IMPROVING ORGANS FOR TRANSPLANTATION

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INTELLECTUAL PROPERTY STATEMENT

I confirm that the work submitted is my own, except where work which has formed part of jointly authored publications has been included. My contribution and the other authors to this work has been explicitly indicated below. I confirm that appropriate credit has been given within the thesis where reference has been made to the work of others.

The literature search used in chapter two of this thesis, specifically evidence base for existing preservation solutions was used in 'Abdominal organ preservation solutions in the age of machine perfusion. *Ramos P, Williams P**,** Salinas J, Vengohechea J, Lodge JPA, Fondevilla C, Hessheimer A. Transplantation. Aug 2022'.* The work detailing the role of existing and novel cold storage preservations solutions is directly attributable to me, other sections of this article examining the role of machine perfusion were made by the other authors.

PUBLISHED PAPERS/ABSTRACTS

Abdominal organ preservation solutions in the age of machine perfusion. Ramos P, **Williams P,** Salinas J, Vengohechea J, Lodge JPA, Fondevilla C, Hessheimer A. *Transplantation* *107(2):p 326-340, February 2023.*

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Abstract

Transplantation has grown exponentially with 4,533 life-saving UK transplants in 2022-23. Static cold storage (SCS), using University of Wisconsin solution (UW) is the gold standard. Despite increasing transplants, demand outstrips supply necessitating use of marginal organs.

Here a novel preservation solution, Leeds solution for abdomen (LS-A) is examined, with SCS and Hypothermic oxygenated machine perfusion (HOPE) applications. A battery of biomarkers: HMBG-1 marker of necrosis; caspase-3 marker of apoptosis; syndecan-1 marker of glycocalyx function and Flavin mitochondrial mononucleotide (FMN) a putative marker of post-transplant function were examined.

This was a pre-clinical, prospective, randomised controlled trial in a porcine model. Normothermic machine perfusion (NMP) simulated the post-transplant milieu. Livers were assigned to LS-A SCS or control (UW or IGL-1).

A proof of principle comparison compared LS-A for both SCS and as a HOPE perfusate with UW for SCS and UW-MPS (UW Machine Perfusion Solution) for HOPE. Livers were interrogated for perfusate blood gases, biochemistry and histology.

50% of SCS LS-A livers met NMP viability criteria versus 0% of controls. LS-A shows significantly lower lactate and potassium. LS-A at 8-hours shows no significant differences from UW at 4-hours. When used for HOPE 40% of LS-A livers met viability criteria whilst no controls did. LS-A and UW-MPS showed no significant differences in lactate, but significantly lower potassium was seen in LS-A HOPE. There were significantly higher levels of apoptosis and necrosis with UW than LS-A in SCS but, no difference in HOPE. FMN showed no correlation with outcome.

This study is the first to show LS-A performed better than UW in marginal organs. No difference existed between LS-A 8-hours versus UW 4-hours SCS. No significant difference exists between LS-A and UW-MPS in HOPE. FMN cannot predict viability, with no correlation between its pre-NMP levels and outcomes. LS-A provides exciting opportunities to improve organ preservation and live animal trials should follow.

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Abbreviations

ABG: Arterial blood gas

ALT: Alanine transaminase

ANOVA: Analysis of variance

ASPA: Animals for scientific procedures act

AST: Aspartate aminotransferase

ATP: Adenosine triphosphate

AWERB: Animal welfare and review Body

BUN: Blood urea nitrogen

Ca2+: Calcium ions

CIT: Cold ischaemic time

CO2: Carbon dioxide

DAMP's: Damage associated molecular patterns

DBD: Donation after brainstem death

DCD: Donation after cardiac death

DGF: Delayed graft function

D-HOPE: Dual hypothermic oxygenated machine perfusion

DNA: Deoxyribonucleic acid

EAD: Early allograft dysfunction

ELISA: Enzyme-linked immunoassay

FMN: Flavin mitochondrial mononucleotide

HES: Hydroxy-ethyl starch

HLA: Human leucocyte antigen

HMGB-1: High motility group box 1

HOPE: Hypothermic oxygenated machine perfusion

HTK: Histidine-tryptophan-ketoglutarate

ICAM: Intercellular adhesion molecule

IGL-1: Institute George-Lopez solution 1

IGL-2: Institute George-Lopez solution 2

IRI: Ischaemia-reperfusion injury

IVC: Inferior vena cava

K+: Potassium ions

LDH: Lactate dehydrogenase

LFT's: Liver function tests

LS-A: Leeds solution for abdomen

MANOVA: Multivariate analysis of variance

MP: Machine perfusion

MTP: Mitochondrial permeability transition

Na+: Sodium ions

NADH: Nicotinamide adenine dinucleotide

NAFLD: Non-alcoholic fatty liver disease

NET's: Neutrophil extracellular traps

NHS: National health service

NMP: Normothermic machine perfusion

PAS: Periodic Acid-Schiff

PEG: Poly-ethylene glycol

PNF: Primary non-function

RCT: Randomised controlled/clinical trial

ROS: Reactive oxygen species

SCS: Static cold storage

SMA: Superior mesenteric artery

TNF-α: Tumour necrosis factor alpha

Tukey's HSD: Tukey's honestly significant difference

USSR: Union of soviet socialist republics

UW: University of Wisconsin solution

UW-MPS: University of Wisconsin solution for machine perfusion

VCAM: Vascular cell adhesion protein

WIT: Warm ischaemic time

1.0 Introduction

1.1 History of Transplantation

The first successful transplant is credited to Emerich Ullman who performed a kidney autograft in a dog and a dog-to-goat xenograft in 1902. These early attempts, although demonstrating a successful flow of urine ended with the recipient death within a few days[1]. These were followed by the first human transplants by Jaboulay (1906) and Unger (1909) using a xenograft model, again demonstrating initial function but quickly progressed to organ failure and death. Alexis Carrel went on to show great successes in solid organ transplantation. On his move to Chicago, he partnered with the physiologist Charles Guthrie producing a huge volume of work in transplants of kidney, thyroid, and even small bowel in animals. The success of this work lead to Nobel prize for Carrel, much to the chagrin of Guthrie who would not share in it. This era of intense research at the Rockefeller institute, was interrupted by World War I and would not resume postbellum. Upon Carrel's return after the Great War, his research would take a different turn: in collaboration with the famed aviator Charles Lindbergh, and Otto Hopf, they designed a pump oxygenator which would allow fixed perfusion for individual organs and form the basis of the heart lung by-pass machine[2].

In the 1930's organ transplantation developed in the USSR although due to their publication in Russian this would not be available to the Western world until the 1950's. This work, conducted by Voronoy, was the first series of human-to-human transplants. All these grafts failed rapidly, attributed to the lengthy ischaemic time and blood group mismatch[2]. Work in the USSR reverted to animal models with Lapchinksy transplanting limbs and kidneys stored at 2◦C and 4◦C in the 1950's, thus showing the first use of hypothermia as a preservation strategy. In his animal model, autograft organs and limbs were transplanted with up to 28 hours of preservation. Pharmacological immunosuppression was yet to be discovered and so immunological

suppression was induced by sub-total whole blood exchange. One of the subjects survived for seven years, with the transplanted limb still showing weight bearing capability in the animal's last year of life[3].

In 1954, the world's first successful human-to-human kidney transplant was performed by Joseph Murray in Boston. This was a living donor kidney transplant between a set of monozygotic twins following Dr Murray's research into rejection in skin grafts. The patient made a full recovery from the surgery and the implanted kidney survived for a full eight years until the recurrence of his original renal pathology. For this pioneering approach and further work detailing the nature of rejection in skin grafts Dr Murray won the Nobel prize in 1990[4]. He would continue to develop kidney transplantation in Boston with the first allogenic related donor graft in dizygotic twins. To combat the effect of the recipient immune system a total body irradiation strategy was employed. It would be in 1962 in conjunction with Gertrude Elion, George Hitchings and his research fellow Roy Calne that Dr Murray was able to perform the first cadaveric kidney transplant using the new drug 6-mercaptopurine and its derivative azathioprine[2,5,6].

In the 1960s work in the United States helped to define the role of storage solutions and hypothermia for transplant organs. If a kidney, following heparinisation and flushing, was removed from the body and was re-implanted within an hour, then storage strategies were not necessary. However, for those organs needing to be kept for longer some method of preservation would be needed. This led to the first postulation of an 'organ bank' concept, which could become a reality with a selection of organs frozen and stored for use when clinically needed. Unfortunately, further work into the role of hypothermia showed that reducing temperature exponentially reduced the metabolic requirements and oxygen consumption of tissues until 2◦C but in frozen organs irreversible tissue damage occurred. The next step in the research was the addition of hyperbaric oxygen which helped to meet the metabolic demands of

the cooled tissues; the two methods in conjunction showed much improved organ function than that produced by either alone[7].

By 1963 cold infusion of solutions into either the renal artery or aorta was standard practice during organ procurement, with the eventual production of Collins' solution. This allowed the development of organ sharing networks, although these were informal and local at first, they soon grew to be formally regulated and covering a larger geographical area. The major advancements in kidney transplantation during this time, however, lay in the development of a reliable Human Leucocyte antigen (HLA) cross match test, which showed great success identifying patients at risk of hyperacute rejection along with helping to identify the best donor within a family for living donor transplantation[2]. Throughout the 1960s and the first half of the 1970s a period of knowledge consolidation and dissemination occurred in the kidney transplantation community with no single 'landmark discovery'. Instead the origin of multiple international and national transplant societies and journals propagated expertise gained in individual centres or transplant networks, allowing a prompt dissemination of techniques and clinical knowledge not seen in other fields[2].

Whilst the fast-paced development of kidney transplantation was slowing, the science of transplantation for other organs was beginning to develop. The first successful human heart, liver, pancreas and lung transplants all took place between 1963 and 1973[8]. The stories and development of heart, lung and pancreas transplants are beyond the scope of this document but key issues in transplant science along with the evaluation of the scientific model described below were defined by the advent of liver transplantation. The world's first human liver transplantation was performed by Thomas Starzl whilst working at the University of Colorado in 1963. He had developed the technique following animal work in canine portal circulation. The first liver recipient died intra-operatively from haemorrhage with the subsequent four recipients surviving the operative phase but dying thereafter from infections and emboli[8]. There were further unsuccessful attempts at liver transplantation both in Boston and

Paris but none of these patients survived longer than 22 days postoperatively. The causes of these failures were multi-factorial: prolonged warm ischaemia, coagulation abnormalities, immunosuppressant regimen, and the early technique of caval bypass. These early operations were facilitated by use of a whole body hypothermic machine perfusion technique with cooling times varying between 45 and 104 minutes to reach 15 ◦C which will have contributed significantly to the failure rate[9].

The first successful human liver transplant did not take place until 1968, performed again by Thomas Starzl. There was improved donor selection, donors were not subjected to lengthy periods of resuscitation worsening warm ischaemic damage. The technique of cardio-pulmonary by-pass was introduced, in some cases, to allow some organ cooling whilst maintaining perfusion, allowing rudimentary hypothermic machine perfusion whilst recipient hepatectomies took place[10]. Consolidating on the improved operative technique, Starzl introduced anti-lymphocyte globulin and steroid to act as an immunosuppressants along with azathioprine, introduced by the kidney transplant surgeon Roy Calne [8,10]. The relationship between Starzl and Calne was described later as 'a trans-Atlantic alliance helping to make immunosuppression better for patients[8]. Calne later went on to establish the world's second liver transplant unit in Cambridge, UK.

Starzl and Calne would continue to work to improve immunosuppression and graft survival in transplant patients, the greatest improvement coming in the form of cyclosporine, first introduced in 1976. The initial impression of cyclosporine in 1979 was that it was more potent than the current standard azathioprine but equally carried more toxic side effects. The early findings were moderate at best, and it was not until a combined protocol of cyclosporine with steroids was developed in 1980 that it was established as the mainstay of treatment. In 1989 a new drug, tacrolimus, was shown to be able to reverse the rejection process in organs that were resistant to the established cyclosporine-

steroid regime, thus ushering in tacrolimus as the immunosuppressant of choice for kidney and liver transplants which it remains.

The 1990's saw a renewed interest in the concept of chimerism in relation to solid organ transplant. Chimerism, the concept of a single organism composed of cells with two distinct genotypes was a popular research subject amongst transplant groups throughout the 60's and 70's with some evidence in murine models that inoculating irradiated adults could allow chimerism and the transplantation of solid organs without the need for chemical immunosuppression. The success of subsequent generations of immunosuppression had seen chimerism fall out of vogue however, in 1992 following histological analysis of skin and lymph node samples of 30 patients with solid organ transplant persistent chimerism was seen in all patients. As none of these patients had been pre-conditioned with circulating haemopoietic cells pretransplant as was the case in the murine experiments, a hypothesis of passenger haemopoietic stems cells carried at the time of implantation was put forward. This led Starzl to conclude that chimerism was essential to successful long term graft outcomes [2].

