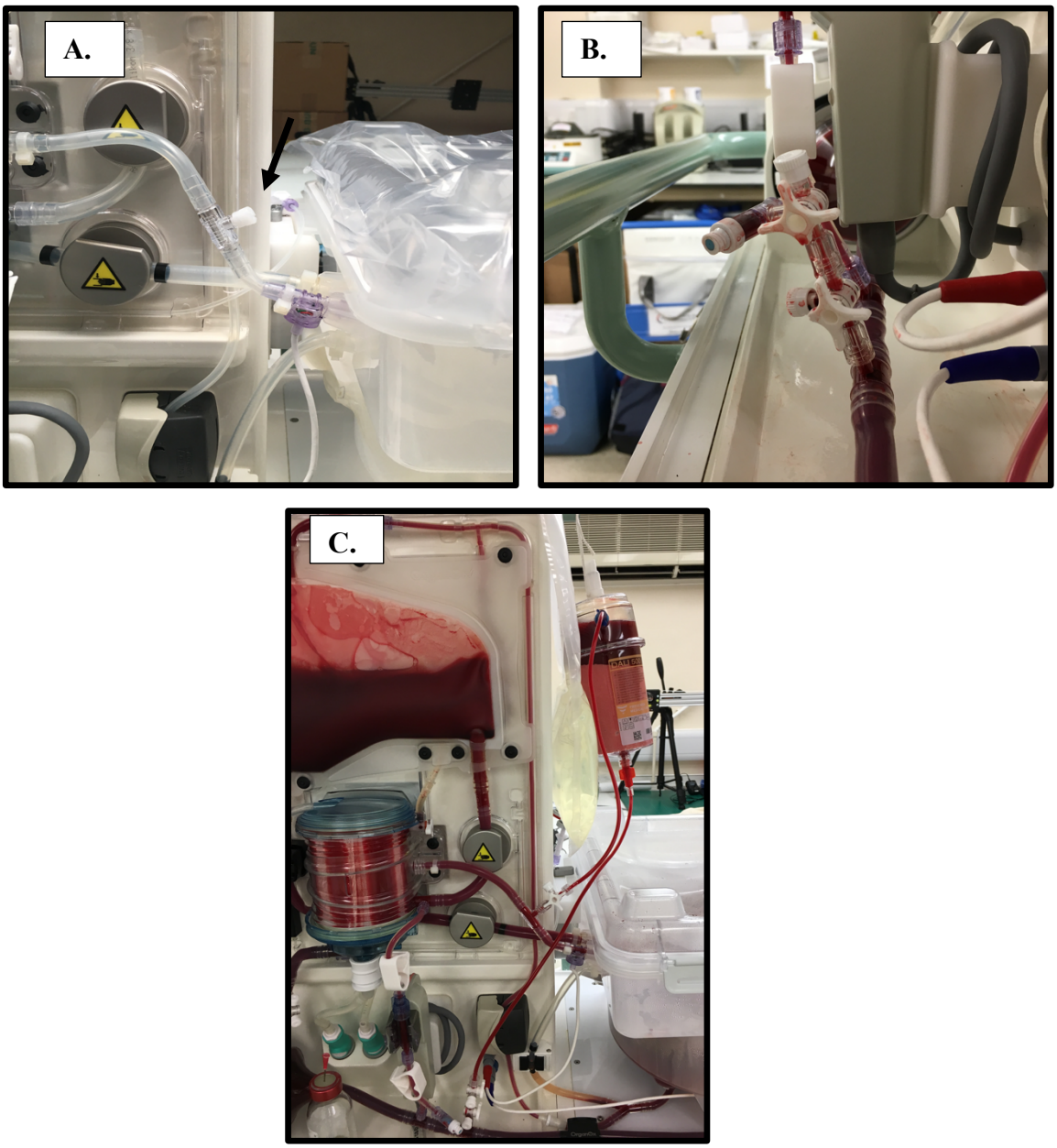


Figure 5.1. Incorporation of lipoprotein apheresis filter into NMP circuit. A sterile equal and reducing straight ¼ inch connector with luer lock (LivaNova UK Ltd, Brockworth, UK) was incorporated into the arterial line **(A)**. A 3-way tap was connected to the arterial line allowing isolation of the filter. Two, 3-way taps were connected to the priming port on the IVC line **(B)**. The use of 2 connectors facilitated the priming of the device without interrupting the perfusion, by siphoning off the priming solution and discarding it. When the 3-way taps on the arterial and IVC lines were opened, perfusate was allowed to shunt through the filter **(C)**. The flow rate through the filter was 133 ml/min.

5.2.2.3 Group 3 – NMP + Lipid Apheresis Filtration + De-fatting interventions

In this group, as well as the filter, l-carnitine hydrochloride and forskolin were included as de-fatting interventions and glucose and insulin concentrations altered.

5.2.2.3.1 L-carnitine hydrochloride

L-carnitine increases the rate of fatty acid transport to the mitochondria and has been shown to be important in β-oxidation of fatty acids from the mitochondrial membrane (166). The perfusate was supplemented with 1 g of l-carnitine hydrochloride (Sigma-Aldrich, Dorset, UK), in 20 ml of 0.9% sodium chloride (B Braun). This dose was based on *in vivo* human studies (206-210). As l-carnitine has a half-life of around 15 h (211), a further 1 g was added at 24 h into the perfusion.

5.2.2.3.2 Forskolin

Forskolin is a glucagon mimetic cAMP activator which results in increased lipolysis of lipid droplets and fatty acid oxidation (162). The perfusate was supplemented with 1 mg NKH477 hydrochloride (Caymam Chemical, Michigan, USA) (a water-soluble version of forskolin), dissolved in 2 ml of 0.9% sodium chloride (B Braun). This dose was extrapolated based on data from rat and canine models (212, 213).

5.2.2.3.3 Reduced insulin

DNL is stimulated by insulin (150). In the absence of peripheral fat stores or dietary fat in this model, the only source of fatty acid production in the liver is via the DNL pathway. In an attempt to reduce DNL (namely the production of palmitic acid), the amount of insulin delivered during the perfusion was reduced by 50%. The perfusate was therefore

supplemented with 100 units of insulin (Actrapid) (Novo Nordisk, West Sussex, UK), dissolved in 30 ml 0.9% sodium chloride (B Braun), and delivered at a rate of 1 ml/h.

5.2.2.3.4 Reduced glucose

Glucose acts as a non-lipid precursor for DNL (152). In an attempt to further reduce the liver's ability to *de novo* synthesise fatty acids during preservation, the glucose threshold to commence the TPN (Nutriflex Special, B Braun) infusion was reduced from 10 mmol/L to 5 mmol/L. This aimed to achieve a lower perfusate glucose concentration during perfusion.

5.2.3 Functional Liver Assessment

5.2.3.1 Flow dynamics

Hepatic arterial pressure and flow and portal flow was assessed at 30 min, 1 h, 4 h, 12 h, 24 h, 36 h and 48 h. Data were recovered from perfusion files stored on the OrganOx *metra* (OrganOx Ltd) device and the mean of each value per minute preceding the time-point of perfusion was obtained. Flow dynamics were then compared between the groups.

5.2.3.2 Blood gas and perfusate analysis

Frequent blood gas sampling was performed (Table 5.1) and analysed as described in Chapter 2, section 2.5. For functional assessment, lactate, pH and glucose levels were compared between groups. To further explore hepatocellular function, perfusate was processed and analysed to compare levels of ALT and haemolysis (haemolysis index) between groups (Chapter 2, section 2.5.1). The total amount of 8.4% sodium bicarbonate (B Braun) required to maintain a physiological pH (7.25-7.35) and bile production were also compared between groups.

5.2.3.3 Glycogen Synthesis

The liver's ability to synthesize glycogen during preservation and replenish the glycogen stores which had been utilised during cold storage was assessed through histological staining with PAS and image analysis software (ImageJ) (Chapter 2, sections 2.3 and 2.4). The percentage of glycogen, as represented by positive PAS staining, was recorded over the course of the perfusion and compared between groups.

5.2.4 Assessment of hepatic lipid metabolism

5.2.4.1 Perfusate lipid metabolites

Circulating levels of TG, total cholesterol (TC), 3-OHB and apolipoprotein B (ApoB) were measured in the perfusate (Chapter 2, sections 2.5.2-2.5.5) at each time point (Table 5.1) and compared between the 3 groups.

5.2.4.2 Fatty acid composition

In order to investigate perfusate fatty acid composition, lipid extraction was performed and gas chromatography (GC) undertaken.

5.2.4.2.1 Lipid extraction

500 μL of perfusate was thawed at room temperature and vortexed for 30 s. Total lipid was then extracted from the perfusate using the Folch extraction method (152). This involved the addition of 5 ml chloroform:methanol (2:1) (VWR International Ltd, Leighton Buzzard, UK) and 20 μL of TG internal standard (Pentadecanoic acid, 15:0). After the addition of 1 M NaCl and mixing, samples were centrifuged for 10 min at 2000 rpm at 14 (±4)°C. The top aqueous phase was aspirated and discarded, ensuring that pellet or solvent phase was not

disturbed. The solvent phase was then evaporated to dryness at 50 (±10)°C under nitrogen using an automated evaporation system (TurboVap® Classic LV) (Biotage, Hengoes, UK).

Next, Isolute®, 100 mg/ml solute solid phase extraction (SPE) columns (Biotage, Hengoed, UK) were used to elute TG (214). Briefly, 1 ml of chloroform (VWR International Ltd) was added to the dry lipid extract and vortexted. SPE columns (Biotage) were pre-washed with acetone (VWR International Ltd) and chloroform (VWR International Ltd) before eluting cholesterol ester (CE) and TG with chloroform (VWR International Ltd). Samples were then dried down under nitrogen (TurboVap® Classic LV), new SPE columns (Biotage) preconditioned with hexane (VWR International Ltd) and then further washed with hexane (VWR International Ltd) to elute the CE. TG was then eluted by passing hexane:chloroform:ethyl acetate (VWR International Ltd) through the SPE columns (Biotage). The lipid fractions were once again dried down under nitrogen (TurboVap® Classic LV).

For the methylation stage, toluene was added to the dried lipid fractions and vortexed and 1.5% sulphuric acid in methanol (VWR International Ltd) was then added and samples placed in a water bath at 80ºC for 1 h. After allowing to cool, neutralising solution and cyclohexane were added (VWR International Ltd) and mixed. After centrifuging for 10 min at 2000 rpm at 14 (±4)°C, the upper solvent phase was transferred and dried down under nitrogen (TurboVap® Classic LV). 190 µl of chloroform (VWR International Ltd) was added to the dried down fatty acid methyl ester sample and transferred to a GC vial containing 20 µl of external standard (Tricosanoic acid, 23:0).

5.2.4.2.2 Fatty acid composition

Fatty acid composition was determined by GC. Peaks were identified by comparison of retention times with known fatty acid methyl ester standards and the fatty acid compositions (μmol/100 μmol total fatty acids (mol%)) calculated based on the area of the respective peaks. Comparisons were made over time in the perfusion and compared between groups.

5.2.5 Quantification of Steatosis

In order to quantify steatosis and compare the effect of the interventions over time, histological and tissue TG analyses were performed.

5.2.5.1 Histological Analysis

Formalin-fixed paraffin embedded (FFPE) sections were prepared and stained with H&E as described in Chapter 2, section 2.3 for each biopsy obtained during the perfusions (Table 5.1). Whole slides were scanned using a digital slide-scanner (Hamamatsu NanoZoomer 2.0-HT whole slide imager) (Hamamatsu UK Ltd, Welwyn Garden City, UK). Each image was subsequently processed using automated image analysis software, validated for the quantification of small and large droplet macrovesicular steatosis (Visiopharm application 10119, H&E liver steatosis) (Visiopharm Ltd, Egham, UK) (Figure 5.2). Quantitative output variables produced by the software for total percentage of macrovesicular steatosis as well as the percentage of small and large droplets were recorded for each section (obtained every 12 h throughout NMP). Any change over the duration of perfusion was recorded and compared between the groups.