In the late 2000s and into the 2010s, transplant research focus began to shift back to machine perfusion of organs retrieved for transplantation. The first clinical series of hypothermic and normothermic machine perfusion studies were published with encouraging results especially in extended criteria organ donors (see below). The research focus has remained in these areas with exciting opportunities for functional assessment pre-implantation as well as the 'intensive care' of individual organs. The current challenges and benefits are discussed in greater detail in subsequent chapters.

1.2 Donor types and the ischaemia-reperfusion injury

There are three types of organ donation used in clinical practice in the UK.

1.2.1 Living Donor

A person who donates all or part of an organ during their lifetime. This is currently the least common form of organ donation[11] but carries significant advantages for the recipient. In kidney transplant it is considered the optimum management as it usually offers a better matched kidney than other forms of donation[12].

1.2.2 Donation after brainstem death (DBD)

A person who donates their organs after confirmation of brainstem death. In the UK this is currently the most common form of donation[11]. It allows organs maintenance using the donor's circulatory system until the organs are explanted.

1.2.3 Donation after circulatory death (DCD)

A person who donates their organs only after circulatory arrest. Since the 1990s the number of this type of donor has steadily increased in an effort to increase the donation pool. DCD donation comes with an inevitable warm ischaemic insult to any organs retrieved due to the haemodynamic instability inherent with cardiac arrest.

1.2.4 'Extended criteria' organ donation

The term extended criteria organ donation is used to describe any features of the donor or organ retrieval process that have been associated with negative outcomes in the recipient. An exhaustive list of criteria, contrary to what the name would suggest, does not exist. The factors below are recommendations from the most recent BTS guidelines of indicators of an organ prone to dysfunction or graft loss[13].

Liver	Kidney
Age >50	Age >60
BMI >25	>50 with a least 2 of below

Sodium >165mmol/l	Hypertension
ICU care and ventilation > 5days	Stroke as cause of death
Cold ischaemic time >8 hours	High pre-retrieval serum creatinine
DCD retrieval	DCD retrieval
Steatosis >15% (Macroscopically or biopsy)	

Table 1: Extended criteria organ donation, reproduced from BTS guidelines[13]

1.3 The Ischaemia-reperfusion injury and putative targets

For any transplant organ there is a temporary cessation of blood flow, constituting the ischaemic phase and a restoration of blood supply constituting the reperfusion injury. The ischaemic phase is variable in length depending on the organ retrieval procedure, donor haemodynamic instability and the storage time of the organs. There are two components to the ischaemic phase, the first is the warm ischaemic time (WIT) defined as the time from circulatory arrest to the infusion of cooled storage solution. The cold ischaemic time (CIT) then begins with perfusion of the cold storage solution into the feeding vessels and surface cooling by packing the chest and abdomen with ice. The CIT lasts throughout organ storage and ends when the organs returns to body temperature for re-implantation. Longer WIT is known to correlate with poorer short- and long-term graft function especially in the DCD setting[14]. In liver transplant especially, differences as small as a few minutes in the WIT increase risk of graft failure. Variations in the CIT are less severe. Longer preservation times are thought to lead to greater derangement of post-transplant liver function, increased need for blood transfusion and increased length of stay. This effect is seen most with CIT time over 8 hours in liver transplant.

1.3.1 Reactive Oxygen Species (ROS) and innate immunity

During the ischaemic phase, lack of oxygen causes aerobic respiration to cease leading to the accumulation of nicotinamide adenine dinucleotide and hydrogen

(NADH) and succinate within cells. When the oxygenated blood flow is restored a build-up of reactive oxygen species (ROS) within mitochondria occurs marking the hyperacute phase of an ischaemia-reperfusion injury (IRI)[15]. The surge in ROS formation activates both necrotic and apoptotic pathways within hepatocytes; there is contention over which process plays the larger role in hepatic IRI with some studies suggesting that up to 60% of hepatic IRI is apoptotic[16,17]. Adenosine triphosphate (ATP) depletion leads to dysfunction of the Na+/K+ ion transporter which in turn causes intra-cellular Ca2+ levels to rise leading to cell oedema and plasma membrane disruption (oncotic necrosis). A secondary necrotic pathway is activated in hepatocytes known as mitochondrial permeability transition (mPT). The process characterised by leakage of mitochondrial contents into the cytoplasm, activates apoptotic pathways but in the ATP depleted hepatocytes there is not enough cellular energy to execute apoptosis and as such necrosis ensues[15].

The consequence of both forms of necrosis is the release of damage associated molecular patterns (DAMPs) into the hepatic circulation. DAMPs are intracellular molecules that gain immunogenic properties after release and are a heterogeneous group of molecules comprising: high mobility box 1 (HMGB-1) proteins, purine metabolites, hyaluronan and mitochondrial or nuclear DNA[17]. DAMPs activate the innate immune response mediated by Kupfer cells, neutrophils, NK cells and T cells[16]. Similarly, hepatocytes undergoing apoptosis can also activate Kupfer cells: once apoptosis is complete, cell remnants form membrane bound structures known as apoptotic bodies that signal to Kupfer cells to initiate phagocytosis, which activates them in a similar fashion to DAMPs[18]. Kupfer cells are the first mediator in a cascade promoting the release of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin 1 (IL-1) causing migration of neutrophils and T-lymphocytes into the liver parenchyma. A key player in the activation of Kupfer cells is Ca2+ which has its effect via an L-type calcium channel on the cell surface, with a pre-clinical study in rat livers showing that blockade of this channel offers protective effects during IRI[19]. Once Kupfer cells are activated they facilitate ROS production via

direct activation of monocytes and neutrophils mediated by pro-inflammatory cytokines. Additionally, further neutrophil activation and recruitment is facilitated by TNF-α action on endothelial cells increasing expression of vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM) [15,17]. In reality, these processes occur simultaneously and act as a positive feedback loop propagating the IRI.

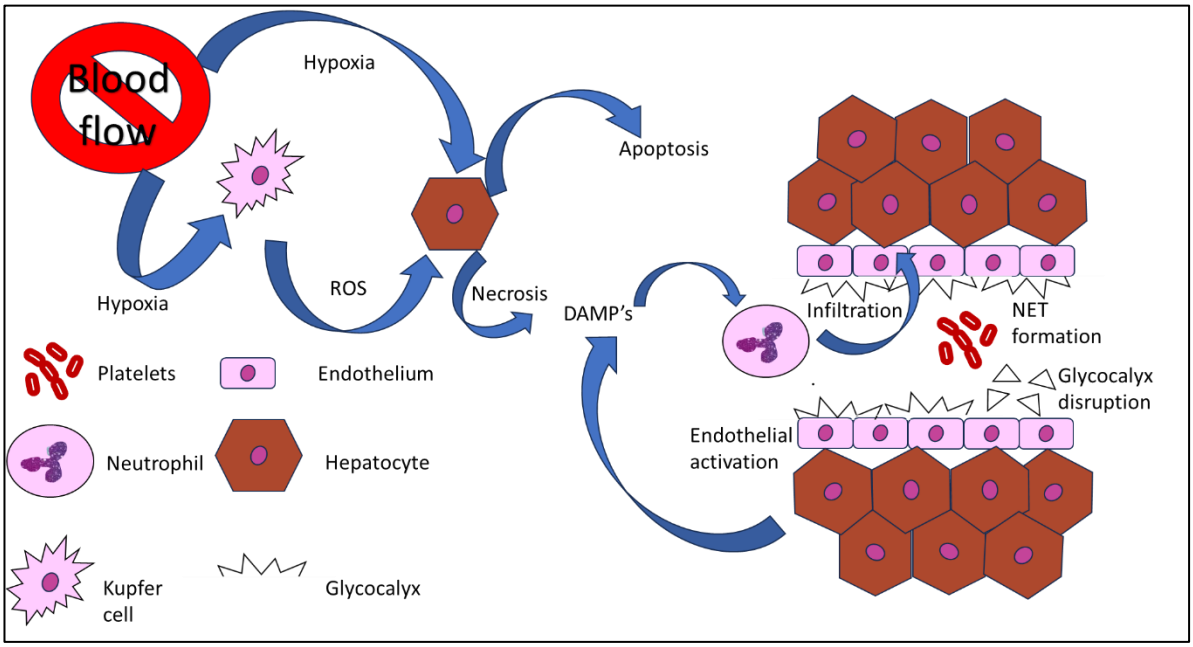

Figure 1:Summary of the ischaemia reperfusion injury. The various roles played by each cell type are highlighted above but special significance is given to the role of necrosis and DAMPs in the propagation of the IRI

1.3.2 Platelet function

Platelets have an integral role in IRI, it would seem intuitive to suspect their role in coagulation. I.e., formation of micro-thrombi, as the putative mechanism for their contribution to IRI. Pre-clinical data using a rat NMP model infused with activated platelet isolates found no evidence of microthrombi on histological evaluation despite clear IRI in the absence of an adaptive immune system[20].

Other experimental models have implicated the formation of neutrophil extracellular traps (NETs) in platelet aggregation and microthrombus formation[17]. Thus, the role of platelets in the formation of microthrombi in liver

IRI is dependent on other immune cells. As with most elements of the IRI there is a positive feedback loop of platelet activation increasing the action of neutrophils and activated neutrophils inducing more platelet activation. The consensus from the two conflicting points above is that a decrease in platelet activation should lead to a reduction in the severity of IRI.

1.3.3 The glycocalyx

The glycocalyx is a thin reticular layer of proteoglycans coating the vascular endothelium, during IRI the glycocalyx can be damaged through enzymatic cleavage or through direct oxidative stress induced by ROS. One of the main functions of the glycocalyx is mediating membrane permeability and as such dysfunction or disruption leads to increased vascular permeability and oedema. Damage to the glycocalyx also exposes adhesions molecules such as cadherins to circulating immune cells and platelets with the circulating fragments of the glycocalyx acting as DAMPS, allowing the processes described in the previous paragraph to propagate. In animal models, protection of the glycocalyx with steroids or antithrombin reduces both platelet activation and leukocyte adhesion, which theoretically should reduce the severity of IRI[21,22]. In transplantation, preservation solutions based on PEG 35 have been shown to induce glycocalyx protection in animal models[23]. The human literature shows the glycocalyx exhibiting changes in various illnesses ranging from cancer to severe sepsis and has a defined a role as described above however, there is very little evidence showing that glycocalyx protection provides measurable clinical benefit.

1.4 Principles and history of static cold storage

In current UK practice static cold storage (SCS) is the method most organs for transplantation are stored whilst in transit or awaiting re-implantation. The technique involves cannulation of the major inflow vessel, and as most donors are multi-organ donors, both the aorta and the superior mesenteric vein are canulated for infusion of a cold preservation solution along with rapid cooling using ice packed into the abdominal cavity. The organs are packed into containers filled with preservation solution and kept on ice which maintains the temperature at between 0 and 4◦C.

The hypothermic element of SCS is explained by the Arrhenius equation which is a formula for the temperature dependence of reaction rates shown below:

$$k = Ae\frac{-Ea}{RT}$$

k= rate constant

A= pre-exponetial factor

Ea= activation energy

R= universal gas constant

T= absolute temperature

In liver tissues this usually equates to a 50% reduction in cellular metabolic rate for each 10◦C of cooling[15].

The hypothermia induced as part of SCS reduces metabolic demands of tissues as enzymatically controlled reactions producing ROS and depleting ATP are curtailed following ischaemia. Hypothermia however is a double-edged sword: the rapid cooling involved causes cell membranes to become much stiffer and lose their selective permeability allowing water to enter the cell causing oedema.

The key features of an effective preservation solution were outlined by Belzer and Southard in 1988 as a composition that:

(1) minimizes cell swelling induced by hypothermia

(2) prevents intracellular acidosis

(3) prevents the expansion of interstitial space during flush-out

(4) prevents injury from reactive oxygen species (ROS)

(5) provides substrates for regenerating high-energy phosphate compounds during reperfusion[24,25].

The key components to a preservation solution are summarised in Fig. 2 below from Ramos et al[26].