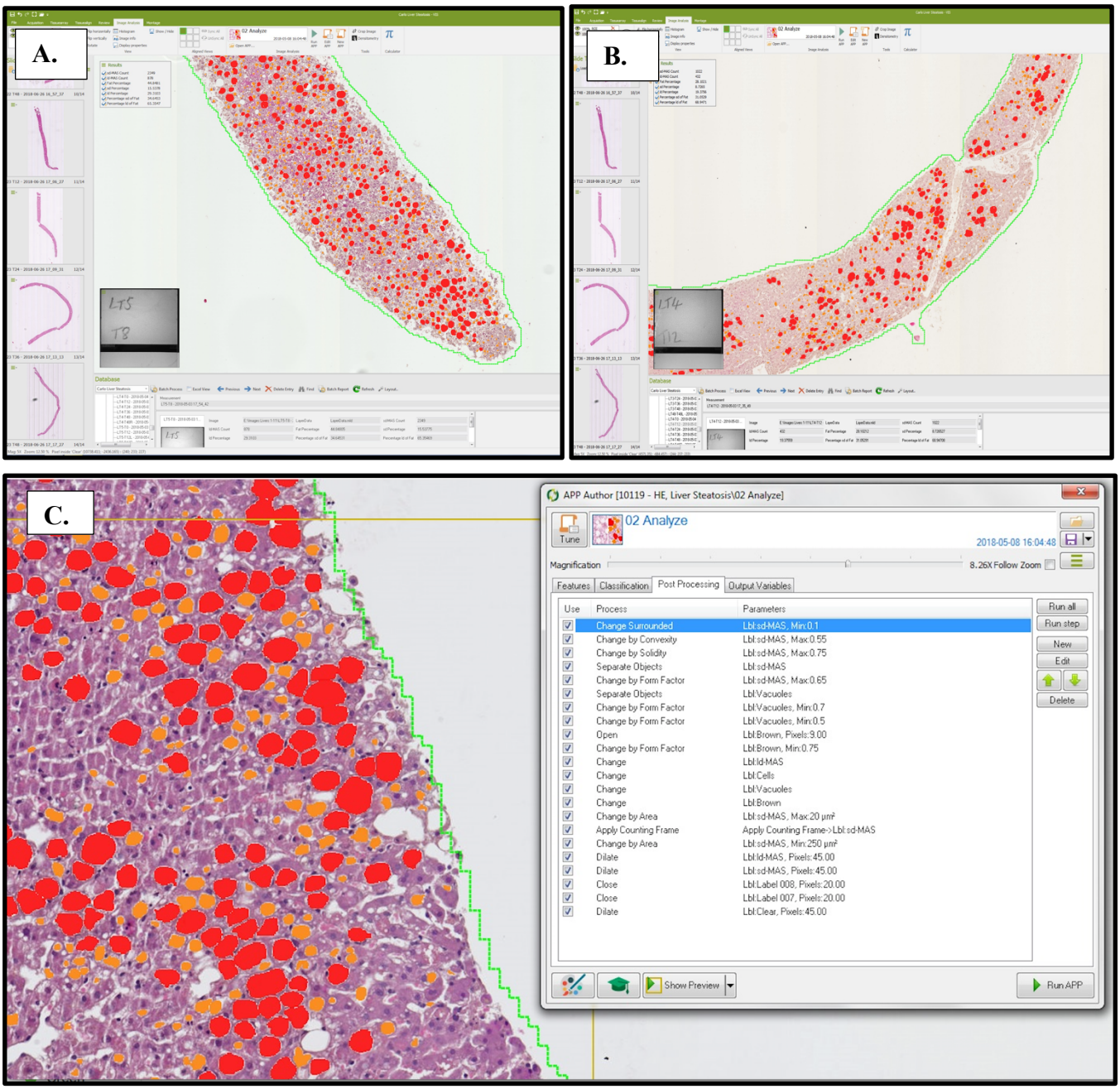


Figure 5.2A-C. Automated image analysis of H&E sections for hepatic steatosis. The first imaging processing step involves a segmentation of the liver tissue by defining a region of interest as shown by the green line circulating the tissue **(A-C)**. The software then identifies lipid droplets within the liver tissue and characterises them based on their size and shape. Large droplet macrovesicular steatosis (ld-MaS) is labelled red and small droplet macrovesicular steatosis (sd-MaS) is labelled orange. The software uses post-processing thresholds to distinguish the lipid droplets based on their size and shape **(C)**.

5.2.5.2 Tissue fatty acid composition and TG Measurement

Frozen tissue obtained during the perfusion (Chapter 2, section 2.3.1) was weighed and added to 1.5 ml of methanol (VWR International Ltd). The sample was then sonicated in a water bath for 5 min and further broken up with a spatula. 3 ml of chloroform (VWR International Ltd) was then added. The Folch extraction, SPE and methylation steps were then performed for TG as described above (section 5.2.4.2.1). After fatty acid composition was obtained (section 5.2.4.2.2), the total fatty acid concentration was calculated and expressed as μg/mg of tissue. This was then plotted over the course of each perfusion and the groups were compared.

5.2.6 Quantifying DNL

5 ml of $^{2}H_2O$ (heavy water) was added to the perfusate prior to the commencement of all perfusions. *In vivo*, heavy water rapidly equilibrates with the body water pool (151), producing a homogenous precursor pool for any reactions involving water (e.g. condensation/hydrolysis), potentially allowing the investigation of several metabolic processes (215). As VLDL TG is suggested to represent hepatic TG, the incorporation of ^{2}H atoms into VLDL-TG palmitate (either from intracellular $^{2}H_2O$ or incorporation into nicotinamide adenine dinucleotide phosphate or acetyl-CoA first) can be used to estimate hepatic DNL (216-220).

Following lipid extraction (section 5.2.4.2.1), perfusate and tissue DNL was measured by the incorporation of deuterium from $^{2}H_2O$ (Finnigan GasBench-II, TermoFisher Scientific, UK) into perfusate and tissue TG [^{2}H]-palmitate. [^{2}H]-palmitate enrichments were determined by GC-mass spectrometry (GC-MS) using a 5890 GC coupled to a 5973N MSD

(Agilent Technologies, CA, USA). Ions with mass-to-charge ratios (m/z) of 270 (M+0) and 271 (M+1) were determined by selected ion monitoring (220).

5.3 Results

5.3.1 Donor demographics and liver characteristics

A total of 21 steatotic livers were recruited during the study period. Three livers did not demonstrate any evidence of metabolic or synthetic liver function therefore perfusions were terminated early (non-functions 1-3, NF1-3). These livers were excluded from further analysis in order to ensure valid comparisons were made between the groups. When the main aim is to explore the effect of interventions on hepatic lipid metabolism, livers should all demonstrate evidence of function to ensure fair conclusions are being made regarding the intervention. Donor and perfusion characteristics from these 3 livers are shown in Tables 5.2 and 5.3.

Table 5.2. Donor demographics for non-functioning livers.

	Age (years)	Gender	Type	BMI (kg/m^2)	Girth (cm)	Cause of death	ALT (U/L)	Glucose (mmol/L)	CIT (h & min)	% total MaS	% sd-MaS	% ld-MaS	Perfusion Protocol	Perfusion duration (h)
NF1	67	Female	DBD	25.8	100	ICH	32	8.9	21h 33min	49	16	33	Group 1	12
NF2	57	Male	DBD	31.6	124	Hypoxia	227	9.8	8h	47	26	21	Group 2	24
NF3	56	Male	DCD	21.9	98	Seizure	46	5.6	15h 03min	42	32	10	Group 2	12

Abbreviations: ALT, alanine aminotransferase; BMI, body mass index; CIT, cold ischaemia time; DBD, donor after brain-stem death; DCD, donor after circulatory death; ICH, intracerebral haemorrhage; ld-MaS, large droplet macrovesicular steatosis; MaS, macrovesicular steatosis; NF, non-functioning; sd-MaS, small droplet macrovesicular steatosis.

Table 5.3. Perfusion characteristics for non-functioning livers.

	Mean arterial flow (L/min)	Mean portal flow (L/min)	pH start (30min)	pH end	Glucose start (mmol/L)	Glucose end (mmol/L)	Lactate start (mmol/L)	Lactate end (mmol/L)	ALT start (U/L)	ALT end (U/L)	TG start (µmol/L)	TG end (µmol/L)	Total bile (ml)
NF1	0.12	0.62	6.34	6.84	31	34	12.4	23	14724	19670	326	189.1	0
NF2	0.05	0.83	6.69	6.89	7.3	11.3	8.4	25	10066	12540	95.3	162.4	0
NF3	0.02	0.81	6.84	7.06	34	40	15	20	13854	17693	51.5	79.3	0

Eighteen livers were therefore included in the study and perfused for 48 h with 6 livers assigned to each group. Donor demographics and liver characteristics for these organs are shown in Table 5.4.

Table 5.4. Comparison between donor demographics and liver characteristics between groups

		Group 1 (NMP)	**Group 2 (NMP + filter)**	**Group 3 (NMP + filter + de-fatting interventions)**	**p-value**
Age (years)		53 (41-69)	64.5 (30-72)	45.5 (40-74)	0.66
Gender	Male	5	3	5	0.33
	Female	1	3	1	
Type	DBD	4	4	4	>0.99
	DCD	2	2	2	
BMI (kg/m^2)		28.5 (23.4-41.5)	30.1 (27.8-34.9)	27.6 (23.4-37.7)	0.90
Girth (cm)		104 (92-129)	105.5 (83-125)	105.5 (96-128)	0.94
ALT (U/L)		95 (25-119)	34 (16-104)	148 (16-352)	0.08
TG (μmol/L)		1267 (778-3124)	1777 (1513-2109)	1040 (853-4274)	0.96
Glucose (mmol/L)		10.2 (5.6-14.9)	9.8 (2.8-14.3)	6.6 (5.3-20.3)	0.94
Baseline total MaS (%)		29 (8-54)	14 (7-71)	36 (6-46)	0.87
Baseline tissue TG (μg/mg)		64.3 (45.0-132.7)	68.0 (12.4-204.7)	67.3 (21.3-267.9)	0.70
CIT (h & min)		12h 32min (8h 10min-13h 33min)	14h 43min (9h 6min-18h 42min)	11h 7min (7h 41min-14h 28min)	0.26

Data presented as median (range).

5.3.2 Functional liver analysis

5.3.2.1 Flow dynamics

Arterial pressure remained stable throughout the perfusions with no significant differences observed between mean pressures over the course of 48 h between the groups (Table 5.5).

Table 5.5. Mean arterial pressure over course of perfusion

	Group 1	Group 2	Group 3	p-value
Arterial pressure (mmHg)	66 ± 2	67 ± 1	67 ± 1	0.18

Data presented as mean ± SD.

A significant reduction in arterial flow rate from 0.18 ± 0.03 L/min to 0.09 ± 0.03 L/min ($p = 0.0003$) was observed in group 1 over the duration of the perfusion (Figure 5.3). However, in groups 2 and 3, an increase from 0.32 ± 0.06 L/min to 0.36 ± 0.15 L/min ($p = 0.55$) and 0.43 ± 0.11 L/min to 0.52 ± 0.18 L/min ($p = 0.31$) was observed, respectively.

Over the course of the whole perfusion, the mean arterial flow was significantly higher in group 2 (0.35 ± 0.12 L/min) than in group 1 (0.14 ± 0.02 L/min) ($p = 0.0006$) and in group 3 (0.48 ± 0.12 L/min) compared to group 1 ($p < 0.0001$). The increased mean arterial flow in group 3 compared to group 2 did not reach statistical significance ($p = 0.07$) (Figure 5.3).

A slight increase in portal flow was seen over the course of the perfusion in all 3 groups with no difference between groups (Figure 5.4).

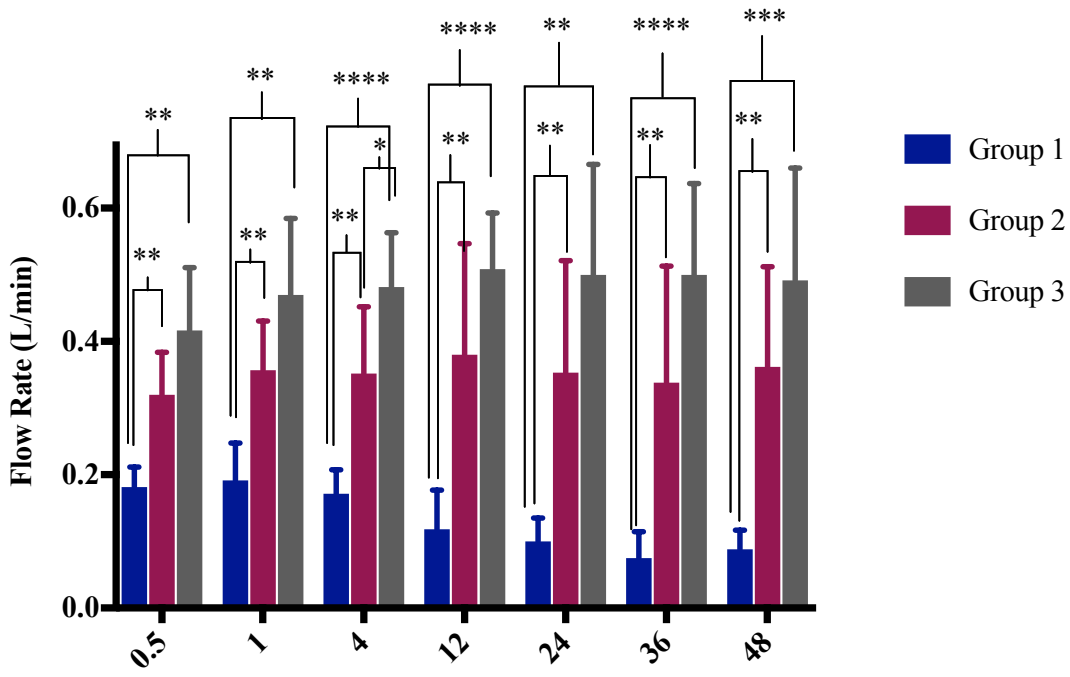


Figure 5.3. Arterial flow rates (L/min) in the respective treatment groups. Arterial flow is significantly higher at each time point between groups 2 and 3 and group 1. Arterial flow is also higher in group 3 compared to group 2 and this is statistically significant at 4h into the perfusion. Data presented as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

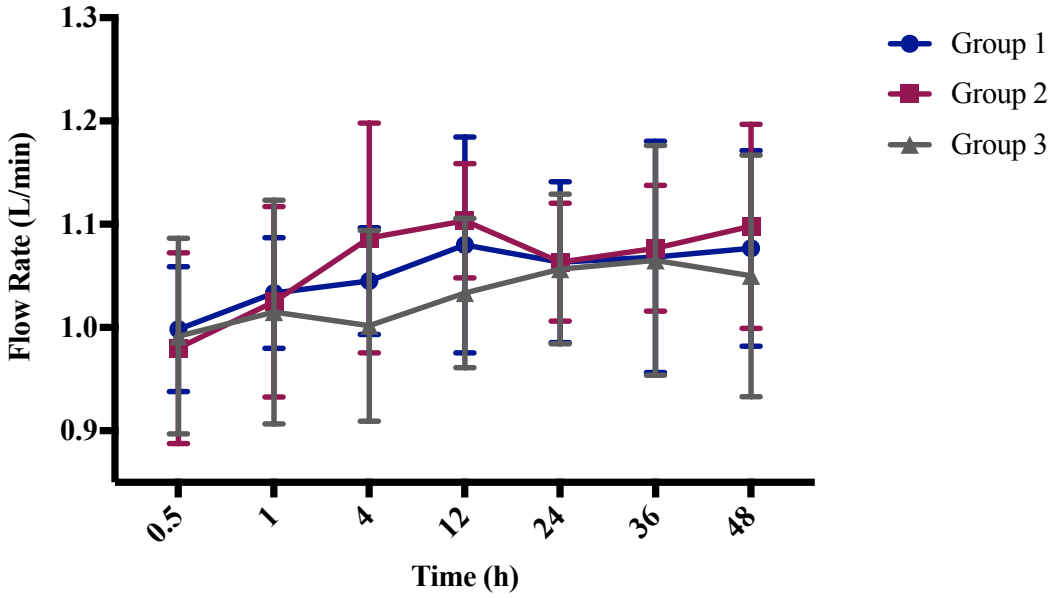


Figure 5.4. Effect of treatment on portal flow (L/min) between groups over time. The mean portal flow over 48h was comparable between groups 1 (1.05 ± 0.07 L/min), 2 (1.06 ± 0.07 L/min) and 3 (1.03 ± 0.09 L/min) ($p = 0.82$). Data presented as mean ± SD.

5.3.2.2 Blood gas analysis

5.3.2.2.1 Perfusate pH

All livers normalised pH in a similar pattern with no differences observed between groups (Figure 5.5). However, after an initial bolus of 30 ml 8.4% sodium bicarbonate (B Braun) during priming in all groups, group 3 required less sodium bicarbonate supplementation (45 ml (20-70 ml)) than both groups 1 (65 ml (40-90 ml)) ($p = 0.25$) and 2 (60 ml (40-90 ml)) ($p = 0.36$).

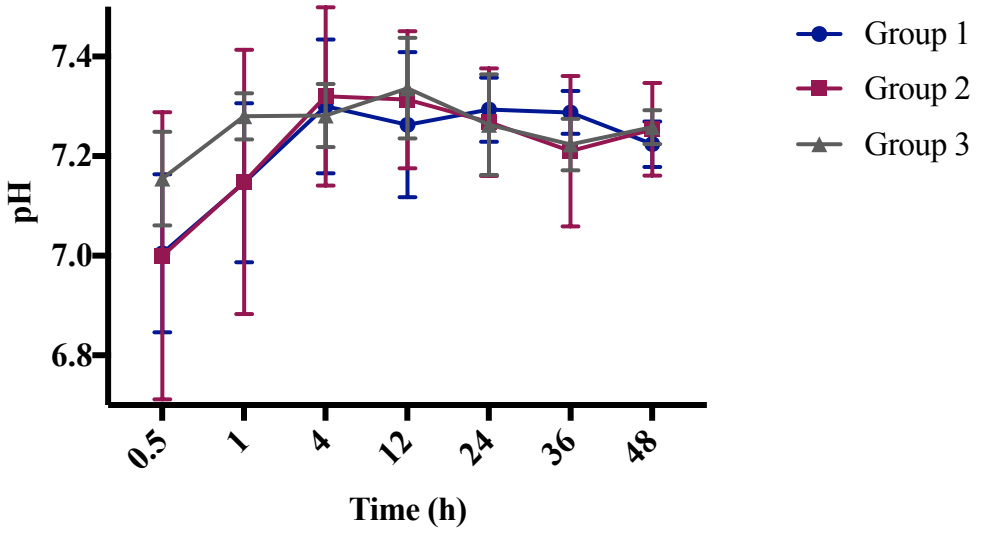


Figure 5.5. Perfusate pH over time in the different treatment groups. pH increased in all groups and remained fairly stable until the end of the experiment. Data presented as mean ± SD.

5.3.2.2.2 Perfusate lactate

A significant, spontaneous reduction in lactate was seen from the beginning of perfusion until 24 h in all 3 groups (group 1: 9.78 ± 4.81 mmol/L to 0.8 ± 0.78 mmol/L, $p = 0.001$; group 2: 10.67 ± 4.27 mmol/L to 2.50 ± 3.48 mmol/L, $p = 0.005$; group 3: 7.77 ± 3.20 mmol/L to 1.78 ± 2.68 mmol/L, $p = 0.006$). However, from 24 h to 48 h a non-significant increase in perfusate lactate was seen and this was consistent between the groups (Figure 5.6).

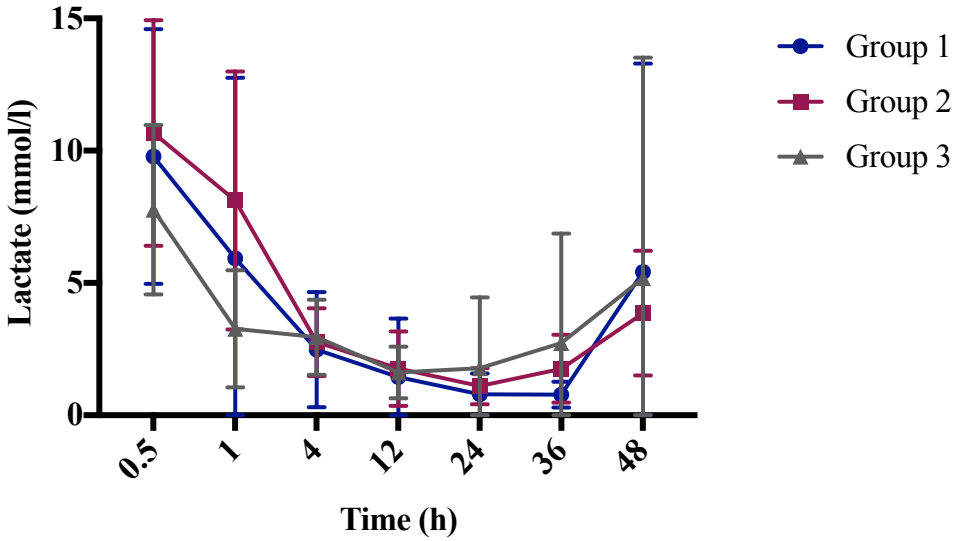


Figure 5.6. Lactate concentrations (mmol/L) in perfusate over time in the different treatment groups. Data presented as mean ± SD.

5.3.2.2.3 Perfusate glucose

All livers metabolised glucose and a significant reduction from 30 min until 48 h was seen in groups 1 (32.78 ± 10.99 mmol/L to 14.95 ± 14.55 mmol/L, $p = 0.04$) and 2 (29.28 ± 10.74 mmol/L to 14.47 ± 3.49 mmol/L, $p = 0.02$) (Figure 5.7). The effect was greatest in group 3 where the mean glucose level fell from 38.50 ± 2.95 mmol/L to 8.18 ± 2.7 mmol/L ($p < 0.0001$) over the course of the experiment. In group 3, where a lower glucose threshold was targeted, the perfusate glucose at 48 h was significantly lower than in group 2 ($p = 0.008$).

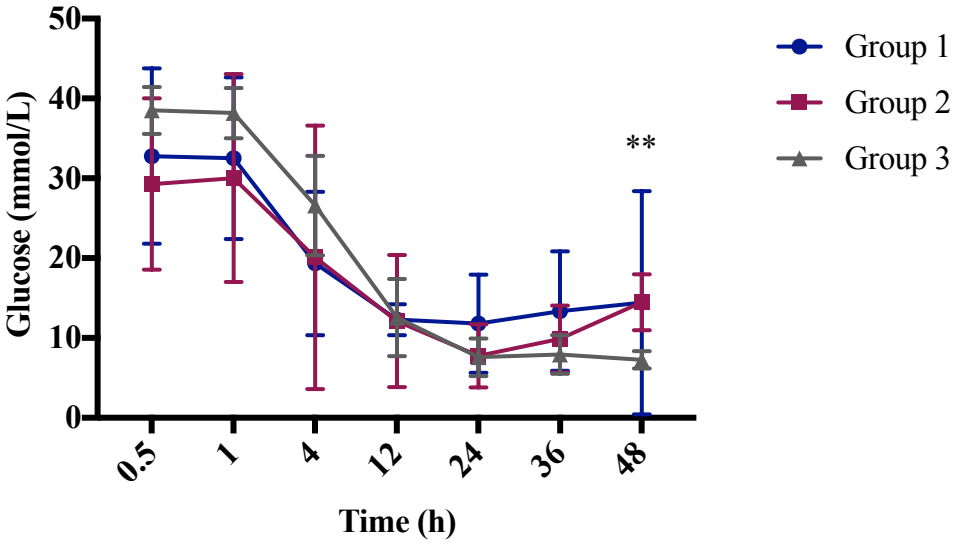


Figure 5.7. Glucose concentration (mmol/L) in perfusate over time in the different treatment groups. Data presented as mean ± SD. ** $p < 0.01$ group 3 vs. group 2.

5.3.2.3 Bile production

Cumulative bile production was comparable between groups with a median of 235 ml (0-400 ml) in group 1, 185 ml (0-400 ml) in group 2 and 195 ml (0-410 ml) in group 3 ($p = 0.98$).

5.3.2.4 Perfusate markers of hepatocellular function

5.3.2.4.1 ALT

There was an increase in perfusate ALT in all 3 groups, however this was greatest (and only significant) in group 1 where the ALT increased from a median of 2430 U/L (695 – 4473 U/L) to 6031 U/L (2614 – 12679 U/L) ($p = 0.04$) (Figure 5.8). The change in perfusate ALT over the course of 48 h in group 1 (+ 4328 U/L (751 – 8346 U/L)) was greater than both groups 2 (+ 1738 U/L (340 – 5899 U/L)) ($p = 0.31$) and 3 (+ 2505 U/L (847 – 10341 U/L) ($p = 0.70$).

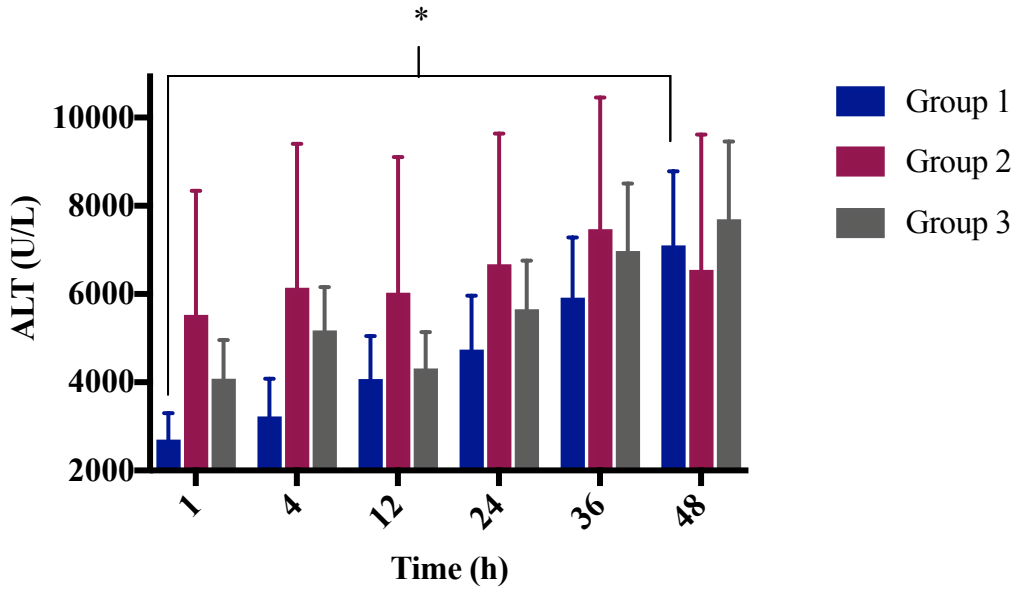


Figure 5.8. Perfusate ALT (U/L) concentration over time in the different treatment groups. Data presented as mean ± SD. * $p < 0.05$.

5.3.2.4.2 Haemolysis index (HI)

A significant increase in HI was seen in all groups, with HI going from 0.82 ± 0.66 to 9.98 ± 6.04 ($p = 0.004$) in group 1, 0.64 ± 0.42 to 7.51 ± 5.60 ($p = 0.01$) in group 2 and 0.57 ± 0.43 to 8.03 ± 4.70 ($p = 0.003$) in group 3 over the course of the 48h (Figure 5.9). Up until 24 h, the degree of haemolysis was attenuated in groups 2 and 3 compared to group 1 with a mean change of + 2.21 ± 3.55 and + 2.8 ± 3.36 in groups 2 and 3, respectively and + 4.76 ± 5.59 in group 1 ($p = 0.57$). A pictorial representation of the progressively increasing haemolysis (as characterised by a deeper shade of red) is shown in Figure 5.10.