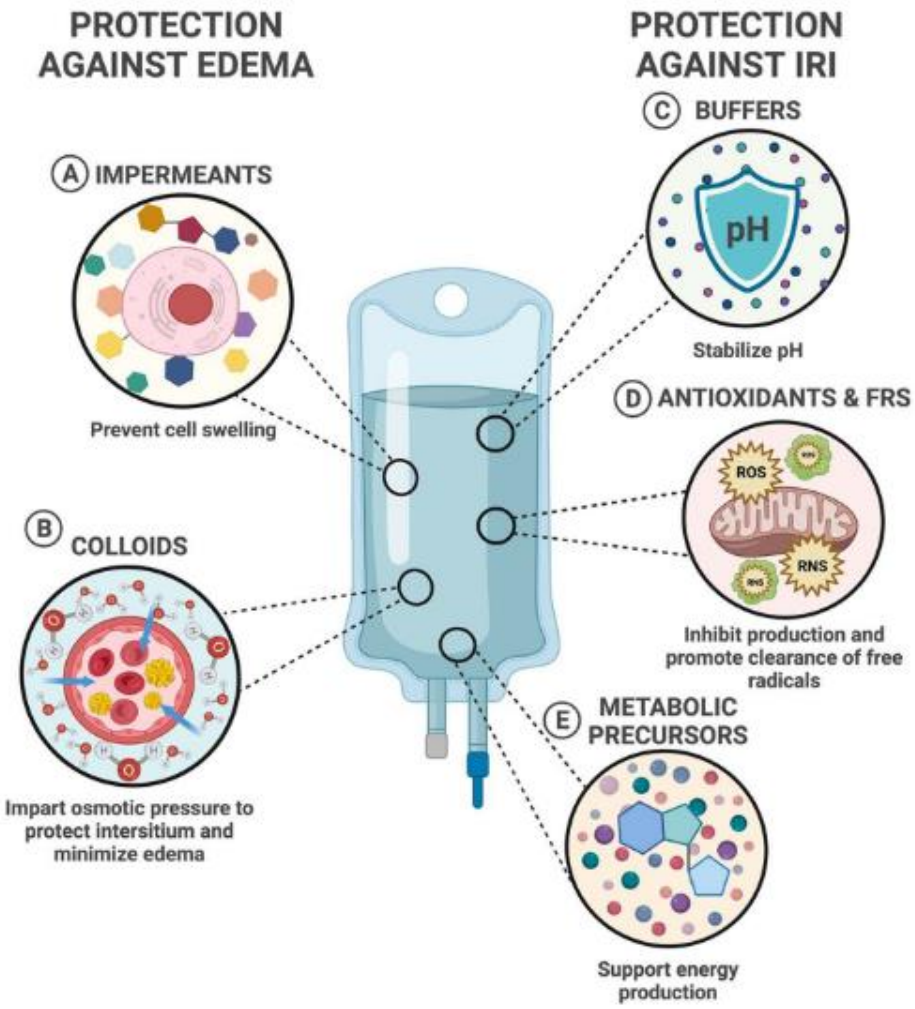

Figure 2: Key components of preservation solutions. This infographic highlights how different components of a preservation solution fulfil their function. Recreated from Ramos et al.

1.4.1 Collins' Solution

In 1969 the first commercially available organ preservation solution came to clinical practice: Collins' solution. Designed to mimic the intracellular ion balance by having high K+ and low Na+ concentrations with respect to the serum, a high concentration of glucose provided an osmotic balance between the organ and the solution thus preventing fluid and electrolyte shifts and reducing oedema. A phosphate buffer was also present to reduce intracellular acidosis caused by ongoing anaerobic respiration in the now ischaemic organ. The first Collins' solution provided a simple and effective storage of a retrieved kidney for up to twenty-four hours allowing for the advent of tissue typing and organ sharing [2,27,28]. Due to the simplicity of the method and its facilitation of unsupervised transport SCS became the mainstay of clinical organ preservation. Collins' enjoyed eleven years as the preservation solution of choice expanding its role from kidneys to cover heart, lung and liver grafts[28]. In 1980 Collins' solution was modified by increasing the glucose concentration to guard against fluid shifts. Further to this magnesium sulphate was excluded from the composition to prevent formation of magnesium phosphate precipitates. The now EuroCollins' solution continued to be used in clinical transplantation for many years[29].

Marshall's solution or hyperosmolar citrate has been used for a large number of kidney transplants in UK and has been shown to have similar outcomes to kidney preserved in UW and other widely available preservation solutions[30]. Its role in the liver preservation is less well defined with UW having better functional outcomes and allowing a longer SCS period[31]. The use of Marshalls solution is now largely dictated by practices of multi-organ retrieval. For example, if only liver and kidneys are retrieved then Marshall' solution may be used as an aortic flush, but the liver is still back-table perfused and stored with UW to improve preservation. However, if the pancreas is also retrieved then the use of Marshalls solutions is precluded reverting to a UW aortic flush technique[32].

1.4.2 Introduction of UW

The next generation of SCS solutions entered clinical use in 1987 with the production of University of Wisconsin solution (UW) by Belzer and Southard. Although UW mimics the intracellular matrix and shares its phosphate buffer system with EuroCollins it also delivered several new features targeted to IRI. Glucose was replaced by raffinose and lactobionic acid as impermeants, as neither of these molecules cross cell membranes due to their size and charge, thereby reducing cell swelling. This is especially pertinent in liver transplant as glucose is readily absorbed by liver cells causing both oedema and providing substrate for anaerobic glycolysis. The addition of hydroxyethyl starch confers higher oncotic pressure but at the cost of UW's viscosity and difficult storage. UW also used several mechanisms to deal with the accumulation of ROS and the depletion of ATP. Firstly, allopurinol reduces oxidative burden produced via the hypoxanthine-xanthine-uric acid pathway. To deal with those ROS that were present, glutathione was added as an antioxidant and adenosine was added to function as a substrate for ATP repletion after implantation. The improvements in UW over EuroCollins were reflected in its clinical uptake; UW remains the gold standard in liver SCS today [29,33].

1.4.3 HTK

Other solutions sought to answer Belzer and Southard's principles with different chemical processes. Histidine-Tryptophan-Ketoglutarate Solution (HTK) was originally developed as cardioplegia fluid in the early 1970's by Hans Bretschneider. The solution has an intracellular level of sodium along with an extracellular level of K+, the mechanism of its diastolic cardiac arrest action. The solution uses a histidine/hydrochloric acid buffer to maintain pH during anaerobic respiration. HTK uses mannitol as an impermeant to prevent the cell oedema induced with hypothermia. Tryptophan acts as its primary free radical scavenger; with three actions directly scavenging ROS, stimulating a number of

antioxidant enzymes and finally by stabilising the cell membrane, protecting against free radical damage[34]. Ketoglutarate acts as the substrate for the energy depleting reactions once reperfusion has been re-established. In its current market form, Custodial, glutathione acts as a free radical scavenger in conjunction with tryptophan and lactobionic acid as an additional impermeant. Custodial was being tested in clinical liver transplant throughout the 1990's and was eventually FDA approved in 1999[27,29].

1.5 Evidence base for solutions currently in clinical use

UW and HTK became the most used preservation solutions in the world following two prospective randomised control trials taking place in the 90's. The first in 1992 compared results for UW against the established gold standard Eurocollins. The work was entirely in kidneys: 695 transplants with equal patient distribution to the two groups 50.6% vs 49.4% to UW and Eurocollins, respectively. The results showed a 10% reduction in DGF (delayed graft function) with UW and significantly higher graft survival rates at three and six months. The study incorporated over 50 European transplant centres with variability in the operative and postoperative management protocols used, however, it showed that choice of preservation solution was the most important variable in development of DGF and subsequent graft function[35].

The second published in 1999 compared HTK to Eurocollins and UW solution in a multicentre large-scale trial, examining DBD donors only. For renal transplant, both the newer solutions were superior to Eurocollins but were equivalent in terms of DGF and 3-year graft survival. The rates of DGF were reduced by a similar degree to those in the earlier Eurotransplant work. Between UW and HTK there was no significant difference in rate of DGF and multi-variate analysis showed that donor factors played a more significant role in development of DGF[36].

For liver transplantation the role of UW as the gold standard was further defined by other studies in the early 90's. Several cohort studies found that extended

preservation times with UW gave comparable results to livers preserved in Eurocollins [37–39]. This was supported by animal models; however, no direct randomised clinical trial were carried out. Throughout the 1990s and into the early 2000s a number of cohort studies and two RCT's were performed comparing HTK and UW in liver transplantation. The RCT's had varying methodologies and small samples sizes, the largest being n=30 per experimental group[40,41]. A recent systematic review succinctly summarises these results stating that no significant differences in DGF, overall graft survival or overall graft function were found between the two solutions[42,43,44]. There is still controversy over the benefits of HTK vs UW and their associated costs but no adequately powered RCT with sufficient length of follow up has ever been conducted. A more recent study has explored the role of Celsior, originally developed as cardioplegia fluid, as an agent to provide SCS in liver transplant. The study showed no significant difference between the two solutions considering EAD or PNF but only examined a cohort of 140 patients which is too small from which to draw robust conclusions[45].

Similarly, the newest solution in clinical use, the Institute Georges-Lopez 1 (IGL-1) has never been compared to UW, HTK or Eurocollins in an adequately powered, multi-centre randomised clinical trial in liver transplantation. The small, prospective studies, in humans found that IGL-1 performs comparably to both HTK or UW in terms of DGF, long term graft function and graft survival but with a lower associated cost[46–48]. The small numbers in these trials with low DGF and graft failure rates make it difficult to adequately assess the role played by the type of preservation solution. There is one multi-centre prospective study showing comparable results in of IGL-1 to UW in kidney transplantation, with similar rates of DGF, graft and host survival[49]. By far the largest trial looking at IGL-1 use is a retrospective analysis of kidney transplantation using the French transplant registry. This study looked at over 7000 kidney transplants showing a significantly lower risk of DGF with IGL-1 than with the other preservation solutions. Although this shows an encouraging trend, the risk of type 2 error within this analysis is very large given that 45% of the transplants used IGL-1 whist the remaining 55% of transplants were unequally split between UW, HTK, Celsior and SCOT (Solution de

Conservation des Organes et Tissus)[50]. A recent systematic review found that in comparison to UW, IGL-1 shows comparable early results but that the evidence for medium and long term results need to be further explored in clinical trials[51].

1.6 Novel organ preservation solutions

1.6.1 IGL-2

The IGL-2 solution is similar in composition to IGL-1 with increased concentrations of polyethylene glycol (PEG) and glutathione, along with the removal of magnesium and lactobionic acid and addition of histidine to the buffering system. It is currently being investigated as a SCS preservation solution as well as a hypothermic oxygenated machine perfusion (HOPE) perfusate[2352]. Through the actions of increased PEG and glutathione, it is thought to reduce ROS activation and cell damage. By using PEG instead of hydroxy-ethyl starch (HES), the reduced viscosity of IGL-2 improves its efficacy as a machine perfusion solution[23].

1.6.2 LS-A Leeds solution for abdomen

LS-A is a new solution based upon PBSL (Phosphate buffered sucrose for Liver) it uses a different impermeant to the established preservation solutions in the form of sucrose. Mammalian cells, except for small bowel mucosa, lack the necessary enzymes to breakdown sucrose thus, maintaining its impermeant effect for longer. Various papers have shown reduced tissue damage from IRI in organs and cell lines preserved with sucrose[53–55]. LS-A also uses PEG as an impermeant; this offers a putative secondary immunological role, reducing lipid peroxidation in hepatocytes and repairing cell membranes, which is thought to reduce the influx of extracellular calcium[56,57]. PEGs are non-immunogenic, non-toxic, and water-soluble polymers which show no electric charge and no affinity for any specific organ. PEG has been shown to reduce hepatocyte damage during storage and transplant, especially in

steatotic tissues[52,57]. The mechanism of action is thought to be via increased production of nitric oxide (NO) which allows smooth muscle relaxation in the microcirculation. Furthermore, PEG has been shown to interfere with the coagulation cascade by reducing platelet aggregation through formation of a barrier over the glycocalyx[57]. PEGs vary in molecular weight and are usually identified numerically i.e. PEG20 or PEG35. LS-A contains PEG 20 which has also formed a part of other SCS preservation solutions and has been shown to reduce alloantigen recognition in comparison to HES, found in UW. There is ongoing debate regarding the ideal molecular weight PEG for SCS [57].

The ion buffering and chelation systems used by LS-A are similar to those used in both UW and IGL-1 using phosphate and lactobionate. LS-A is classed as an extra-cellular solution in terms of ion content, with an aim to avoid the detrimental effects of large potassium shifts that can occur in liver transplant recipients.

There are a number of secondary components to LS-A which reduce the IRI. It contains both allopurinol and glutathione to act as free radical scavengers similar to UW. However , it has been designed to maintain its reduced glutathione level without the further addition of reduced glutathione prior to use which is currently recommended with UW[58]. LS-A uses diltiazem as a calcium channel blocker, theoretically reducing endothelial dysfunction, and a thromboxane inhibitor in the form of salicylic acid to reduce prostaglandin activity and platelet aggregation in the microcirculation.

Akin to the IGL solutions LS-A has a low viscosity which should improve the organ flush during retrieval. It is thought that the low viscosity of LS-A will enable hypothermic machine perfusion applications.

1.7 Principles of Hypothermic oxygenated machine perfusion (HOPE)

HOPE refers to machine perfusion in the temperature range of 0-12◦C, it builds upon the concept of reducing metabolic demand by reducing temperature dependent aerobic respiration. The preservation fluid used, carries substrate for the synthesis of ATP and the continuous flow of fluid allows waste products to be

washed from the circulation along with removal of microthrombi or platelet aggregates. The addition of oxygen aims to allow mitochondrial redox and cellular energy metabolism to move towards normal levels while metabolism is reduced secondary to hypothermia. Due to low metabolic demand most solutions used in HOPE rely solely on dissolved oxygen content.