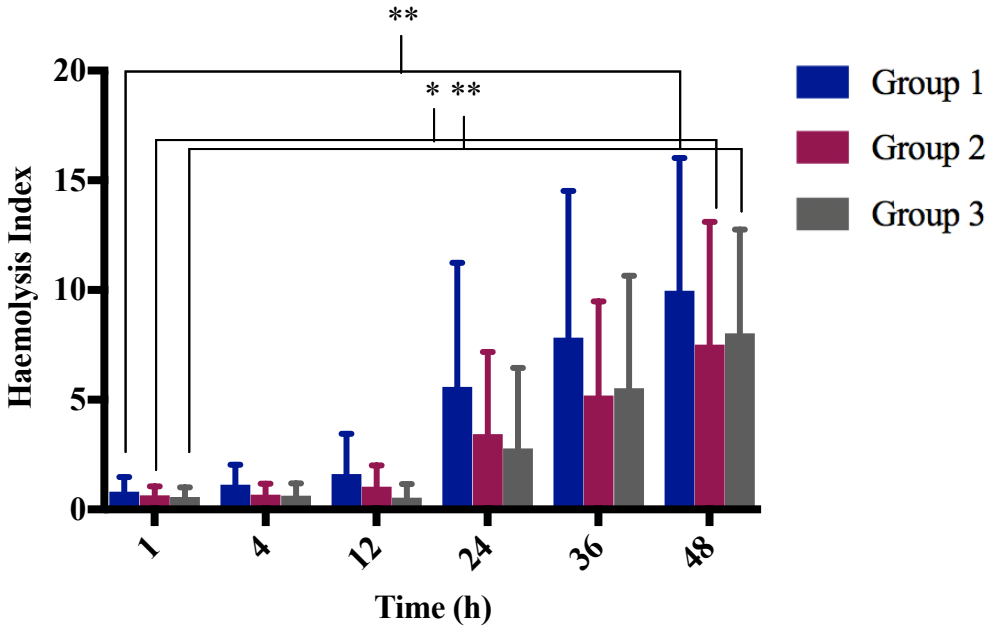


Figure 5.9. Haemolysis index over time in the different treatment groups. Data presented as mean ± SD. * $p < 0.05$, ** $p < 0.01$.

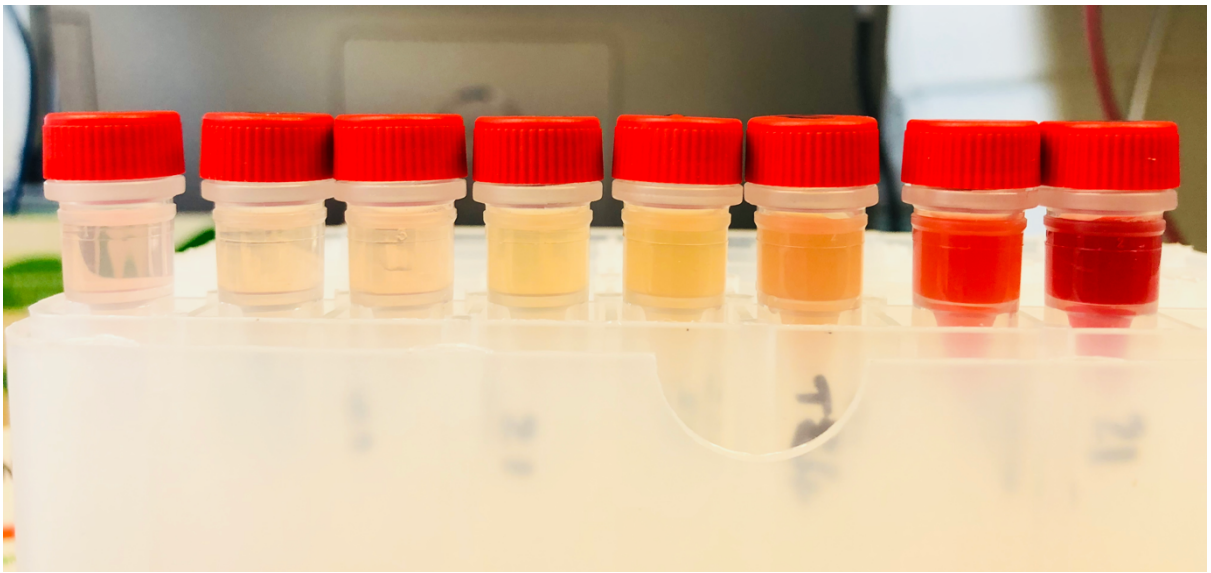


Figure 5.10. Image demonstrating increasing haemolysis in the perfusate (left to right) over the course of 48 h from a liver in group 1.

5.3.2.5 Glycogen synthesis

Between 0 and 12 h, all livers across the 3 groups replenished their glycogen stores (Figure 5.11). In group 1, the percentage of glycogen in the tissue increased from 24 ± 17% pre-perfusion to 42 ± 12% at 12 h ($p = 0.06$). In group 2, hepatic glycogen content increased from 24 ± 16% pre-perfusion to 39 ± 20% at 12 h ($p = 0.19$). In group 3, there was a significant increase from 33 ± 14% to 53 ± 11% at 12 h ($p = 0.02$). After 12 h, glycogen content decreased in group 1 (from 42 ± 12% to 25 ± 14% at 48 h, $p = 0.07$). However, between 12 and 48 h hepatic glycogen content increased and was maintained in groups 2 and 3 with levels of 48 ± 15% and 48 ± 20%, respectively, at 48 h. There was a tendency for a difference in hepatic glycogen content at 48 h between group 1 and 2 and group 1 and 3 ($p = 0.06$ and $p = 0.07$, respectively).

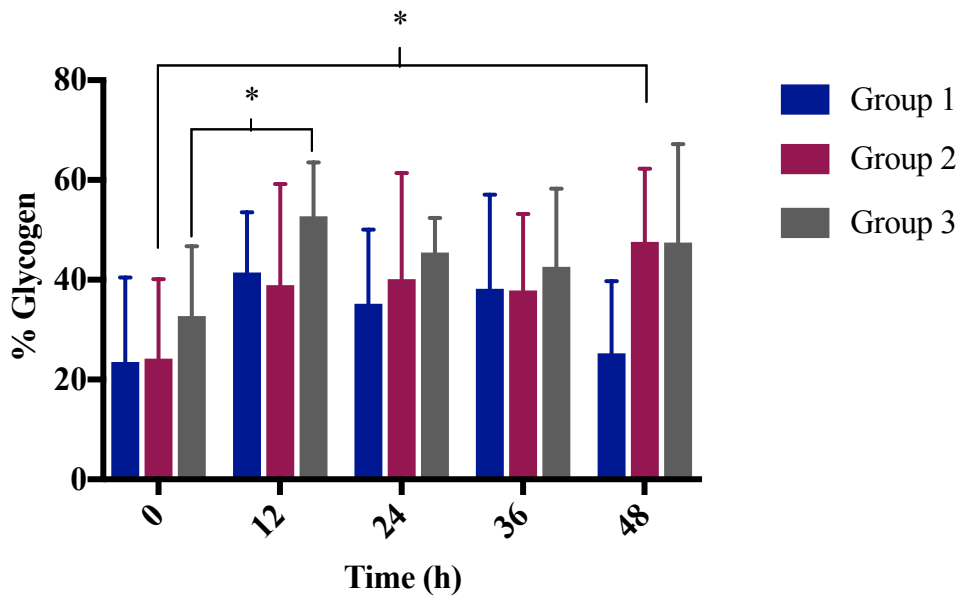


Figure 5.11. Percentage of glycogen on liver sections as defined by positive uptake of PAS stain in the different treatment groups. Data presented as mean ± SD. * $p < 0.05$.

5.3.3 Hepatic lipid metabolism

5.3.3.1 Perfusate TG

A significant increase in median perfusate TG was seen from 1 h to 48 h in all 3 groups (Figure 5.12). However, in group 1, TG increased by 4327 μmol/L (3446 – 12854 μmol/L), which was greater compared to 2016 μmol/L (890 – 5214 μmol/L) in group 2 (Δ group 1 vs. Δ group 2, p = 0.06) and 1559 μmol/L (-103 – 3293 μmol/L) in group 3 (Δ group 1 vs. Δ group 3, p = 0.002). It appears that the addition of the lipoprotein apheresis filter in these groups is effective at removing the TG mobilised by the liver from the circuit. This effect is greatest in group 3 where a significant reduction in perfusate TG was seen compared to group 1 at 24 h (p = 0.03), 36 h (p = 0.004) and 48 h (= 0.009) (Figure 5.12).

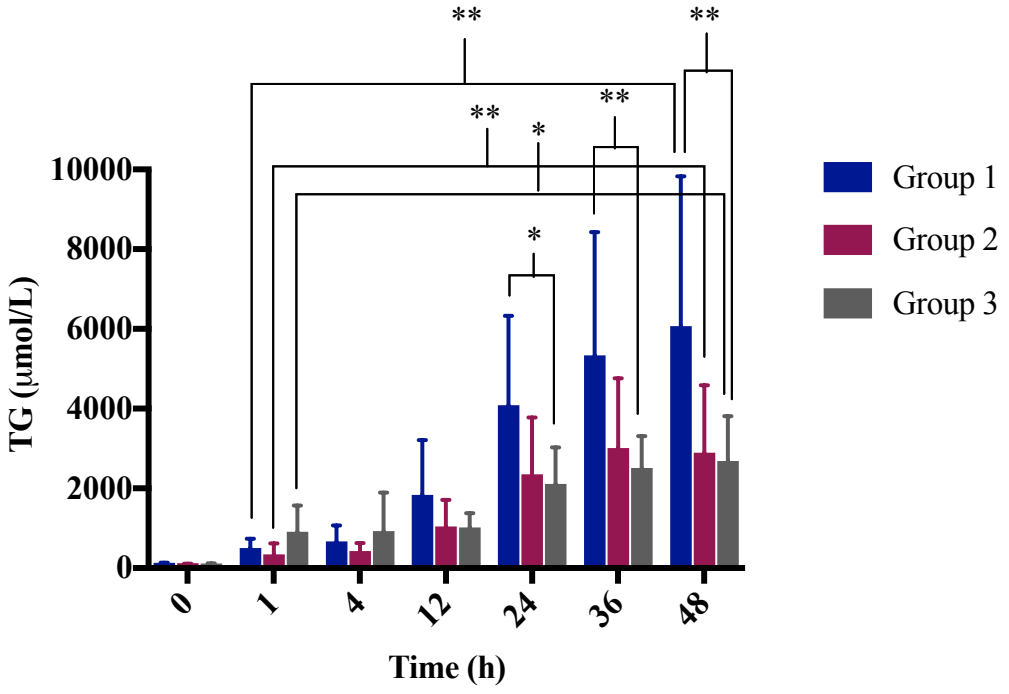


Figure 5.12. Perfusate TG (μmol/L) over time in the treatment groups. Data presented as mean ± SD. * $p < 0.05$, ** $p < 0.01$.

5.3.3.2 Perfusate TC

A significant increase in median perfusate TC was seen from 1 h to 48 h in all 3 groups (Figure 5.13). However, as was observed with TG, the median increase of TC in group 1 (+ 3.9 mmol/L (2.9 – 3.46 mmol/L) was significantly greater than in groups 2 (+ 2.05 mmol/L (1.12 – 2.51 mmol/L)) (p = 0.002) and 3 (+ 2.01 mmol/L (0.64 – 2.76 mmol/L)) (p = 0.002). At 48 h median perfusate TC was significantly (p = 0.04) lower in group 2 than in group 1 (Figure 5.13).

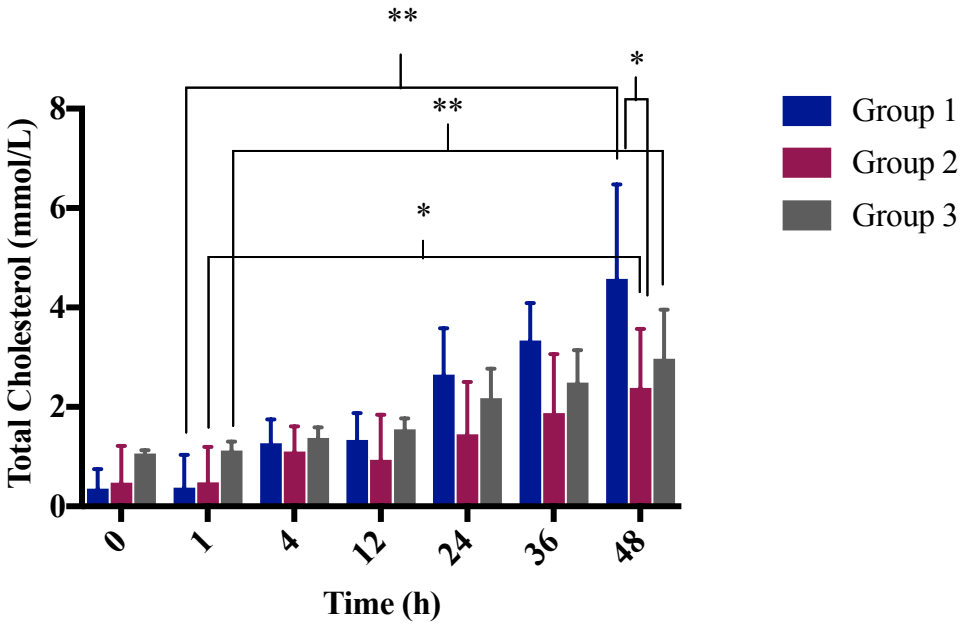


Figure 5.13. Perfusate total cholesterol (mmol/L) over 48 h between groups. Data presented as mean ± SD. * $p < 0.05$, ** $p < 0.01$.

5.3.3.3 Perfusate ApoB

The amount of circulating ApoB remained fairly stable over the course of 48 h in groups 2 and 3 and an increase was seen in group 1 (0.29 ± 0.24 µmol/L to 0.52 ± 0.36 µmol/L, p = 0.30) (Figure 5.14A). At 48 h there was a non-significant reduction in ApoB between group 1 (0.52 ± 0.36 µmol/L) and group 2 (0.26 ± 0.08 µmol/L) (p = 0.12) and group 1 and group 3 (0.25 ± 0.18 µmol/L) (p = 0.14) (Figure 5.14A). There was a comparable increase in the

TG:ApoB molar ratio between all 3 groups, suggesting an increase of particle size over the course of the perfusion. However, there was no difference between groups (Figure 5.14B).

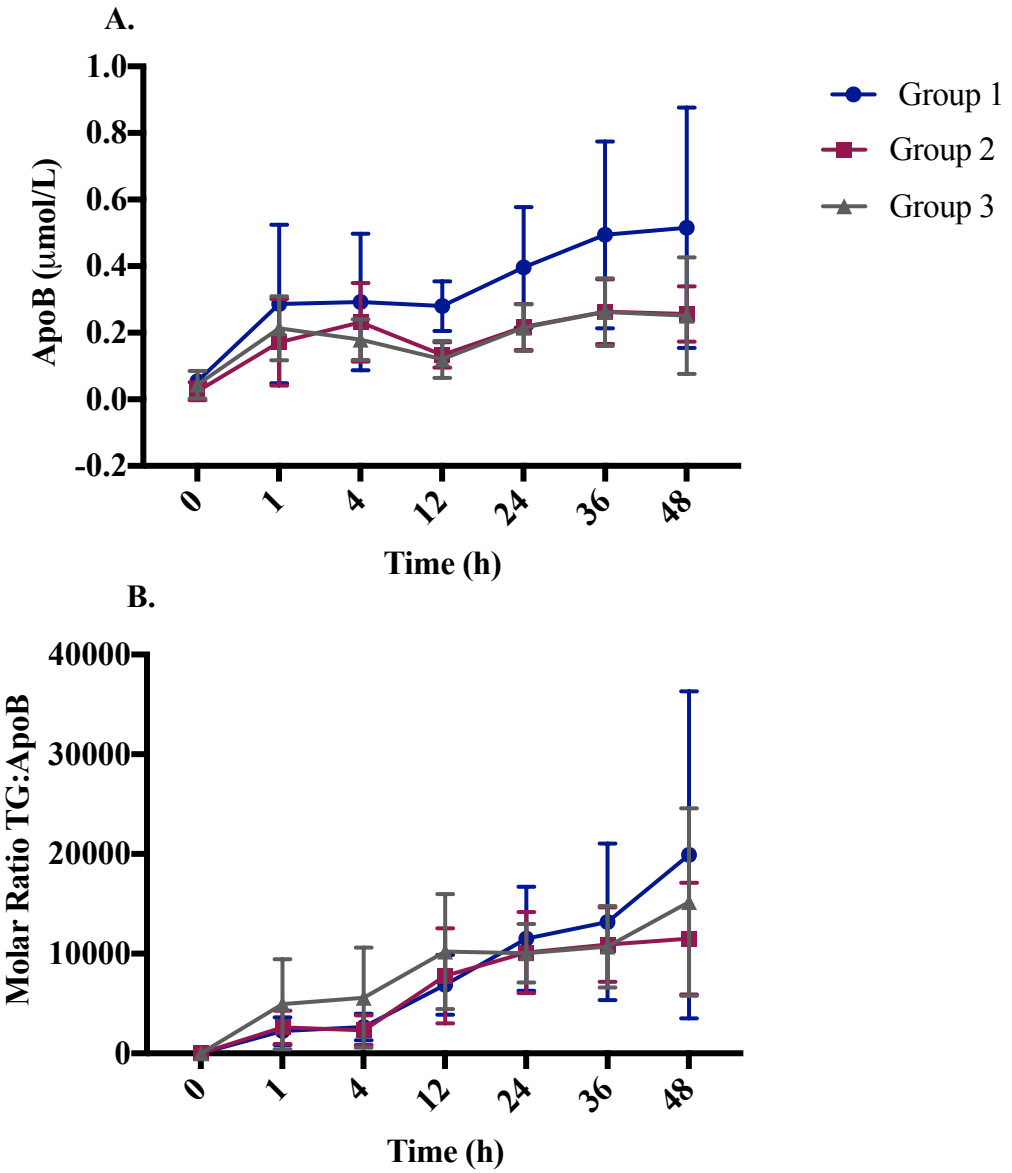


Figure 5.14A-B. Change in ApoB **(A)** and molar ratio of TG:ApoB **(B)** over time between groups.

5.3.3.4 Perfusate (3-OHB)

A significant increase in perfusate 3-OHB was seen in all 3 groups (Figure 5.15), providing evidence of fatty acid oxidation during perfusion. The greatest median increase was seen in group 3 (+ 3121µmol/L (557 – 6084 µmol/L)), where l-carnitine was added to the perfusate. This represented a 28% increase compared to group 1 (+ 2437 µmol/L (534 – 2964 µmol/L)) ($p = 0.24$) and 63% increase compared to group 2 (+ 1918 µmol/L (578 – 4240 µmol/L)) ($p = 0.39$). The levels of 3-OHB were significantly higher at 36 h in group 3 compared to both groups 1 ($p = 0.04$) and 2 ($p = 0.009$) (Figure 5.15).

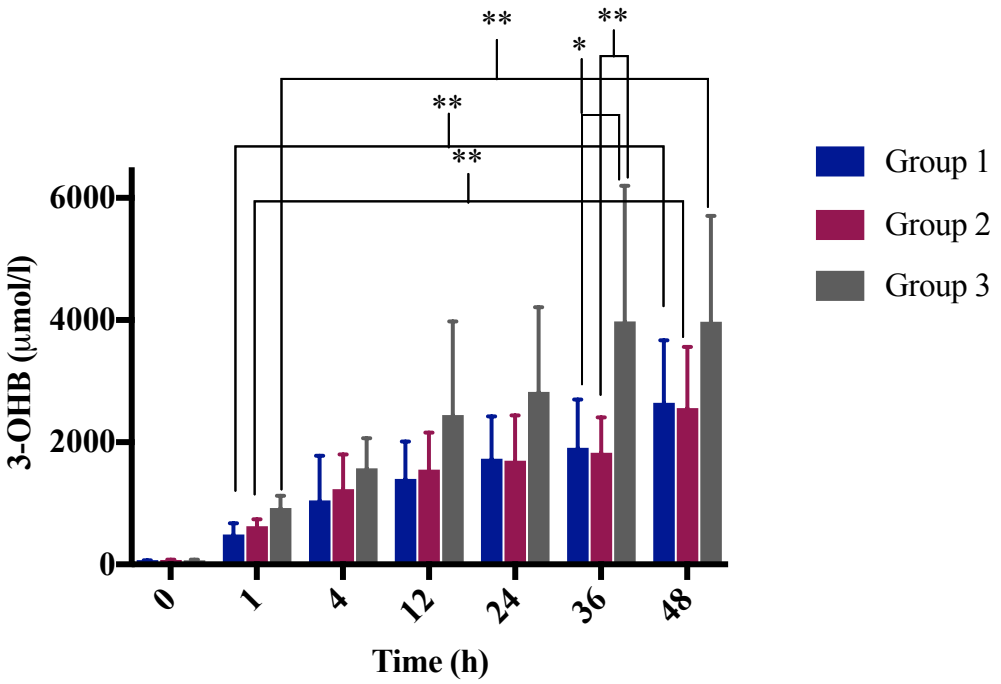


Figure 5.15. Perfusate 3-OHB (µmol/L) over time in the respective treatment groups. Data presented as mean ± SD. * $p < 0.05$, ** $p < 0.01$.

5.3.4 Hepatic Steatosis

5.3.4.1 Histological quantification

There appeared to be no change in the degree of total macrovesicular hepatic steatosis in groups 1 and 2 (Figure 5.16A and B, respectively). However, there was a non-significant reduction from a median of 36% (6 – 46%) to 27% (1 – 36%) (p = 0.31) in group 3 (5.16C). The median reduction in group 3 (-9% (-21 – 3%)) was significant compared to a slight median increase (+ 3% (-2 – 6%)) in group 2 (p = 0.02).

No differences were observed between the degree of small droplet macrovesicular steatosis (sd-MaS) or large droplet MaS (ld-MaS) over the course of the perfusion in groups 1 or 2 (data not shown). The observed reduction of total MaS observed in group 3 was mostly limited to the ld-MaS (11% (1 – 25%) to 4% (0 – 19%), p = 0.28).

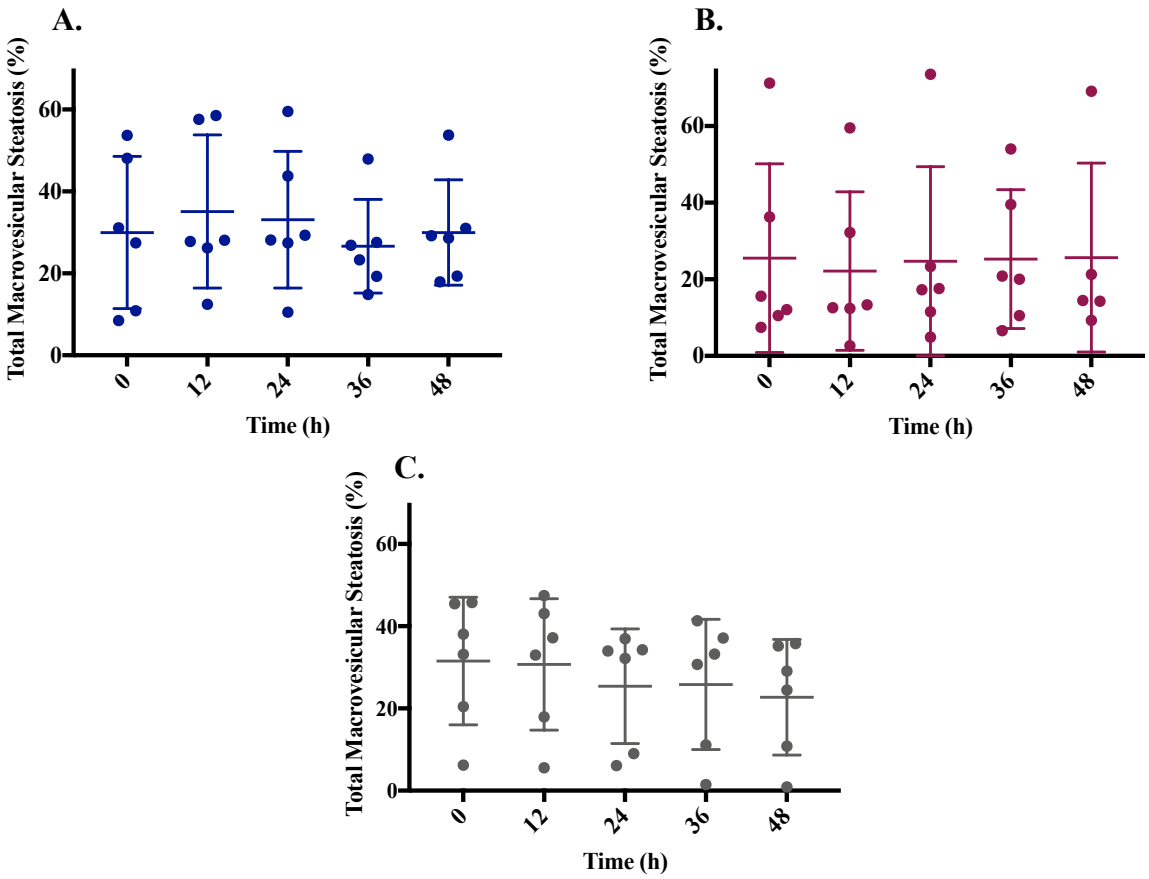
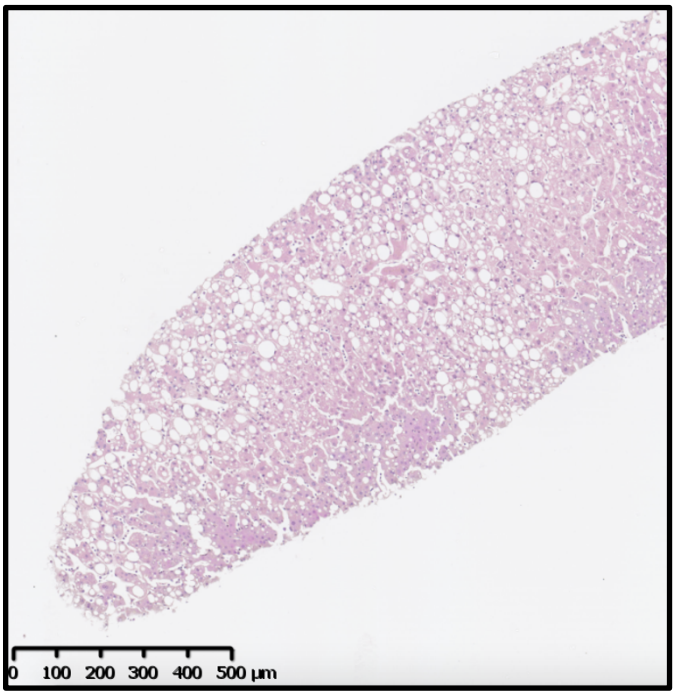


Figure 5.16A-C. Scatter-dot plots demonstrating trend in total MaS over time for group 1 **(A)**, Group 2 **(B)** and Group 3 **(C)**. Data presented as mean ± SD.

Figure 5.17 highlights a demonstrable microscopic reduction in MaS which was observed from a liver in group 3.

A.



B.