The first published clinical trial of hypothermic machine perfusion was in 2010, using non-oxygenated fluid to perfuse the hepatic portal vein and artery in twenty human livers. On implantation these livers showed significantly lower early allograft dysfunction and biliary stricture rates[59]. The University medical centre Groningen research group have a developing body of work examining the effects of D-HOPE (Dual Hypothermic oxygenated machine perfusion). Across their work from small numbers in initial trials[60] to the most recent multicentre randomised controlled trial they have shown lower rates of non-anastomotic stricture and early allograft dysfunction using D-HOPE [61]. In pre-clinical work no difference has been seen between HOPE (portal vein only) and D-HOPE (portal vein and hepatic artery) although study numbers are often small[62].

Although HOPE is beginning to show robust evidence of advantage over SCS it has several limitations compared to other MP techniques. Precisely because the cellular metabolism is in a reduced state during hypothermia, real-time assessments of cellular function using established biochemical testing cannot predict function in the recipient. It remains complicated to perform although much less so than other forms of machine perfusion and requires less specialist training. As HOPE is most often administered following a period of SCS the issue of portability is less complicated. In the not-too-distant future the concept of a self-contained HOPE machine with no SCS transport period is easily applicable given there is no continuous monitoring and intervention required. Reassuringly any pump failure would revert to the current gold standard of SCS.

1.8 Principles of Normothermic machine perfusion (NMP)

Normothermic machine perfusion refers to machine perfusion with perfusates within the normal temperature range of the species being studied, 37◦C for humans and 38◦C in porcine models. The underlying principle is to mimic, as closely as possible, physiological conditions, avoiding or greatly reducing ischaemia and hypothermia. The metabolic demands of normothermic machine perfusion mirror those found *in-vivo* and as such oxygen requirements cannot be met by dissolved oxygen content. Thus, NMP uses blood products or synthetic oxygen carriage molecules as a base for its perfusate[27]. Furthermore, electrolyte and acid base balance must be monitored as these conditions must be controlled exogenously. If prolonged periods of NMP are desired a nutrient supply must be accounted for usually in the form of amino acids[63]. The neurohormonal control of nutrient supply and vascular tone need to be controlled with additions of insulin and vasodilators. NMP of the liver particularly, tends towards hyperglycaemia as gluconeogenesis occurs to facilitate lactate metabolism[64]. The warm, nutrient rich environment provides the ideal bacterial breeding ground and so equipment sterility and judicious antibiotic use become increasingly important with duration of NMP. Strict control is especially difficult in single organ perfusion as specialised tissue functions E.g. proton elimination via the kidneys or immune cell function are not present. Most studies to date have focused on maintaining single organs on a perfusion circuit, however some animal models have examined multi-organ perfusion using the same circuit and found that physiological conditions were easier to achieve[65].

NMP is used as both a translational experimental model and clinically as an organ preservation strategy. Experimentally, it has been used across species and organs examining a broad range of hypotheses. Liver NMP specifically has been used to compare SCS to NMP in animal models and to delineate processes and markers of lactate metabolism and gluconeogenesis in the context of the IRI[66]. A rodent model of NMP has been used previously as a simulated transplant within our own laboratory in the development stages of LS-A[67,68]. The difficulty in its application as an experimental model lies in the complexity and variation of the protocols

involved. Specialised training and equipment are required to perform perfusions, with many biochemical processes that must be continuously monitored. Exogenous administration of insulin, vasodilators, buffers, and nutrients must occur to mimic normal physiology, however designing experimental protocols that allow for naturally occurring variations in physiology is not always clear at the point of administration. It is difficult to discern a natural variation arising from organ failure against that induced by the intervention being examined. During the course of this review several experimental protocols have been identified on which to base NMP experiments[63–65,69–71]. Each group shows significant variation in experimental protocol, equipment and trigger points for correction of physiological disturbance. This lack of uniformity in the established literature makes defining a 'successful reperfusion' during experiments difficult, but all models agree that lactate clearance is an essential marker of liver perfusion. On the contrary the fact that many distinct experimental protocols can lead to meaningful peer-reviewed results shows a robustness to this model not seen in other models. On top of all the technical difficulties of using NMP as an experimental model, high equipment costs and the susceptibility of equipment to malfunction makes these experiments very expensive. The lack of neurohormonal regulation of the clotting cascade along with the presence of multiple thrombogenic factors such as blood stasis, inflammation and contact with foreign material leads to thrombus formation in almost any part of the equipment used.

In the year 2020-2021 the NHS organ usage figures showed that a sizeable portion of livers retrieved were not transplanted. This accounted for 14% of DBD donors and 35% of DCD donors[72]. This number is slightly higher than previous years and likely represents population factors as well as confounding factors due to the COVID-19 pandemic. Most organs are discarded out of fear that they will not provide life-saving function after transplantation based on; haemodynamic instability during retrieval, steatosis or prolonged cold ischaemic storage[64]. The decision for discard is based on a subjective clinical assessment performed by the retrieving or implanting surgical team. The introduction of clinical NMP allows two processes in these discarded livers. Firstly, NMP will provide better storage than

SCS alone either when used exclusively or in conjunction with a period of SCS. Secondly, it allows an objective assessment of function to take place with the evaluation of various biochemical tests or biomarkers. Building on this concept organ specific treatments could be administered to improve suitability for transplant. The first multicentre RCT comparing outcomes after NMP and SCS was published in 2018. This showed encouraging results with no significant difference in graft or recipient survival but a significantly lower discard rate (20%) using NMP. In addition the preservation period in the NMP group was significantly longer than that in the SCS group[73]. These findings are particularly important for the long-term implications of NMP, a longer preservation period means functional assessment can be carried out with little impact to recipients and a lower discard rate will mean more organs are available for transplant. Although the results of Nasralla et al are encouraging the sample size is relatively small for the frequency of the major complications that need to be characterised.

The limiting factors to the wide-spread clinical adoption of NMP are akin to those faced in the academic world. Specialised staff and equipment are needed to maintain each organ whilst undergoing NMP which greatly increases the cost compared to the simple SCS method. The parameters which need to be monitored and treated will become increasingly complex with increasing experience of NMP and apparently beneficial organ treatments may translate into poorer post-transplant outcomes, as was seen in one UK experiment[64]. Allocation of blood products, required to maintain organs, will place further strain on blood donation services or expensive, synthetic oxygen carriage solutions will be required. Preventing catastrophic infectious or thrombotic sequalae of NMP will pose significant challenges especially if NMP is used in a transport setting. Specifically in UK transplant services, NMP provides a unique challenge. As the retrieving team are rarely involved in subsequent transplant of retrieved livers one must ask how an equitable distribution of machine perfusion technology can be allocated?

Over the coming decade NMP will have an increasing clinical and academic application. Clinically NMP is likely to be used in conjunction with SCS at first, providing a form of pre-conditioning before implantation. If subsequent trials show

better patient outcomes along with an increase in the transplant pool NMP may represent the preservation strategy of choice. Simultaneously, treatments for organs undergoing NMP are being developed. These include 'defatting' treatments for steatotic livers[74]. If clinical NMP can be shown to safely increase the preservation period as seen in animal[63] and human trials[75] then the concept of 'organ intensive care' can be developed with a range of pharmacological, immunological, genetic or stem cell treatments. For this concept to be fully realised reliable, cheap, and specific biomarkers must be identified and validated.

1.9 Novel biomarkers in transplantation

With the advent of machine perfusion many new opportunities present themselves. In the medium term a novel biomarker, detectable at the machine perfusion stage can help prognosticate response to transplantation or potential complications. In the longer term, a reliable biomarker that can be measured in real time will allow progress in two important goals for transplant research. Namely, extension of the donor pool by either more robust and objective selection criteria for organs or in judging the response of 'organ treatments' that can be administered during machine perfusion.

Biomarkers usually constitute a product of the IRI. It logically follows that those organs experiencing the greatest degree of ischaemia undergo the greatest cellular damage and therefore suffer the largest degree of functional impairment. Specifically in liver transplant it is important to consider which outcome a putative biomarker will prognosticate. It is reasonable to assume a whole battery of tests will be needed to assess for early allograft dysfunction or primary non-function, extent of rejection and development of biliary complications. The ideal biomarker will:

1. Change in real-time.
2. Need a single or very few measurements.

3. Be specific to an outcome.
4. Be affordable and easily measurable.

A myriad of molecules have been interrogated for their role in prognostication these can be broadly categorised into: MicroRNA's, interleukins/cytokines and metabolites.

1.9.1 MicroRNA's

MicroRNA's have long been a subject of interest in oncology research, examining their post-transcriptional gene regulation function and how serum levels of these small molecules can be raised in dysregulated cellular processes. The hallmark of the liver IRI is increased oxidative stress in hepatocytes and there is an increasing body of evidence that suggests specific MicroRNA's (miR1225p, miR1925p, miR2233p, and miR12245p) show raised serum levels in response to oxidative stress[76]. In particular miR122 has been the subject of further work showing increased serum concentrations following liver injury and specifically following 45 minutes of ischaemia with varying length of reperfusion up to 24 hours[77]. Interestingly, this work suggests that the primary mechanism of hepatic IRI is necrosis which as discussed in earlier chapters is not widely accepted[16,17]. Although miR122 shows early promise there are currently no validated levels showing irreparable damage and the vast majority of evidence is in animal models examining IRI extent and not early allograft dysfunction (EAD) or primary non-function (PNF), significant work would need to be undertaken before it could be useful in a clinical machine perfusion setting[78].

In both porcine and human models miR146b has been shown to correlate with extent of IRI and EAD, respectively. The human samples taken were graft biopsies, taken in a single centre Chinese study, following 90 minutes of *in-situ* perfusion after transplant which showed good correlation with the risk of EAD. The generalisability of these data to western populations is, however, difficult as the

Chinese organ donation system allows for organ procurement in donation after brainstem death with subsequent cardiac death situations. This is not an entity that is seen in UK donors and as such the high levels of miR146b detected in these donors may not be seen[79].

1.9.2 Interleukins/Cytokines

There are several interleukins and pro-inflammatory cytokines associated with liver injury these include IL- 6,17, 23 and 33 along with cyclin D1, fibroblast growth factor 21 and endothelin-1. Interleukin 17 (IL-17) is released from a subset of CD4+ T -cells in response to IL-23. This pathway has been linked to autoimmune conditions and other forms of sterile inflammation. A recent study showed elevated levels in a murine model during warm IRI associated with more severe injury[80]. This is difficult to rationalise in a clinical context as IL-17 and its downstream markers are present in many inflammatory processes so if it progresses into machine perfusion studies its utility may be limited to use within a closed circuit.

IL-33 and cyclin D1 expression are intricately linked to one another, increased levels of Il-33 lead to decreased expression of cyclin D1 in liver cells. Rat models show that steatotic livers undergoing IRI express less IL-33 and more cyclin D1, these rats either did not survive warm ischaemia or developed long term macro-steatosis following resumption of a low-fat diet. Higher cyclin D1 expression is also correlated with delayed graft function in these rats[81]. From the study data, it is difficult to ascertain if the increased levels of cyclin D1 represent greater IRI because of steatosis or simply represent a baseline poor functioning of the liver due to steatosis and as such this pathway may play a greater role in assessing before NMP rather than allowing intervention during NMP.

Fibroblast growth factor 21 regulates adipocyte glucose uptake and is primarily secreted by hepatocytes. Its expression is elevated after induction of IRI with peak levels reached at 2 hours post-cessation of blood supply, which is earlier than damage would be seen with traditional biochemical markers. It is thought that

levels reliably correlate with severity of IRI. The levels stayed raised with serum samples taken at 2 hours post-implantation up to 20-fold seen in heathy individuals[82]. The main limiting factor of the trial is that its outcome measures did not include EAD or PNF and so the clinical utility of fibroblast growth factor has not yet been shown. Other studies have also shown that fibroblast growth factor 21 shows elevated serum levels in a whole host of hepatic and systemic disease from NAFLD to type 2 diabetes[83], this limits its utility in donor and recipient testing but its role in MP is still unclear. It also raises a very interesting question regarding machine perfusion and systemic disease that will need considerable exploration as the technology becomes more widespread: what are the effects of systemic disease on the perfused organ, and can these be adequately assessed and even treated during the preservation process? Further work is needed examining the specific post-transplant outcomes with exploration of commonly occurring confounders such as NAFLD and type 2 diabetes.

1.9.3 Lactate/Lactate clearance.

Lactate is the product of anaerobic respiration within cells and up to 70% of its metabolism takes place within the liver where it is converted back to circulating glucose[84,85]. This makes it an especially important marker for liver IRI given that there will be over production by the ischaemic liver cells and a resultant reduction in clearance given reduction in liver metabolic rate. There is emerging consensus that healthy livers undergoing MP should show an ability to clear lactate before they can be considered for transplantation, yet there remains controversy over the extent to which lactate clearance occurs and within what time period [64,66,78,86–88]. Lactate clearance as a measure has several disadvantages, it requires multiple readings and construction of a trend line, so the cost of the MP period is required to measure it. Although its association with poor outcome measures i.e. EAD and PNF show strong relationships the underlying cause of this can be distorted by the quality of the MP itself, as can be seen in the methods section of this thesis. For example, if there is a failure of circulation or even a significant airlock within the

NMP circuit the lactate will rise exponentially suggesting a more severe IRI but is not reflective of the pre-MP potential function of the liver. Furthermore lactate clearance is clearly present in NMP where the full metabolic function of the liver is restored but has no use in HOPE where the object of the treatment is reduction in free radicals and restoration of mitochondrial function [89–91]. In short, the perfusate lactate level is especially useful in monitoring the livers response, especially during NMP but can be affected by several temperamental perfusion protocols. If solely relying on lactate clearance the situation could arise where a viable liver receiving poor NMP becomes seemingly unviable during perfusion and is mistakenly discarded.

The role of lactate metabolism in both the donor and the recipient must also be considered. In the donor the key considerations will be time from asystole, with higher times being consistent with higher serum lactate levels. It is logical that this will influence the starting level during perfusion, it is not yet clear how this is linked to PNF or EAD. The increasing prevalence of hepatic steatosis and the subsequent increase in transplant of these organs will also impact the donor lactate level both *in-situ* and immediately after explant. The literature suggests that steatosis predisposes these livers to free radical and mitochondrial injury to a greater extent than normal liver tissue thus the IRI of liver transplantation would manifest to a much larger extent in steatotic livers undergoing NMP[92]. There is a paucity of evidence on this subject in human livers however a large animal trial suggests that NMP may even reduce the amount of steatosis in these grafts if performed over an extended period (40+ hours). This study did not measure perfusate lactate clearance and as such the effects of steatosis on lactate clearance are not directly seen, it did however measure perfusate glucose[93] which is closely linked to the perfusate lactate level in all other published studies, presumably as it makes up the primary substrate of gluconeogenesis. That these livers were hyperglycaemic for much of their perfusion is a sign usually associated with concurrent high perfusate lactate, it is unfortunately impossible to know this for certain and the higher perfusate glucose could also be accounted for by using triglycerides as a substrate for gluconeogenesis.

In the recipient, the baseline lactate level may be elevated secondary to the liver pathology for which they require transplantation, a large proportion of patients with liver cirrhosis will have elevated serum lactate[94]. As lactate is a nonspecific marker it is of little usefulness in the post-operative period especially in the presence of complications or vasopressor drugs which would see its production from other sources. All in all lactate clearance is one of the best NMP predictors of post-transplant function that is currently available hence its inclusion in most of the studies detailing outcomes[64,65,86,88]. It does have major flaws as a marker in that it can easily be falsely elevated, it requires multiple measurements that must be conducted whilst the technically challenging and costly machine perfusions continues running.

1.9.4 Flavin mitochondrial mononucleotide (FMN)

FMN is a small molecule bound to mitochondrial complex I which plays a role in oxidising NADH freeing electrons in the electron transport chain. Under physiological conditions it is very tightly bound to the electron transport chain, when halted in the ischaemic phase of IRI the bond between FMN and mitochondrial complex I becomes weaker, then on reperfusion it readily dissociates generating ROS and causing mitochondrial dysfunction[95]. Once reperfusion has been established and viable mitochondria have resumed near normal function FMN dissociation ceases thus measuring the expressed levels in the explanted organs generates a specific biomarker of the extent of IRI before other recipient factors can cause interference.

Of all the discussed molecules FMN holds the most promise in the context of the normothermic isolated organ machine perfusion. It has been shown to be measurable in the normothermic setting using a regional *in-situ* perfusion of abdominal organs[96]. It also shows promise in isolated human liver models as shown in the hypothermic machine perfusion setting in predicting post-transplant function and early graft loss [97]. While this correlation shows promise some critics of FMN believe a more thorough assessment of the bioenergetic mitochondrial function is

required; this has been shown to be possible and reliable but comes at the expense of time consuming and technically challenging methods[87].

Building from these two studies it is logical to surmise that a normothermic isolated liver model should show a similar expression of FMN which can then be contrasted to defined functional parameters. One of FMN's most attractive qualities is real-time measurement using fluorescence spectrometry which if supported by robust data, could easily be incorporated into an existing hypothermic or normothermic machine perfusion device. This would greatly improve processing time from the ELIZA method which is currently in use and allow real time modifications of organ treatment protocols.

2.0 Materials and Methods

2.1 Animals, ethics, and organ retrieval

2.1.1 DBD experiments

70-90kg Landrace white pigs were obtained from Leeds. United Kingdom home office guidelines (ASPA 1986) were strictly adhered to throughout this work.

2.1.2 DCD experiments

70-90kg white Yorkshire pigs were obtained from Joseph Morris butchers, Leicester. Animals were killed by a licensed slaughterman. Organs were retrieved immediately *post-mortem.*

2.1.3 Ethical considerations

All steps were taken to minimise animal pain and suffering during this work. Specifically, in the DBD setting experiments were performed under terminal anaesthesia with Animal Welfare and Ethical Review Body (AWERB) approval and home office approved personal and project license in keeping with university regulations. All experiments were carried out at premises with a valid home office

establishment license after site inspection by the personal and project license holder. Specifically, this work was performed at the Griffin institute in London which has an excellent track record for supporting pre-clinical research.

2.1.4 The 3R's of animal research

All animal research is governed by the 3R's as set out below specific to this project. These study design and ethical principles aim to reduce duplication of work and use alternative methodologies to minimise the number and suffering of animal involved in pre-clinical research.

In this study a large animal model was used following successes in a rodent model with earlier projects. This step was necessary due to structural and functional differences between rodent and large mammal livers. Out width the primates, on who animal research is prohibited, the porcine liver most closely resembles the human with regard to both anatomy and function.

Reduction: Other groups have required extra cross-matched animals to supply surplus blood during reperfusions, but by developing the exsanguination technique via the aorta I was able to eliminate the need for any further animals. We were able to eliminate the need for repeated procedures including biopsy by taking these samples during through the NMP phase of the experiments.

Refinement: By using a significantly large effect size and aiming the severity of the ischaemic insult to discriminate between failing and functioning livers, the model I developed is refined enough to use small numbers of animal subjects. In key variables, detailed below, failure and function can be separated by as much as a 2-3-fold increase but can often be significantly higher at extremes being near to 10-fold. The use of NMP in the study design allows it to be conducted under terminal anaesthesia and as such the suffering of the animals through the recovery period is eliminated.

Replacement: Using this study design, we were able to negate the need for a transplant model, immediately halving the number of pigs used in the study.

Invasive monitoring that would be needed during any transplant model is also replaced with perfusion parameters during NMP.

2.2 Composition of organ preservation solutions used

For the purposes of this study UW, UW-MPS and IGL-1 were obtained commercially through their respective UK suppliers. LS-A was produced by Huddersfield pharmacy specials (HPS, Huddersfield, UK) to the specifications set out in the table below. All preservation solutions were stored in accordance with manufacturers recommendations and used within their known shelf-life. Put simply they were stored in a monitored fridge at between 2 and 4◦C. As LS-A is not a commercial product, it is still undergoing stability analyses which are included in this thesis as appendix 1. This work was carried out by the HPS team to examine for shelf-life and none of it was performed by the primary researcher, it appears only to validate the use of LS-A at a given shelf life.

Characteristics and compositions of current and future solutions for static and hypothermic perfusion preservation of abdominal organs

	UW SCS[28]	HTK[29]	Celsior[30]	IGL-1[31]	UW MPS[32,33]	Vasosol[34]	Polysol[35]	HTK-N[36]	IGL-2[37]	LS-A
pH	7.4	7.02–7.2	7.3	7.4	7.4	7.4		7.0	7.4	6.75
Osmolarity, mOsm/L	320	310	320–360	320	300	300	320	305	360	340
Viscosity, cP[a]	5.70	1.8	1.15	1.28	2.40		1.8		1.7	1.1
Colloids										
HES, g/L	50	–	–	–	50	50	–	–	–	–
PEG, g/L	–	–	–	0.03	–	–	10	–	5	20
Impermeants										
Gluconate, mmol/L	–	–	–	–	90	90	10	–	–	–
Glucose, mmol/L	–	–	–	–	10	10	16	–	–	–
Lactobionate, mmol/L	100	–	80	100	–	–	–	–	100	50
Mannitol, mmol/L	–	30	60	–	–	30	–	–	60	–
Raffinose, mmol/L	30	–	–	30	30	–	–	–	–	–
Sucrose, mmol/L	–	–	–	–	–	–	–	33	–	100
Buffers	Phosphate Sulfate	Histidine	Bicarbonate Histidine	Phos-phate	HEPES Phosphate	HEPES Phosphate	Bicarbonate HEPES Phosphate Sulfate	Histidine N-acetylhisti-dine	HEPES Histidine Phosphate Sulfate	Bicarbonate Phosphate
Electrolytes										
Calcium, mmol/L	–	0.015	0.25	–	0.5	0.5	2	0.02	–	–
Chloride, mmol/L	–	50	41.5	20	1	1	109	30.04	–	–
Magnesium, mmol/L	5	4	13	5	5	5	14	8	5	–
Potassium, mmol/L	120	10	15	30	25	28	5	10	25	5
Sodium, mmol/L	25	15	100	120	100	110	135	16	125	45
Zinc, mmol/L	–	–	–	–	–	–	–	–	0.091	–
Metabolic precursors	Adenosine	α-ketoglutarate	–	Adenosine	Adenine Adenosine Ribose	Adenine L-arginine α-ketoglutarate Ribose	Adenine Adenosine Amino acids Pyruvate Vitamins	Alanine Arginine Aspartate Glycine α-ketoglutarate	Adenosine Sodium nitrite	–
Antioxidants and free-radical scavengers	Allopurinol Glutathione	Tryptophan	Glutamic acid Glutathione	Allopurinol Glu-tathione	Allopurinol Glutathione	NAC	Allopurinol Glutathione	Deferoxamine LK-614 Tryptophan	Glutathione	Allopurinol Glutamic acid Glutathione Salicylic acid
Other additives	Dexamethasone Insulin Penicillin G	–	–	–	–	NTG PGE_1	Phenol red	–	–	Diltiazem

Figure 3: Table taken from Ramos et al showing composition of preservation solutions used. This table was produced as part of writing this thesis and adapted for publication in the cited article

2.3 Governance

As previously stated, the DBD experiments where performed at the Griffin institute in London, who had a valid establishment license. As the primary researcher I held a personal license and attended all the necessary mandatory training set out by the university regulations. The Home Office regulation were strictly adhered to at all times to reduce potential for any animal harm.

2.4 Anaesthesia (DBD only)

For each animal:

Pre-medication: Ketamine (5 mg/kg)/ Xylazine (1 mg/kg) was administered intramuscularly.

Diazepam tablets (10 mg/10kg) and Acepromazine (ACP) tablets (10 mg/10kg) were administered orally in a piece of banana, apple, or diet, approximately 30-60 minutes prior to other administrations.

Following the above sedative administration, general anaesthesia was induced with oxygen over Isoflurane, delivered via a close-fitting face mask. The animal was transferred to the operating theatre, intubated with a cuffed endotracheal tube. After intubation, anaesthesia was maintained using oxygen over isoflurane, with respiration controlled via a ventilator.

2.5 Surgical procedure: Retrieval from donor in DBD setting and preservation

The animals underwent bilateral nephrectomy and hepatectomy simulating the clinical practice of multi-organ retrieval. Organs were preserved in one of the three study solutions. Which were randomised on the day of retrieval. The intra-operative monitoring included heart rate, oxygen saturations, non-invasive blood pressure monitoring and temperature.

2.5.1 Blood retrieval

A catheter placed into the distal abdominal aorta post anaesthesia provided the means of blood retrieval. Once cannulation occurred a maximum of 2.5L of blood was taken dependent on the animal's weight. This blood was stored in heparinised glass containers at 4◦C

2.5.2 Multi-organ retrieval

A thoraco-abdominal incision was performed using a scalpel and pencil, monopolar diathermy to access the peritoneal cavity. A self-retaining abdominal retractor was used. The large and small bowel were mobilised to give access to the retroperitoneum. The retroperitoneum was opened to visualise and isolate the distal abdominal aorta at its bifurcation. Three heavy silk ties (1.0) were passed around the aorta and clipped. These helped to secure the cannula and tie off the distal end of the aorta when cannulation took place. The retroperitoneum was opened from distal to proximal until the superior mesenteric artery was identified. At this point both renal arteries were identified.

The connective tissue from stomach and liver was divided and the supero-lateral connective tissue from the liver's edge was mobilised. The portal vein was isolated in the hepatoduodenal ligament and followed to its junction with the splenic vein, the portal vein was isolated and cannulated with a 28french catheter and secured in place. The thorax was entered via the diaphragm to give access to both the aorta and supra-hepatic part of the IVC. The thoracic aorta was isolated on a nylon tape.