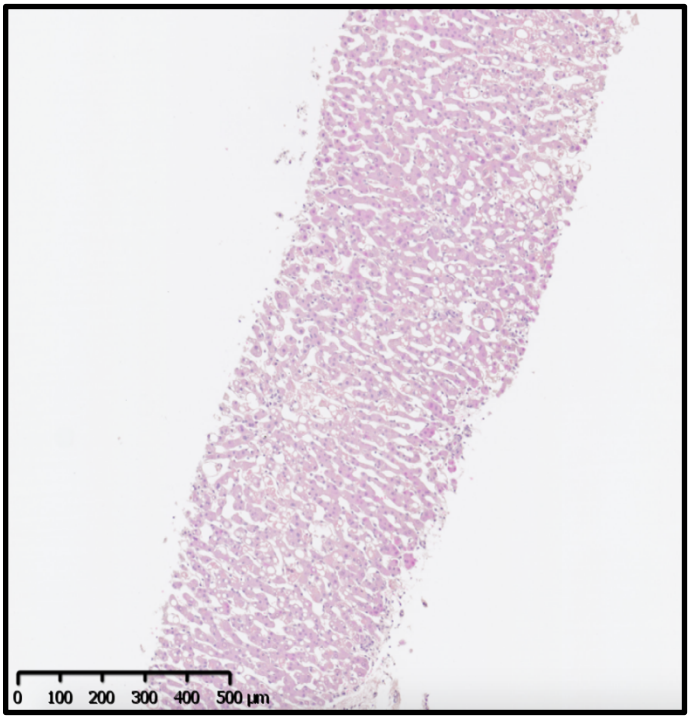


Figure 5.17A-B. H&E stained section demonstrating a greater degree of MaS before preservation (45%) **(A)** and a demonstrable reduction at 48 h to 24% **(B)** following intervention with lipoprotein apheresis filtration and pharmacological intervention (group 3).

5.3.4.2 Tissue TG

Intrahepatocellular tissue TG content was quantified by GC, as an internal standard was added and therefore total fatty acid content could be calculated. In group 1, a slight increase in tissue TG was seen over the course of 48 h from a median of 64.52 µg/mg (48.65 - 134.1 µg/mg) to 72.96 µg/mg (39.60 - 154.30 µg/mg) (P = 0.39) (Figure 5.18). The livers in group 2 saw a fluctuation in tissue TG during the 48 h experiment and saw no great change over the whole time period (p = 0.79) (Figure 5.18). However, livers in group 3 saw a reduction from a median of 67.98 µg/mg (19.45 - 260.5 µg/mg) to 54.18 µg/mg (6.57 – 119 µg/mg) (p = 0.26) (Figure 5.18). The reduction in group 3 was statistically significant compared to the increase in group 1 (p = 0.04).

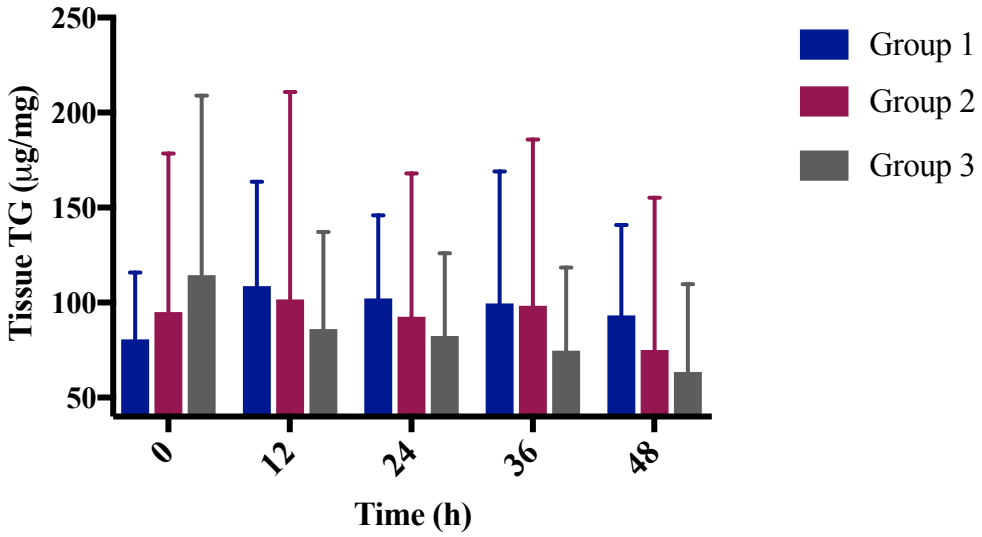


Figure 5.18. Change in tissue TG (µg TG / mg tissue) over time for each group. Data presented as mean ± SD.

I assessed association between the two methods (histological and biochemical) used to determine intrahepatic fat and found a strong, positive correlation (r = 0.77, p < 0.0001) between % total MaS and tissue TG (Figure 5.19), making this a useful method of quantifying steatosis and to explore any intervention-based changes.

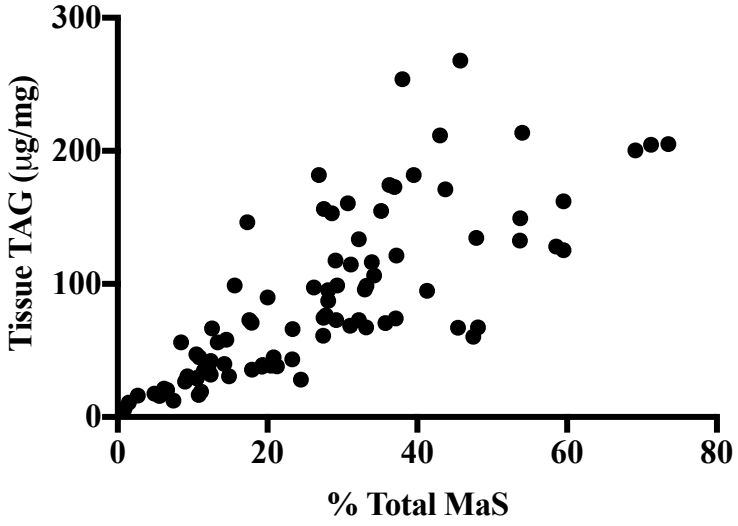


Figure 5.19. Association between liver fat content assessed histologically (percent of total macrosteatosis) and liver TG content assessed biochemically (µg TG / mg tissue). $r = 0.77$, $p < 0.0001$.

5.3.4.3 Liver and perfusate TG fatty acid composition

The most abundant fatty acids in liver tissue and perfusate TG were identified and the change in their proportions over time are shown in Tables 5.6 and 5.7, respectively. The fatty acid composition of the perfusate was similar between groups and remained stable over the course of the perfusion. However, a non-significant reduction in the percentage contribution to the total fatty acid pool of the essential polyunsaturated omega-6 fatty acid, linoleic acid (C18: 2n-6), was seen in each group over the 48 h duration for both perfusate (Table 5.6) and tissue (Table 5.7). The most abundant fatty acids in tissue and perfusate were: myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1n-7), stearic acid (C18:0), oleic acid (18:1n-9) and linoleic acid (18:2n-6).

Table 5.6. The change in proportion (mol%) of perfusate TG fatty acid composition over time.

	12h				24h				36h				48h		
Fatty Acid	**Group**				**Group**				**Group**				**Group**		
	1	2	3		1	2	3		1	2	3		1	2	3
C14:0	1.86 ± 0.67	2.35 ± 0.49	2.18 ± 0.62		1.95 ± 0.68	2.44 ± 0.52	2.14 ± 0.59		1.98 ± 0.64	2.48 ± 0.57	2.08 ± 0.57		1.99 ± 0.59	3.74 ± 2.30	2.09 ± 0.52
C16:0	31.10 ± 4.16	33.67 ± 3.16	33.98 ± 2.83		30.76 ± 4.45	33.40 ± 3.57	33.00 ± 2.48		30.41 ± 4.52	33.38 ± 3.59	32.40 ± 2.69		29.88 ± 4.91	33.19 ± 4.58	32.64 ± 2.78
C16:1*n-7*	2.85 ± 1.36	3.79 ± 0.89	5.05 ± 2.34		3.91 ± 1.25	4.53 ± 0.60	5.29 ± 1.93		3.87 ± 1.52	4.89 ± 0.53	5.37 ± 1.75		3.95 ± 1.51	4.79 ± 0.54	5.54 ± 1.63
C18:0	5.23 ± 1.40	5.71 ± 1.82	5.54 ± 1.12		5.00 ± 1.42	5.51 ± 1.69	5.23 ± 0.84		4.99 ± 1.31	5.46 ± 1.70	5.23 ± 0.77		4.99 ± 1.26	5.15 ± 1.48	5.45 ± 0.97
C18:1*n-9*	37.25 ± 4.83	36.70 ± 3.70	38.47 ± 4.26		38.54 ± 4.62	37.64 ± 3.70	39.94 ± 3.59		39.13 ± 4.56	37.82 ± 3.96	40.78 ± 4.13		39.88 ± 5.76	37.24 ± 4.17	40.48 ± 3.52
C18:2*n-6*	14.88 ± 3.75	11.64 ± 3.18	10.11 ± 2.40		13.98 ± 3.51	10.79 ± 3.00	9.60 ± 2.45		13.34 ± 3.45	10.26 ± 3.10	9.24 ± 2.32		12.93 ± 3.46	10.64 ± 2.5	9.03 ± 2.16

Data presented as mean ± SD.

Table 5.7. The change in proportion (mol%) of tissue TG fatty acid composition over time.

	0h				12h				24h				36h				48h		
Fatty Acid	**Group**				**Group**				**Group**				**Group**				**Group**		
	1	2	3		1	2	3		1	2	3		1	2	3		1	2	3
C14:0	2.55 ± 1.18	2.76 ± 0.83	2.70 ± 1.00		2.46 ± 1.10	2.81 ± 0.82	2.62 ± 0.91		2.42 ± 1.08	2.76 ± 0.83	2.74 ± 0.79		2.50 ± 0.93	2.74 ± 0.82	2.52 ± 0.78		2.50 ± 0.96	2.56 ± 0.89	2.54 ± 0.68
C16:0	32.16 ± 2.77	33.24 ± 3.79	33.18 ± 2.64		33.50 ± 3.38	33.93 ± 4.18	33.36 ± 2.47		32.66 ± 3.57	34.44 ± 4.17	33.33 ± 2.82		33.12 ± 2.95	34.26 ± 4.67	33.18 ± 2.88		32.86 ± 2.98	33.62 ± 5.99	32.98 ± 3.94
C16:1*n-7*	3.93 ± 1.63	5.84 ± 1.48	5.32 ± 2.55		3.66 ± 1.35	5.41 ± 1.88	5.01 ± 2.37		3.91 ± 1.25	5.19 ± 1.71	4.95 ± 2.17		4.19 ± 1.26	5.49 ± 1.85	4.85 ± 1.96		3.87 ± 1.06	5.06 ± 0.89	4.78 ± 1.92
C18:0	4.69 ± 0.83	4.37 ± 1.31	4.77 ± 0.54		4.74 ± 0.86	4.76 ± 1.47	5.13 ± 0.58		4.92 ± 0.89	4.91 ± 1.34	5.23 ± 0.53		5.02 ± 0.98	5.28 ± 1.22	5.51 ± 0.61		5.45 ± 0.89	5.82 ± 1.12	5.94 ± 0.98
C18:1*n-9*	41.12 ± 4.80	40.70 ± 3.37	41.70 ± 3.75		41.01 ± 4.96	40.22 ± 3.01	41.07 ± 3.39		41.45 ± 5.01	40.61 ± 2.90	41.77 ± 3.78		41.08 ± 4.44	40.47 ± 3.46	41.68 ± 3.63		41.35 ± 4.65	40.66 ± 4.79	41.66 ± 4.39
C18:2*n-6*	12.64 ± 2.05	9.76 ± 4.09	8.69 ± 2.89		12.29 ± 1.91	9.56 ± 4.13	8.92 ± 3.19		11.80 ± 1.76	8.86 ± 3.72	8.50 ± 2.92		11.18 ± 1.81	8.41 ± 3.59	8.17 ± 2.82		11.07 ± 2.05	8.94 ± 2.43	7.69 ± 2.71

Data presented as mean ± SD.

5.3.4.4 DNL

There was a significant increase in the amount of fatty acids synthesised from non-lipid precursors via DNL in the perfusate over the duration of perfusion in all groups (Figure 5.20A). However, there was no significant difference between groups at each time point or change over time (Figure 5.20A).

When exploring the amount of fatty acids synthesised via DNL in the tissue, there was a non-significant increase over time in each group. However it is clear that DNL was attenuated in group 3 (Figure 5.20B) with a median increase of only 2.51 μg/mg (-11.09 – 10.3 μg/mg) in group 3 compared to a 7.15 μg/mg (2 – 41.8 μg/mg) increase in group 1 ($p = 0.18$) and 11.34 μg/mg (1.78 – 22.43 μg/mg) increase in group 2 ($p = 0.09$). At 48 h there was a significant reduction in DNL in group 3 compared to group 2 ($p = 0.03$) (Figure 5.20B).

There was a weak (although statistically significant) correlation between perfusate and tissue DNL ($r = 0.37$, $p = 0.001$) (Figure 5.21).