25,000 units of heparin were administered intravenously and allowed to circulate for three minutes. After three minutes minimum had elapsed, the most distal tie on the aorta was secured, and the distal aorta was divided above and cannulated with a urinary catheter. The aortic cannula was secured with the two proximal ties. Blood collection took place for a maximum of three minutes to minimise the WIT. The proximal aorta was cross clamped and immediate perfusion began with the

chosen organ preservation solution, with 3L delivered via this route. The catheter was secured with a tie. The distal IVC was divided for venting and the abdominal cavity was packed with ice. Once 5L in total preservation solution had been perfused the beginning of SCS was considered to have taken place and the time was recorded. A cuff of proximal abdominal aorta was taken along with the coeliac trunk to allow easy canulation of the hepatic vessels during bench work. The liver was then divided from remaining attachments. The liver was removed, weighed, and placed in a bag in a bowl of iced sterile preservation solution. The aorta was divided below the superior mesenteric artery and then transected in the sagittal plane until exposed vertebrae were seen. The kidneys were removed, taking care to preserve an adequate length of renal vein, renal artery, and ureter. The kidneys were weighed and placed in bags of cooled preservation solution.

2.5.3 Back bench preparation: Liver

A visual inspection of the liver was performed on the back bench to assess length of residual artery, portal vein, IVC and bile duct along with any aberrant anatomy. The inspection also searched for any retrieval related injury and vessels that needed to be trimmed or repaired; if repair was necessary a 5.0 prolene suture was used. In the event of aberrant arterial anatomy an aortic segment was retrieved, and an aortic tube formed to allow perfusion of multiple small vessels via this route.

The livers were bench flushed and stored in the preservation solution in double plastic bags on ice as in clinical transplantation.

After storage but prior to the commencement of NMP, livers preserved in UW or IGL-1 underwent a flushing protocol. This involved washout of the inflow vessels with up to 1L of normal saline, this is performed in clinical practice to washout the large volume of potassium held by those solutions to prevent dumping into the circulation and subsequent cardiac dysrhythmia.

2.6 Surgical procedure: Retrieval from donor in DCD setting and preservation

2.6.1 Blood retrieval

After humane termination in the abattoir setting by a licensed slaughterman, the jugular vein was opened bilaterally and up to 2L of blood were collected into a heparinised non-pyogenic container. Time zero for measurement of WIT was started at the end of the termination step.

2.6.2 Liver retrieval

A large midline thoraco-abdominal incision was made from manubrial notch to symphysis pubis to expose the thoracic and intra-abdominal organs. The supra-hepatic inferior vena cava was divided as was the first part of the duodenum and the small bowel retracted downward and towards the left iliac fossa. This allowed isolation of the portal vein and hepatic artery in the hepato-duodenal ligament. The hepatic artery was cannulated with a 10french catheter in situ to ensure anatomical orientation. The remaining hepato-duodenal ligament was divided with the common bile duct isolated using arterial forceps. The remaining diaphragmatic attachments are taken down taking care to preserve the IVC. Lastly the infra-hepatic IVC was divided, and the liver removed from the abdomen. All of these retrievals were performed in a working abattoir with the meat production line continuing in the background. Each experimental day lasted from 08:30am with randomisation and preparation of equipment and solutions through to 23:00 when the second perfusion ended, and equipment/lab space was cleaned. Therefore, each retrieval was highly pressured, as they could only be performed one morning each week if WIT was outside acceptable limits or variant anatomy was not appreciated the entire experiment had to be abandoned.

2.6.3 Back bench preparation: Liver

The liver was placed onto a bed of ice and the immediate perfusion of 1L of chilled (0-4◦C), heparinised (5000 units/L) preservation solutions began via the cannulated hepatic artery. The portal vein was then cannulated with a 28french catheter and secured. Then 2L of chilled, heparinised preservation solution was infused via the portal vein. Only those retrievals with a WIT less than 20 minutes progressed to normothermic machine perfusion. After storage but prior to reperfusion those livers stored in UW or IGL-1 were flushed out of any remaining preservation solution using 1L of normal saline as clinical transplant and previous study protocols[98–100]. Those livers preserved in LS-A were not flushed before perfusion. The commercially available solutions were flushed with saline as previously mentioned to remove large volumes of potassium from the circulation. As LS-A contains substantially less potassium it was hypothesised that this flush would be unnecessary.

2.7 Isolated perfused porcine liver model

This validated model aims to mimic re-implantation by restoring an arterial and venous flow. It works by attaching the liver into a physiological circuit comprised of a blood reservoir, membrane oxygenator and a humidified chamber. The membrane oxygenator used was (Trilly paediatric oxygenator, Eurosets Ltd, Medolla, Italy) with Carbogen gas (95% 02, 5% C02) to mimic physiological acid base balance. We used a whole blood-based perfusion fluid which consisted of two units (roughly one litre) of whole, autologous porcine blood with 500mls of Gelofusin dilutant. Porcine blood is hypercoagulable in comparison to human blood and so to prevent clotting 25,000 units of unfractionated heparin was added. The harvested blood is depleted of calcium and bicarbonate, and these were added to act as buffering and osmotic regulation systems. In the below table a full list of additives details the role of each drug.

Drug	Rationale	Mechanism of action
Verapamil	Flow rate is governed by internal vascular resistance and blood pressure	Inhibits calcium entry into smooth muscle cells
Epoprostenol	Flow rate is governed by internal vascular resistance and blood pressure	Binds to prostanoid receptors, inhibits endothelin
Insulin	Governs glucose and potassium homeostasis	Increases cellular uptake of glucose and potassium
Calcium carbonate	Maintains capillary oncotic pressure, maintains normal cellular functions	Prevents large fluid shifts
Sodium Bicarbonate 8.4%	Blood needs buffering systems to maintain acid-base balance.	Provided starting block carbonic acid-bicarbonate buffering system

Table 2: Drug therapy during NMP

In establishing a working model there were many challenges to overcome, both operatively and with the model itself. Over the course of twelve weeks, technical issues were systematically worked through as they arose, eventually arriving at a working model. All in all, this required two post-mortem and ten DBD retrievals in order to exclude various cause of experimental failure and refine the model.

2.7.1 Perfusate: Technical issues

Initially, the main problem was finding an appropriate method to harvest the required volume of blood from the animal without compromising the DBD element of the study design or creating the need to use blood typed, cross matched, blood

donor animals. The previous studies examining fixed organ perfusion used a DCD model, with up to 30 minutes of asystole before organ perfusion with preservation solution. Each minute of asystole adds to the initial severity of the IRI and so to maintain minimal injury we tried to eliminate the time between asystole and organ perfusion with the cooled preservation solution. Landrace pigs have a circulating volume of 65ml/Kg and >70kg, they were chosen to provide enough blood volume without causing circulatory arrest. In the first experiment the vena cava was cannulated to provide the blood for perfusion, the flow in the vena cava is 1.2(+/-0.5)L/min which is thought to be higher than the aorta[101] Due to positioning of the animal and the reduction in caval flow as bloodletting began, the necessary amount of blood was not able to be obtained in an acceptable timeframe. Collection was also attempted via the internal jugular vein using blood bags before the surgical procedure, but this caused haemodynamic instability despite aggressive fluid resuscitation and so was abandoned. A method using an aortic cannula of collecting around 2.5L of blood before applying the proximal aortic cross clamp was developed followed by immediate reperfusion with cooled preservation solution.

2.7.2 Gas and pH balance: Technical issues

The acid-base balance of the blood is strictly maintained between pH 7.35-7.45. This is the optimal pH for many biological processes but most importantly the oxygenation of haemoglobin[102]. Haemoglobins affinity for oxygen and thus its saturation changes with any deviation from physiological norms, an effect known as the Bohr shift. This means that for a given partial pressure of oxygen, decreases in pH, increases in temperature or an increase in partial pressure of C02 reduces haemoglobins affinity for oxygen and releases it to the tissues.

Such changes are regulated by most tissues however the two largest contributors to this are the lung and the kidneys. The lungs work to eliminate excess C02 produced via respiring tissues, preventing the formation of carbonic acid. As this system can eliminate C02 on a breath-by-breath basis this allows for pH change within minutes

to hours. The renal system acts by reabsorbing bicarbonate ions filtered into the urine and excreting fixed acids[102]. This effect can take much longer, sometimes many hours and often days to occur. In this fixed organ perfusion model, it is necessary to simulate the function of both lungs and kidneys. The lungs are replaced by a membrane oxygenator fed with an artificial gas. Currently there is controversy about which gas should be used. Some authors advocate the use of medical air, a sterile gas mixture comprised of 78% nitrogen, 21% oxygen and trace amounts of C02 and water[64]. Whilst other authors advocate for a gas mixture containing up to 5% carbon dioxide such as Carbogen, (BOC UK)[65,103]. Whilst medical air provides a partial pressure of oxygen that is supraphysiological but close to physiological limits, roughly 20kPa, it does not provide C02 to act as a blood buffer and often leads to alkalotic blood. Furthermore, the flow of oxygen through the membrane oxygenator causes CO2 dissolved in the blood to be 'blown off' thus compounding this effect. On the other hand, Carbogen, delivers C02 with its acidic buffer helping to maintain physiological pH of the blood but at the detriment of hyperoxaemia with partial pressures of oxygen far exceeding those seen in clinical practice or physiology at roughly 60-70kPa. Hyperoxaemia is thought to propagate ROS especially in the lungs during periods of critical illness[104]. It is theoretically possible that this would also increase damage done in the IRI. From the literature it seems that effect of hyperoxia tends to occur once organs are removed from the normothermic perfusion machine and manifest once transplant has occurred[64]. There does not seem to be a measurable effect on organ function during the normothermic reperfusion period and as such, Carbogen was employed due to its acid-base regulation properties.

There is no mechanical renal system incorporated into this model, although this has been attempted by other research groups. Some groups have incorporated a liver-kidney reperfusion model showing better homeostatic results[65]. Although this model offers a better physiological window on the factors affecting the liver and kidney such as drug metabolism, the nature of the outputs of this study and the shared blood reservoir precluded its use. Inevitably, as there was no means of excretion, this led to accumulation of waste products in the liver model namely,

urea, BUN, creatinine, and hydrogen ions. With the short perfusion time and small production of urea, BUN, and creatinine there was no need to account for them beyond acknowledging their presence in the model as an expected finding. The hydrogen ions, with no means of excretion, needed to be buffered to prevent the adverse effect of acidaemia on the perfusate. This was achieved via the use of 8.4% sodium bicarbonate which was titrated to keep levels in the physiological range. The dosing protocol was initially taken from work by Chung et al but had to be adapted due to tendency towards alkalosis[65]. The 'intervention' criteria for this model are summarised in the Table 6.

2.7.3 Glucose metabolism and potassium homeostasis: Technical issues

For the duration of the perfusion, the liver produces glucose via glycogenolysis and gluconeogenesis. The conversion of lactate to glucose is one of the key markers of hepatocyte function following transplant. In an effort to mimic the conditions of in-situ transplant a continuous insulin infusion must be provided to prevent uncontrolled serum glucose from causing fluid shifts and cell lysis. Refractory hyperglycaemia tends to occur in many perfusions especially in the first 4-6 hours and the treatment strategies, developed from the literature, are summarised in Table 6.

Potassium homeostasis is intimately linked to the amount of insulin supplied and extent of cell lysis within hepatic tissues and circulating erythrocytes. For the purposes of this study, hypokalaemia was designated as a marker of good tissue preservation given that potassium cannot leave the circuit via any means aside from intra-cellular transport by insulin nor can it enter by any mechanism other than cell lysis. Conversely hyperkalaemia was taken to indicate increased cell lysis and hence poor tissue preservation. Aside from the continuous action of insulin no other attempts to control perfusate potassium were made.

2.7.4 Perfusate pressure, internal vascular resistance, and blood flow: Technical issues

Blood pressure, blood flow and organ internal resistances are intimately related. The liver receives a quarter of cardiac output with 75% of the blood supply being via the portal vein. The overall hepatic blood flow should reach roughly 1.5L/min[63]. The flow rate for the artery lies in the range 200-400mls/min, with the portal flow rate for humans measuring between 1.6-1.8L/min[105]. This is variable depending on size and position and the acceptable limits are between 1 and 2L/min. Porcine anatomy and physiology is sufficiently similar to maintain the flows at an acceptable level for human livers. The physiological pressures of the hepatic artery and hepatic portal vein are 80mmHg and 10mmHg, respectively. The other major factor effecting flow and organ perfusion is the internal resistance or put more descriptively how much blood passes through the capillary beds. The availability of these capillary beds is determined *in- vivo* by a number of hormonal and neuronal influences which are not present in this model. Further the animal is exsanguinated before the harvesting of the organs, the effects of such blood loss are to produce catecholamines and other vasoactive substances which will be present in the perfusate on reperfusion. The vasoactive substances will help to simulate the stress response found in the transplant recipient as a consequence of the major surgery. In the absence of neuronal and hormonal homeostatic regulation of vasomotor tone a vasodilatory drug must be added to allow adequate flows to perfuse the entire organ. The drug of choice is epoprostenol, a prostacyclin analogue[63–65,106]. Epoprostenol has both vasodilatory and anti-thrombotic effects which are mediated by G-protein coupled IP prostanoid receptors in both vascular smooth muscle and thrombocytes, it is also thought to cause vasodilation via the secondary mechanism of inhibition of endothelin-1 production[107]. Due to initial supply issues with epoprostenol an alternative was sought and found in verapamil, this had been used by another research group in isolated perfusion of a kidney [103]. Verapamil is a dihydropyridine calcium channel blocker and causes vasodilation by inhibiting entry of calcium ions into the slow L-type calcium channels[108]. As shown below once

epoprostenol supply was secured I switched to this drug as it is more in keeping with the established literature.