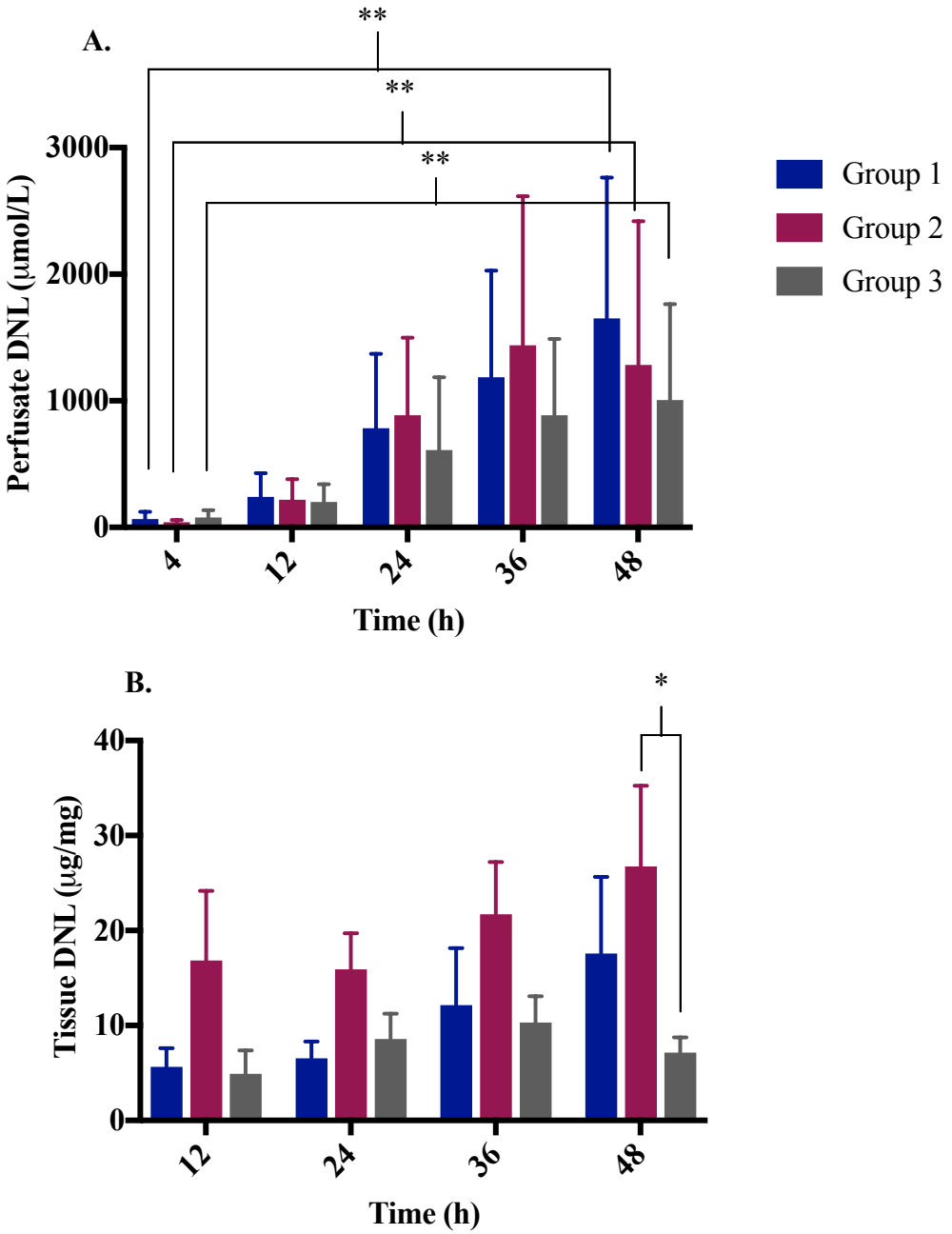


Figure 5.3A-B. Amount of DNL fatty acids in the perfusate **(A)** and tissue **(B)**. Data presented as mean ± SD. * $p < 0.05$

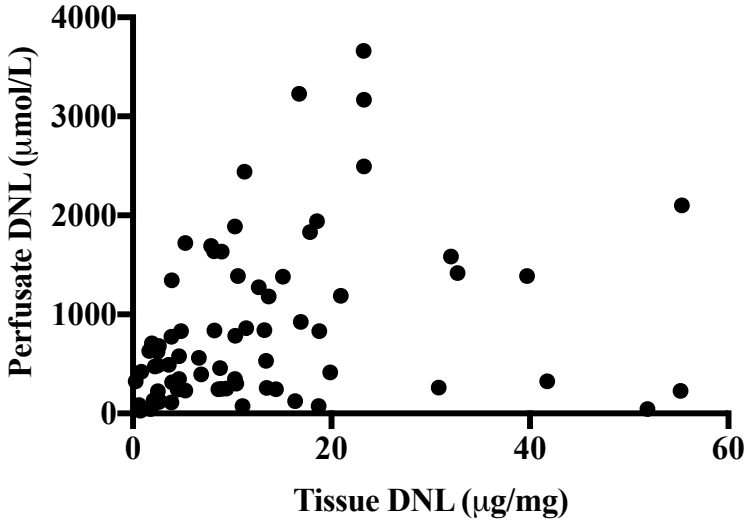


Figure 5.21. The association between tissue and perfusate DNL. r = 0.37, p = 0.001

5.4 Discussion

The success of liver transplantation is limited by a shortage of suitable donor organs. As discussed throughout this thesis, steatotic livers result in particularly poor outcomes when transplanted and as a result, a large number are discarded (79, 133). In Chapter 4, I reported data suggesting that NMP improves post-transplant outcomes from steatotic livers primarily through a reduction in IRI. However, all livers included in that study were deemed suitable for transplantation (despite some high levels of MaS). In order to push the boundaries further and enable the safe and reliable transplantation of severely steatotic livers which are currently discarded, it may be necessary to further enhance their function during NMP and reduce the amount of fat within the liver. If the recipient of a steatotic liver overcomes the initial severe reperfusion injury and avoids a PNF, longer-term outcomes can be satisfactory (146). Furthermore, when the steatotic liver is exposed to a more physiological environment in the recipient, where the imbalance between fatty acid input and disposal is removed, the fat within the liver can disappear (147). This provides the impetus to attempt to optimise severely steatotic grafts through specific interventions during NMP. In this chapter I have demonstrated that hepatic lipid metabolism can be manipulated in the *ex situ* setting in order to create an improved environment for liver preservation with enhanced function. Through the addition of an apheresis filter to the circuit, a significant increase in hepatic arterial blood flow was observed, as well as significant reductions in circulating lipoproteins (namely TG, carried in VLDL, and cholesterol) and increase in hepatic glycogen content. As well as the benefits of lipoprotein apheresis filtration, pharmacological intervention further improved arterial flow, significantly reduced circulating glucose levels, increased mitochondrial β-oxidation and reduced hepatic TG content. It is likely that the reduction in TG content was due to increased fatty acid oxidation and a reduction in DNL. The weaker correlation between liver and perfusate DNL suggests that in some livers the DNL fatty acids are not

being exported in VLDL-TG whilst in others, it would appear that DNL fatty acids are being channelled toward secretory pathways; why there is this difference in export of DNL fatty acids between livers remains unclear.

The livers included in this study demonstrated varying degrees of steatosis although there was no significant differences between groups, which were well matched for baseline (time 0) characteristics. Despite some non-significant differences between the groups such as donor ALT and CIT, all livers demonstrated similar patterns of crude function as defined by lactate clearance, glucose metabolism and pH maintenance (although livers in group 3 required less sodium bicarbonate supplementation to maintain a physiological pH, $p = 0.36$). All livers demonstrated evidence of metabolic activity (albeit to varying degrees), meaning that the effect of interventions on each group in terms of lipid metabolism are comparable. As can be seen from the 3 livers that were excluded, rising lactate levels and the inability to normalise pH or glucose resulted in very little TG being measured in the perfusate. These non-functioning livers were also characterised by very low hepatic arterial and portal flows as well as perfusate ALT levels > 10,000 U/L representing severe hepatocellular injury. The low perfusate TG levels in these livers was in the context of severe steatosis and based on my previous findings, much higher perfusate TG concentrations would be expected, as it has been reported that liver fat is associated with an increase in VLDL-TG secretion rate (221). It would, therefore, be inappropriate to include these livers for further analysis as a perceived alteration in lipid metabolism may simply be attributable to the metabolic inactivity of a failing organ. None of the non-functioning livers were subjected to the de-fatting interventions. One can only speculate as to whether these livers might have been salvaged by the addition of forskolin and l-carnitine and reduction of insulin and glucose.

When no intervention was included (group 1), there was a significant reduction in hepatic arterial flow over the course of the perfusion. This finding was reversed by the addition of the filter in group 2 where arterial flow was significantly higher than in group 1 and a slight increase was observed over 48 h. The effect of hepatic steatosis on blood flow is well documented with lipid droplets within the hepatocytes causing structural injury and obstruction to the microcirculation resulting in impaired blood flow (123-125). Doppler studies have reported reduced hepatic artery resistance indexes in patients with NAFLD (222, 223). It is likely that this manifests as poor arterial flow in group 1 and the notably poor arterial and portal flow in the non-functioning livers. *In vivo* studies have demonstrated the significant reduction of blood viscosity and increased flow after treatment with lipoprotein apheresis (224, 225) as well as endothelium-dependent vasodilatation (226). It is possible that the same effect is observed in the *ex situ* liver. An even greater improvement in hepatic arterial flow was seen in group 3 compared to group 2 and this was statistically significant at 4 h. As well as the benefits of apheresis filtration on the circulating perfusate, by removing excess TG from the liver, it appears that the structural injury caused by lipid droplets and resultant impairment in sinusoidal blood flow can be restored. These findings were limited to the arterial flow, with similar portal flow rates observed across the groups, suggesting that the difference could be resistance-related.

A significant increase in perfusate ALT was seen only in group 1 over the 48 h perfusion. It is likely that the baseline perfusate ALT level is representative of the pre-existing injury sustained during liver procurement and prolonged cold storage and the change over the course of the perfusion is indicative of on-going or worsening hepatocellular injury (58, 61). Although not statistically significant, the change over time was greater in group 1 livers than those in groups 2 and 3 suggesting a trend towards increased hepatocellular injury in this group. Nasralla *et al* explored haemolysis during NMP (58) and demonstrated livers with

minimal preservation injury (as defined by post-operative peak serum AST < 250 U/L) had significantly reduced haemolysis over the course of a perfusion compared to those with significant preservation injury (post-operative peak serum AST > 1000 U/L). It may be that a liver with good *ex situ* function can phagocytose lysed red cells in the monocyte-macrophage system (227). The haemolysis index (as measured by the clinical biochemistry analyser) provides an objective and accurate measure of haemolysis which reliably reflects the concentration of free haemoglobin (227). In all groups, there was a significant increase of haemolysis from baseline to 48 h. However, there was a non-significant trend towards less haemolysis in groups 2 and 3 compared to group 1. At 24 h, the haemolysis index was 50% lower in group 3 than group 1.

It is important to note that liver function in all groups had deteriorated by 48 h as evidenced by increasing lactate, ALT and haemolysis; although the deterioration appears to have been attenuated in groups 2 and 3 compared to group 1. It is possible that in its current form, the NMP system is not ideally equipped to preserve severely injured livers for greater than 24 h. This issue may be addressed by using a non-blood-based oxygen carrier as has been described in the literature (228-230). It must also be taken into consideration that these livers had considerably prolonged cold ischaemia prior to NMP, further contributing to the injury and limiting their potential for prolonged perfusion. Although in Chapter 3, I reported comparable post-transplant outcomes when adopting a post-SCS approach, this was in the context of non-discarded livers with considerably shorter cold ischaemia times. The situation with these much more marginal donor organs and greater levels of damage may be different.

As was observed in Chapters 3 and 4, all livers across the groups replenished their glycogen stores within the first 12 h. In the current study, a significant, 60% increase in liver glycogen occurred only in group 3. A common finding in NMP is elevated perfusate glucose levels at the start of the perfusion (58, 61). This is likely, in-part, to be caused by glycogenolysis during SCS, which is an ATP-independent process (193, 231). Packed red cells are also known to possess a high glucose concentration, attributable to preservation with citrate-phosphate-dextrose and sodium-adenine-glucose-mannitol (232). Upon commencement of NMP, the high levels of circulating glucose should inhibit glycogenolysis and stimulate glycogenesis – as evidenced by the repletion of glycogen stores in this study (and in the studies detailed in Chapters 3 and 4). At the end of perfusion glycogen depletion has recurred in the context of high perfusate glucose levels in group 1 only. This is likely to represent pan-lobular injury and the liver's inability to phosphorylate glucose as glucose-6-phosphate and stimulate glycogenesis, a situation which appears to be preserved in the livers in group 3 which had significantly reduced perfusate glucose levels and increase glycogen content.

Although perfusate TG levels significantly increased over the course of the perfusion in all 3 groups, at 48 h the perfusate TG in groups 2 and 3 was significantly lower than in group 1. The same is true for total cholesterol. It appears that the filter is slightly more effective in this setting than *in vivo* where plasma TG and TC levels are reduced by 40% and 60%, respectively (203). The NMP system benefits from the ability to prolong filtration (compared to *in vivo*) and there may be a difference in efficacy when filtering packed red cells rather than whole blood. It is likely, however, that the filter does reach a saturation point although what this might be has yet to be established. It appears that the addition of the filter may aid in improving liver function; however in isolation (group 2) it has no effect on overall liver fat. It may be that by simply improving blood flow to the liver, its liver

function can be optimised. However, it is believed that hypertriglyceridaemia may cause lipotoxicity and induce oxidative stress and a pro-inflammatory response within the liver (233). In a porcine study performed by Jamieson *et al*, it was speculated that an increase in circulating TG may have been responsible for excess hepatic lipid deposition in the control arm of their experiments (156). In the murine NMP study by Nagrath *et* al, a 30% reduction in liver fat was observed when no de-fatting agents were added but they replaced the perfusate each hour (157). However, in the group of human livers perfused by Liu *et al* (164), although a significant increase in perfusate TG was observed, there was no correlation with ALT, AST, bile production or histological markers of injury, suggesting that increasing TG does not result in greater liver injury. Therefore, lipoprotein apheresis filtration's role in potentially reducing lipotoxicity (by reducing the concentration of TG in the perfusate) and improving liver function remains unclear.

ApoB consists of 4,536 amino acids and is one of the largest proteins secreted by the liver in the form of VLDL and is the carrier of TG being secreted by the liver (234, 235). It is therefore, unsurprising that the concentration of perfusate ApoB follows the same trend as TG. As there is exclusively one ApoB particle per VLDL (which remains with it for its life-span), the TG:ApoB molar ratio provides an indication of particle size and this did not appear to change over time between the groups.

A significant increase in perfusate 3-OHB is seen in group 3 compared to groups 1 and 2. As discussed in Chapter 1, 3-OHB is a ketone body formed from the mitochondrial oxidation of fatty acids within the liver and can therefore serve as a surrogate marker for hepatic fatty acid oxidation (236). The reason for the increase is likely to be multifactorial. Within the liver the transportation of free fatty acids from the cytoplasm to the mitochondria is reliant

upon the generation of intra-mitochondrial acetyl-CoA and its transportation by way of the 'carnitine shuttle' (regulated by carnitine-palmitoyl-transferase 1 (CPT1)), into the mitochondria where it enters the Krebs' Cycle for oxidation to CO_2 (generating ATP) or ketogenesis (150). L-carnitine potentiates this pathway and facilitates the transport of fatty acids across the inner mitochondrial membrane (237). Boteon *et al* recently published the successful reduction of steatosis in primary human hepatocytes where a "de-fatting cocktail" supplemented with l-carnitine resulted in a 35% reduction of intracellular TG (161). This was associated with a significant increase in ketone body secretion (161). In group 3, the concentrations of glucose and insulin were reduced, both of which promote DNL – glucose as the non-lipid precursor and insulin as the hormone which acts both acutely and on gene expression (238). In this intervention group, there was a clear reduction in DNL over time which would lead to a reduction in malonyl-CoA, an intermediate in the DNL pathway and is a potent inhibitor of CPT1 (239). Therefore, when malonyl-CoA concentrations are high, such as under conditions of carbohydrate excess, fat oxidation is suppressed through malonyl-CoA inhibition of CPT1. It is therefore likely that the increased oxidation in the group 3 livers was not due to the supplementation of l-carnitine alone but as also due to a shift in cellular metabolism, away from fatty acid synthesis (DNL) and esterification, toward oxidation.

Only livers in group 3 demonstrated a reduction of intrahepatocellular fat and although this was not particularly marked histologically, with only a 9% reduction on image analysis, a 45% decrease in the tissue TG was seen compared to a 16% increase in group 1 livers ($p = 0.04$). The increase in tissue TG in group 1 livers is likely to be attributable to DNL, where the median DNL fatty acids increased, although not significantly, around 3-fold. Without input from other fatty acid sources, such as fatty acids from peripheral or visceral adipose

tissue lipolysis or input from dietary fat, the only fatty acid source in this *ex situ* NMP model is through DNL. The group 2 livers displayed more active DNL with a significant increase compared to group 3 livers at 48 h, however there was a slight reduction in tissue TG over 48 h. In this context, it could be speculated that the lipoprotein apheresis filter plays a role in preventing a further increase in intracellular fat accumulation as was seen in group 1.

Forskolin may have contributed to the reduction of steatosis in group 3 through indirectly activating protein kinase A which increases the phosphorylation of perilipin 5 on the surface of lipid droplets and promotes lipolysis (157, 240, 241). The lipolysis products (fatty acids) can then be recycled back to form TG within the cytosol unless they enter oxidation pathways or form TG, or other lipids (e.g. cholesteryl esters or phospholipids) that are part of VLDL and are secreted from hepatocytes (242). As the TG concentration is reduced in the circuit due to VLDL being scavenged by the filter, forskolin's precise contribution in the reduction of hepatic TG remains unknown.

The fatty acid composition of both the perfusate and tissue TG was comparable and remained rather stable over 48 h. The composition was also reflective of the *in vivo* situation with the same TG fatty acids being the most abundant (243). This supports the use of the NMP model to explore *ex situ* liver TG metabolism. The data provided by the fatty acid composition also corroborates evidence to support the previous reports demonstrating *in vivo* that the serum TG fatty acid composition is similar to that of the liver (244, 245). In this model, however, a (non-significant) reduction of the polyunsaturated omega-6 fatty acid, linoleic acid (C18: 2n-6) was seen in all groups in perfusate and tissue over 48 h. This is an essential fatty acid which can only be obtained from the diet and therefore it may be of

interest to prevent a reduction in this (and the omega-3 fatty acids) in order to optimise the system and create a more physiological environment.

This study is not without limitations. The heterogeneity of livers in the donor pool and the small sample size make it challenging to draw robust conclusions. The type of liver received was unpredictable and indeed the degree of steatosis varied greatly. However, this is a true reflection on clinical practice and any intervention-based effects remain valid. Although the percentage macrovesicular steatosis levels appear rather low in this cohort for discarded livers, this is due to the fact that quantification has been performed using image analysis software which notoriously underestimates compared to a histopathologist assessment (94). By using a completely automated image analysis method, subjectivity is removed from the assessment and allows a fair comparison of steatosis between groups. The fact that lipid deposition in the liver may be heterogeneous (99) has been addressed by ensuring that each sequential biopsy has been taken from the same point in the liver ensuring a fair comparison over time. It can be assumed that any change occurring is true for that area of the liver and may be applied to the whole organ. Core biopsies are also able to sample a deeper section of tissue which may reflect more of the organ compared to a wedge biopsy which only represents the periphery. During this work, I have also tried to address the problematic issue of steatosis quantification histologically, by also measuring tissue TG biochemically and found a strong positive association with macrovesicular steatosis which allows a valid and important comparison. This study lacks statistical power to truly test the effect of the interventions. However, I have observed important trends and observations between groups which certainly provoke further thought, discussion and research.

From this study alone it is difficult to conclude whether lipoprotein apheresis filtration is required to enhance function. To answer this question, further perfusions would need to be performed with the pharmacological interventions without filtration. This would indeed confirm whether the lipotoxicity of excess TG is indeed detrimental. Equally, it is unclear whether de-fatting is actually required to improve post-transplant outcomes.

Without transplantation of the livers, it is impossible to know whether the interventions would have an impact on post-transplant outcome. Although it appears possible to enhance the *ex situ* function of steatotic livers and even reduce the degree of steatosis, it is unclear whether this is required for successful transplantation. It is unclear whether the steatotic livers that do not function during NMP could be salvaged through intervention. It may be that in the clinical setting, when the CIT can be reduced considerably (or even avoided), interventions may be more effective. Furthermore, if prolonged preservation is required, measures will need to be taken in order to ensure optimal preservation and function for the entire preservation period.

In conclusion, I have demonstrated the *ex situ* preservation of human steatotic livers with NMP for 48 h. Through the addition of lipoprotein apheresis filtration, functional improvement can be derived in terms of significantly increased arterial flow, attenuated liver enzyme release, reduced haemolysis, and increased glycogen synthesis. Significant reduction in perfusate TG and TC can also be achieved. However, in order to achieve a reduction in liver TG content, the addition of l-carnitine and forskolin, as well as a reduction in glucose and insulin were required. This resulted in a significant increase in fatty acid oxidation and significant reduction in DNL. This may be beneficial in the longer term as increased DNL may have significant qualitative implications as the primary fatty acid

product is saturated (palmitoyl-CoA) (246, 247) which may interfere with cellular function (248).

Chapter 6

Overall conclusions and future directions

Liver transplantation has been subject to several innovations in recent years, all which aim to improve post-transplant outcomes and increase organ utilisation. It is an exciting time for a specialty where these innovations are likely to have a transformative, beneficial impact. Understanding how novel technologies can be safely implemented and exploited to maximise the benefit is crucial. In this thesis I have focused on NMP and I have demonstrated that it can be safely instigated at the recipient hospital after cold storage with comparable perfusion parameters and clinical outcomes to livers preserved continuously (including transportation) with NMP. This is likely to facilitate clinical adoption by simplifying logistics and reducing costs. This work also contributes to the body of evidence supporting the beneficial impact of NMP in human liver transplantation and its potential role in increasing organ utilisation (50, 58, 61, 63).

In an attempt to address the issue of hepatic steatosis in liver transplantation, where steatotic livers form an increasing proportion of organ discards (79, 133) and are known to result in poor post-transplant outcomes (106), I explored NMP's potential in this context. Experimental animal studies have previously suggested that NMP may play an important role in the preservation of steatotic livers and may result in a reduction of intra-hepatic fat content which may further improve outcomes (156, 157). I compared clinical outcomes from steatotic livers preserved via NMP and SCS and have demonstrated a significant improvement in early biochemical function in steatotic NMP livers. This is likely to be due to a reduction in IRI as characterised histologically by reduced glycogen consumption and

lipid peroxidation. The systemic inflammatory response is also attenuated compared to SCS livers as evidenced by reduced circulating inflammatory cytokines in the steatotic NMP livers. However, no change in the amount of liver fat was seen during NMP and clear differences in perfusate lipid metabolites were observed between steatotic and lean NMP livers. Furthermore, a significant improvement in early biochemical function in lean compared to steatotic NMP livers was seen. These findings provided the impetus to investigate the potential to optimise these steatotic grafts further.

Finally, I perfused 18 discarded steatotic human livers for 48 h on the NMP circuit and subjected these to various interventions, aiming to alter liver fat metabolism with the goal of improving *ex situ* function and reducing the amount of intra-hepatic fat. I observed a deterioration in liver function in all livers over the course of 48 h. By adding a lipoprotein apheresis filter to the circuit, functional improvements were observed during preservation when compared to NMP alone. Lipoprotein apheresis filtration significantly reduced the amount of circulating TG and cholesterol and significantly increased arterial flow rates. However, no reduction in liver fat content was observed. When pharmacological interventions were added, namely l-carnitine, forskolin and a reduction in glucose and insulin (as well as lipoprotein filtration), the liver's functional ability appeared to be further enhanced and a histological and biochemical reduction in liver fat was observed. A significant increase in fatty acid oxidation (characterised by perfusate 3-OHB concentrations) and reduction in DNL are likely to have influenced the observed changes.

Through conducting this body of research, questions have been raised which warrant further investigation. Is liver de-fatting essential to improve post-transplant outcomes from these livers? Evidence suggests that if the recipient of a steatotic liver overcomes the initial severe

IRI and does not develop PNF, overall survival may be comparable to lean livers (106) and that following transplantation, the fat within the liver can rapidly dissipate (146, 147). Therefore, it may be possible that by simply providing an enhanced environment for liver preservation, without the reduction of fat being the primary intention, outcomes may be improved. As biochemical liver function is often normal in the donor of a steatotic liver, it is clear that the process of ischaemia and reperfusion is the main insult resulting in the subsequent detrimental outcomes. Taking this one stage further, it may be that the risk of steatotic liver transplantation could be further reduced by performing a completely ischaemia-free organ donation, preservation and transplantation procedure as has been described (249). Another important question which remains to be addressed is why some steatotic livers do not function *ex situ* and if these can be salvaged? I believe that the aetiology of a non-functioning steatotic NMP liver lies beyond the degree of steatosis. It may be affected by the genotype of the liver or indeed more subtle differences in the way fatty acids are partitioned and the interplay between metabolic processes. Furthermore, all of the livers discussed in this thesis were originally retrieved with the intention on transplantation. It would be interesting to explore the potential of NMP to enhance livers which would not, at present, be considered for transplantation such as those with non-alcoholic steatohepatitis (NASH) (characterised by elevated transaminases in the donor or a confirmed histological diagnosis). This may require prolonged preservation, possibly for several days. However, it is clear from the studies in this thesis that liver function deteriorates between 24 and 48 h. Identifying mechanisms to enable the stable and prolonged preservation of livers could therefore be important. I have speculated that this may be achieved by using a non-red cell-based oxygen carriers (to reduce haemolysis). There may also be challenges in maintaining sterility, although perfusate contamination does not appear to have been a major reported limitation in the published literature.

On-going work beyond the scope of this thesis is planned. Whole gene sequencing of liver biopsies from the discarded livers (Chapter 5) is being undertaken. This will complement the data presented and will strengthen the evidence pertaining the metabolic pathways involved during de-fatting by identifying the key genes which may be implicated. This may also reveal other injury and/or repair genes which may be altered during the NMP (with or without intervention) process. This work may also help identify potential drug targets for NAFLD or NASH. Cytokine profiling of the perfusate will be undertaken in order to investigate whether the de-fatting adjuncts alter the inflammatory profile of the perfusate. I hypothesise that resident inflammatory cells migrate from the liver into the perfusate upon commencement of NMP, the concentration of which may increase exponentially over the course of the perfusion. It would be of interest to see if this could be attenuated through perfusate filtration or pharmacological intervention.

The data that I have presented in this thesis would support a pilot study designed to investigate NMP and de-fatting interventions in steatotic liver transplantation. This would aim to establish whether clinical outcomes and IRI can indeed be improved by enhancing the *ex vivo* environment and/or removing the fat from the liver. It should establish if it is possible to achieve outcomes comparable to lean livers and if this were shown to be the case, then the concerns regarding the transplantation of steatotic livers could be confined to history.

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