2.7.5 Continuous infusions, boluses, and intervention criteria

Continuous infusions

Drug	Strength	Diluted Volume	Total dose present	Dose required	Infusion rate
Epoprostenol	0.5mg	250ml	0.5mg/250ml	0.04mg/hr	20ml/hr
Insulin act rapid	100IU/ml	50ml	1000IU in 50mls	100IU/hr	Initially 5ml/hr, titrate to blood glucose
Taurocholic acid	Weight in grams	50ml	1g in 50ml	200mg/hr	10ml/hr

Table 3: Drugs provided as continuous infusions during liver perfusions with rates.

Initial Bolus drugs

Drug	Dose	Volume
Epoprostenol	0.08mg	40mls
Insulin	200IU	10ml
Calcium carbonate	10mmol	10mls
Sodium Bicarbonate 8.4%	25mg	30mls
Heparin	25,000 units	Variable dependent on preparation (Usually 5mls of 25,000 in 5ml)

Table 4: Preparation of drugs given as bolus on commencement of NMP.

Intervention criteria

Blood gas measurement	Arterial/ Venous	Issue	Range	Action 1	Action 2 (after >2 boluses)
Acidosis	N/A	pH	<7.25	Bolus 30mL 8.4% Sodium Bicarbonate	Sodium Bicarbonate 8.4% as an infusion at a rate of 40 ml/hr and titrate
Hyperglycaemia	N/A	Glucose	>8mmol	Increase insulin rate to 10mls/hr	Increase insulin rate by 10mls/hr until decrease in glucose
Hypoglycaemia	N/A	Glucose	<4 mmol	Bolus 10 mLs of 10% dextrose	Begin dextrose 10% infusion 10 mls/hr and titrate to blood gases
Hypoxia	Arterial	Pa02	8-10	Check circuit for air leaks/ blocked oxygenator or tubing. Confirm gas cylinder delivering gas	Repeat ABG after 10-15 minutes. Inspect tubing for clots. Change oxygenator if no improvement

Table 1: Actions taken during NMP

2.8 Markers of function: External validity of the model

With such a complicated model there are many outputs that can be monitored and as there is no subsequent transplant, the ultimate test of function is not present. As such externally validated criteria of function are needed to define a successful perfusion i.e. a liver that would be considered fit for transplantation in the clinical setting. The factors considered here are the functional biochemical and physical parameters measurable during NMP.

2.8.1 Lactate clearance

Mergental et al set-out criteria for lactate clearance in their 2018 paper, they showed that lactate clearance in discarded livers undergoing NMP correlated well with their subsequent histological injury. They suggest that a trough lactate level of <2.5mmol/L during the perfusion is the cut-off point that should be used to define a successful or 'transplantable' liver in one of their two major criteria[86]. In a subsequent clinical trial they went on to show that lactate reaching the <2.5mmol/L within 4 hours threshold was useful in predicting function in livers that would otherwise have been discarded but was not as useful in identifying livers that would go onto develop biliary complications[109]. This is supported by the findings of other groups with otherwise discarded livers showing lactate clearance during NMP with no subsequent PNF or EAD following transplant[64]. The controversy lies in the time-period in which it is acceptable for livers to return to a 'normal' level of perfusate lactate. Some groups advocate for levels to drop below 2.5mmol/L within 2 hours of perfusion but Mergental et al have shown a trough level at any time during their 4-hour perfusion correlates with favourable histology. Other groups have suggested a reduction in lactate levels measured as a fall per unit weight of liver[110,111]. Although lactate clearance alone does not signify a liver that will not develop PNF or EAD there is near consensus that a liver that does not clear lactate is non-viable [86,88,109–112]. Given the similarity of this model to that of Mergental a trough lactate level of <2.5mmol/L by 4 hours was accepted as a successful perfusion. This level was subsequently adapted

into the VITTAL criteria[113]. Given the emerging consensus that lactate clearance is a minimum requirement of a functioning liver during NMP it was used as a quality control metric during the method development stage of this research.

2.8.2 Angiodynamics

Hepatic artery and portal vein pressure and flow are dictated by resistance within the liver vascular bed. During the IRI reactive oxygen species and proinflammatory cytokines cause vasoconstriction and tissue oedema which when coupled with the no reflow phenomenon all serve to increase hepatic vascular resistance[114]. Logically it would follow that a more severe IRI should produce higher vascular resistance, and that sub-optimal blood flow will propagate areas of IRI within re-perfused liver. In kidney machine perfusion, the vascular resistance has been a subject of intense study, especially as a marker of post-transplant function, there is some evidence of it being a useful marker in hypothermic machine perfusion as well as in NMP[115116]. It is not however reliable enough to predict post-transplant outcome in isolation. In liver machine perfusion there is controversy over the role that vascular dynamic changes play in predicting outcomes. Mergental et al found that lactate clearing livers correlated with larger incremental increases in portal vein flow throughout perfusion but did not give information about trends in absolute values. They did not find any significant correlation between arterial pressure or flow and histological or functional outcomes[86]. Other groups have not found any significant correlation with arterial or portal flow or pressure during NMP and eventual outcomes[110]. What is important to note is that all studies tried to keep as close to physiological pressures as possible. Early in this experimental model there were a number of failures defined by failure to clear lactate. In short, a piece of equipment failed to supply >0.5l/min via the portal vein and all subsequent experiments failed, despite interrogating various other factors. It was therefore decided to set the portal flow rate at a minimum of 0.5l/min to ensure a working model.

2.8.3 Bile production and pH

A clear and easily measurable marker of hepatic function is bile production, several experimental groups have examined this as a marker of function during NMP with variable results. Sutton et al proposed bile volume measured over 6 hours can be used to stratify extended criteria donor livers as viable vs non-viable, this was a pre-clinical trial making this assessment based on histological and biochemical correlation following NMP. Over a 6-hour perfusion >30g of bile would need to be produced for the liver to be considered viable. These findings were to be correlated with clinical transplant outcomes, but this trial has yet to come to fruition[117]. In a clinical series from another group the absolute volume of bile seemed to have no bearing on the eventual outcome of the NMP, however, this group did not have any livers that failed to produce bile and they concentrated instead on bile chemistry[110]. It seems logical therefore that the complete absence of bile production is a significant marker of non-viability. There is not enough evidence yet to suggest that bile production alone is enough to suggest viability, this is supported by other experimental groups[64,88,110]. Given the remaining evidence a greater bile production should be considered in an amalgamate score as marker of good function[86].

Other groups have considered bile chemistry as a predictor of biliary complications. The Cambridge group have shown, in a succession of clinical studies, that production of alkaline bile (pH >7.4) correlates with better bile duct histology and reduction in stromal injury [64,110]. Whilst this is a key factor to consider in studies where a clinical transplant will take place, this study does not allow adequate follow up time for assessment of cholangiopathy.

2.8.4 Potassium homeostasis

As there is no absorption of potassium from external sources, changes to perfusate levels can only be the result of its release or intake from cells. The sustained use of insulin to control the characteristic hyperglycaemia of liver NMP should therefore result in a state of hypokalaemia unless cells are not undergoing extensive lysis. This

is not the trend that is shown in the literature, many groups do not routinely report their measured potassium levels, and those that do, often show potassium levels at the upper limit of normal or above [64,65,110]. In the clinical setting the post-reperfusion syndrome is characterised by a large drop in mean arterial pressure within 5 minutes of reperfusion. High serum potassium levels have been show as an independent risk factor in its development[118–122]. To see a clinical liver transplant with normal serum potassium is a rarity and this is mirrored in the NMP models of other research groups. Therefore, in this simulated transplant model a lower level of serum potassium is thought to represent a lesser extent of I-RI and is seen as a marker of tissue preservation.

2.8.5 Liver function tests

Most human trials to date examining NMP and function have reported the ALT as a surrogate for hepatocellular damage[64,75,86,88,110,117].This is the traditional marker used in monitoring post-transplant function in-vivo however most groups have not seen a significant link between perfusate transaminase and subsequent function. The Cambridge group had a single episode of PNF in a liver with a significantly elevated ALT at 2 hours and are currently examining this link further[110] to see if a threshold can be identified[110]. In porcine studies the ALT is often reported as a marker of function showing very minimal changes in circulating amount [63,93]. In the pig, AST is a much more sensitive marker of hepatocellular damage with some studies showing even severe hepatocellular necrosis only manifesting as small changes in serum ALT[123]. The absolute values of these markers are difficult to interpret with comparison to clinical values. Firstly, measured level are concentrations and the reduced blood volume in NMP makes comparison with clinical settings very difficult. Secondly, it is not clinical protocol to compare the serum levels repeatedly within the first few hours of transplant as they will understandably remain raised or continue to climb. Therefore, any liver function test in the context of this 4-hour reperfusion study cannot accurately predict future function and are analysed for comparison only.

As for the synthetic function of the liver during NMP, this has been assessed by various groups. The Cambridge group looked at both urea and creatinine metabolism, factor V production and complement activity in porcine models, this was done over 72 hours, and did not show significant changes until after the tenth hour and as such were considered poor markers in this short perfusion model[63]. The Groningen group investigated the use of prothrombin time but showed no correlation within their 6 hour perfusion time-frame[117]. Overall, the literature does not comment on these factors as readily as the other markers but from what little evidence there is, it seems clear the synthetic function would need a greater perfusion period to be accurately assessed.

Parameter	Level	Qualifier
Lactate	<2.5mmol/l	Trough level (4hrs)
Bile production	Absolute volume	Presence/absence
Portal vein flow	Rate in L/min	Maintained above 0.5L/min
Hepatic artery flow	Rate in L/min	Maintained above 0.15L/min
Perfusate pH	≥7.30	At 4 hours

Table 5: Viability criteria during NMP adapted from VITTAL trial.

2.9 Method development

Although the *ex-vivo* perfusion model is validated by various outside research groups and similar rodent models have been attempted by our research group, this is the first time this porcine model has been attempted within the laboratory at Leeds.

The conditions were standardised with single variable changes made in the case of experimental failure. To confirm a working model, the initial CIT was kept at 1 hour in the context of a DBD retrieval, this was to simulate the 'ideal' transplant liver.

Summary of experimental conditions and outcome

Perfusion	Circuit	Vasopressor	Gas	Perfusion type	Issue	Lactate clearance
OP1	1	Standard dose verapamil	Oxygen	Dual	Massive oedema	No
OP2	1	Double dose verapamil	Oxygen	Dual		No
OP3	1	Standard dose Verapamil	Carbogen	Dual		No
OP4	1	Standard dose Verapamil	Carbogen	Portal only		No
OP5	1	Standard dose Verapamil	Carbogen	Dual		No
OP6	1	Standard dose epoprostenol	Carbogen	Dual		No
OP7	2	Standard dose epoprostenol	Carbogen	Dual	Torsion right lobe	Partial
OP8	2	Standard dose epoprostenol	Carbogen	Dual	None	Complete
OP9	2	Standard dose epoprostenol	Carbogen	Dual	None	Complete

Table 6: Summary of experimental conditions during model development

2.9.1 Circuit one

In Figure 3 the layout of the original circuit is shown. This used a single blood reservoir with two separate centrifugal pumps (Hugo-Sachs Elektronik, Germany) to deliver blood at differing flow rates and pressures to both the hepatic artery and portal vein. In this circuit portal venous and arterial pressures were maintained at a constant rate. This was set at 10mmgHg and arterial pressure 90mmHg respectively, subsequent flows fluctuated with intravascular resistance and were measured with flowmeters (TTFM-2, Hugo-Sachs elektronik). Blood was oxygenated using a paediatric extracorporeal membrane oxygenator. Constant infusions were supplied using syringe drivers (pump 11 pico plus elite, Harvard apparatus, Holliston, Massachusetts), drug doses and rates have been specified in Table. The liver is kept in a temperature controlled moist chamber maintained at 39◦C which is basal porcine body temperature.

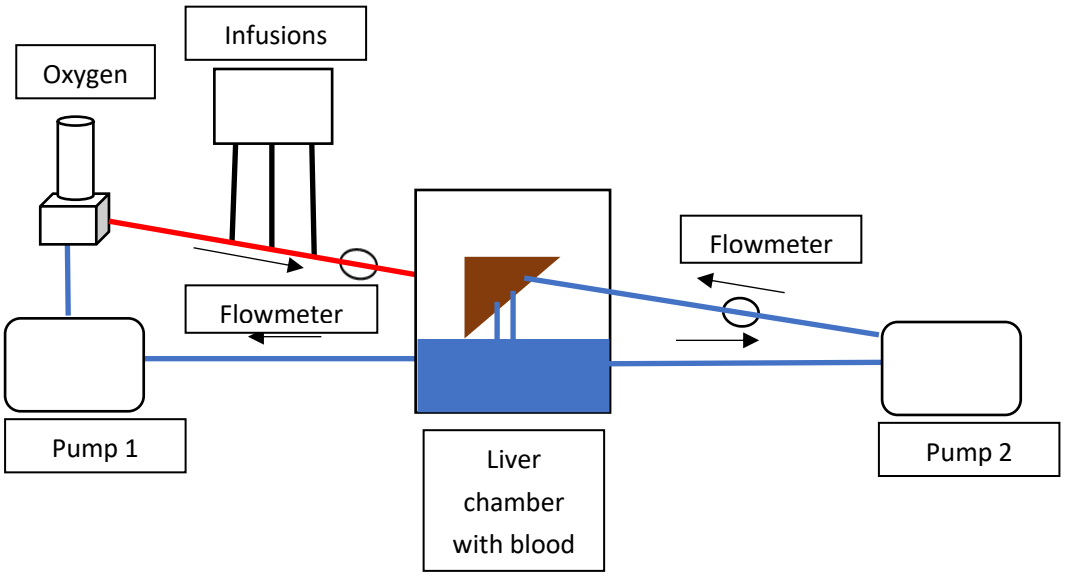

Figure 4: Originally used circuit design, with an individual pump for each inflow blood supply.

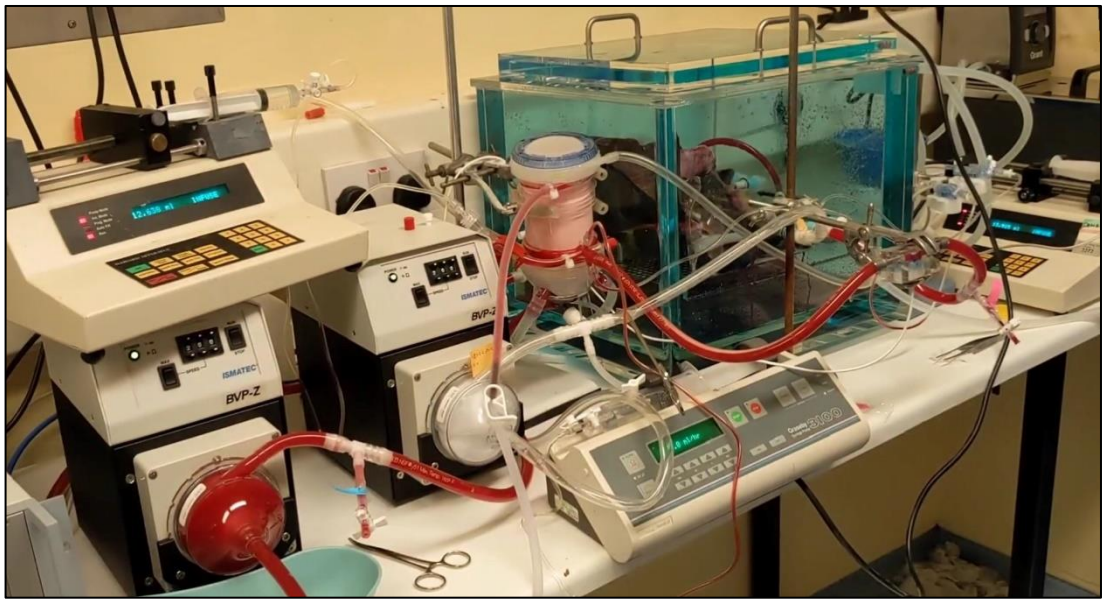

Figure 5: Photograph of initial circuit equipment and layout

2.9.2 Circuit two

Figure 4 shows the changes made after the failed experiments. The most meaningful change is the replacement of the portal vein pump with a gravity driven reservoir bag (MVR 800, Medtronic). Clamp valves on the outflow of the bag were used to dictate flow and pressure in the portal vein allowing near physiological flows. From our previous data and information from the literature we set a minimum portal flow rate of 0.7L/min[117] to ensure adequate blood supply. The continuous infusion site was kept as the arterial inflow.

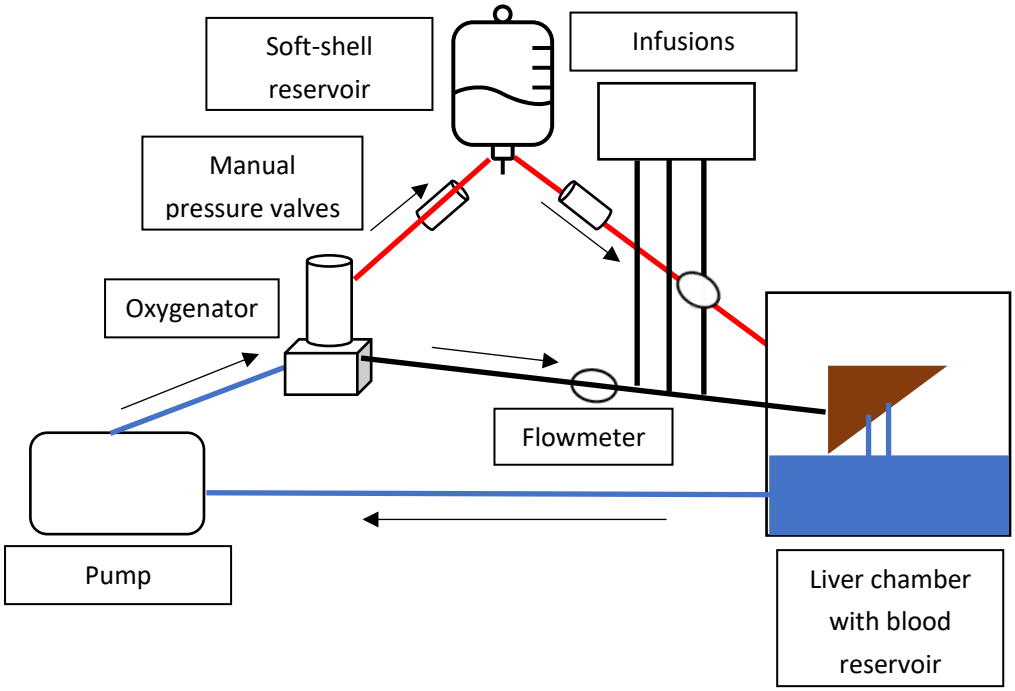

Figure 6: Circuit design used in experimental protocol following initial failures.

Figure 7: Photograph of circuit 2 layout and equipment

2.9.3 Results

Arterial flows remained steady across most perfusions and did not show any discernible trend. The portal flow and pressure can be seen to change in tandem with one another and the large peak with OP6 is the results of a protocol change to explore why the flow rates were so poor. The portal pressure for the other perfusions was kept fixed at 10mmHg for all other unsuccessful perfusions. The two successful perfusions showed greatly increased flows at the same perfusion pressure once the faulty piece of equipment was identified.

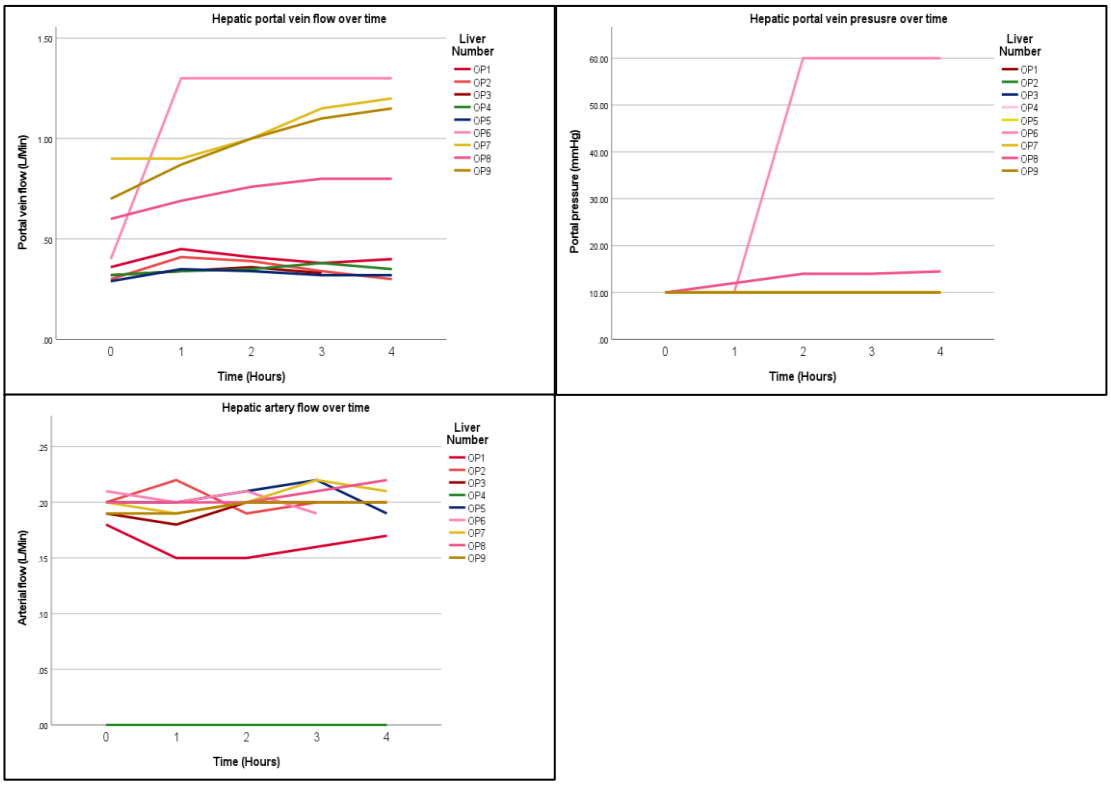

Figure 8: The above graphs show changes in angiodynamics during model development, it should be noted that only OP7,8 and 9 can be considered viable.

The first six livers in our series were perfused with circuit 1 showing a characteristic rapid climb in perfusate lactate. The final three livers were perfused using circuit 2, OP8 and 9 showed return to normal range lactate levels within 2 hours. Unfortunately, OP 7 showed some climb in lactate, examination of this liver during perfusion revealed a torsion of the right lobe. Release of this twist immediately

improved portal vein and hepatic artery flows and the liver returned to a more natural colour.

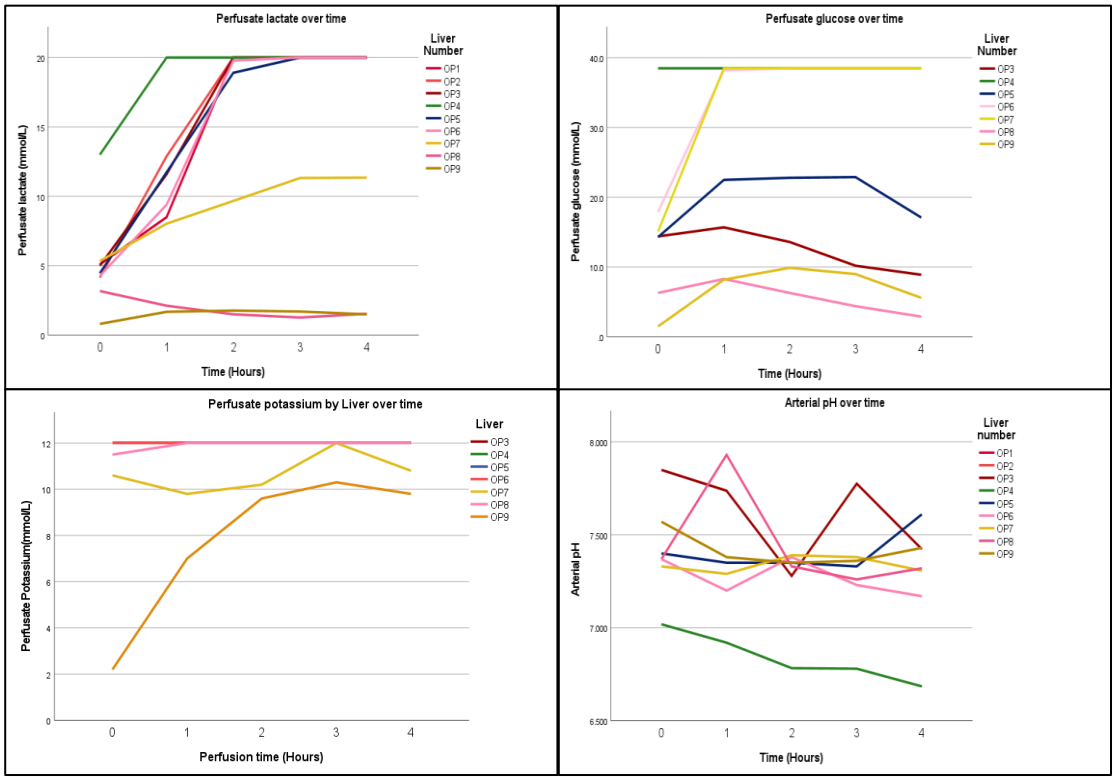

Figure 9: The above graphs show the characteristic climb in lactate of a failing liver along with the expected maintenance of normal levels in successful experiments. The glucose curve shows the how glucose metabolism is intimately link with that of lactate.

There was no recorded bile production present in the first six livers undergoing perfusion, OP7 produced 10mls only in its final hour. The two successful perfusions OP8 and 9 both showed greatly improved bile production from the outset with 90mls and 55mls produced, respectively.

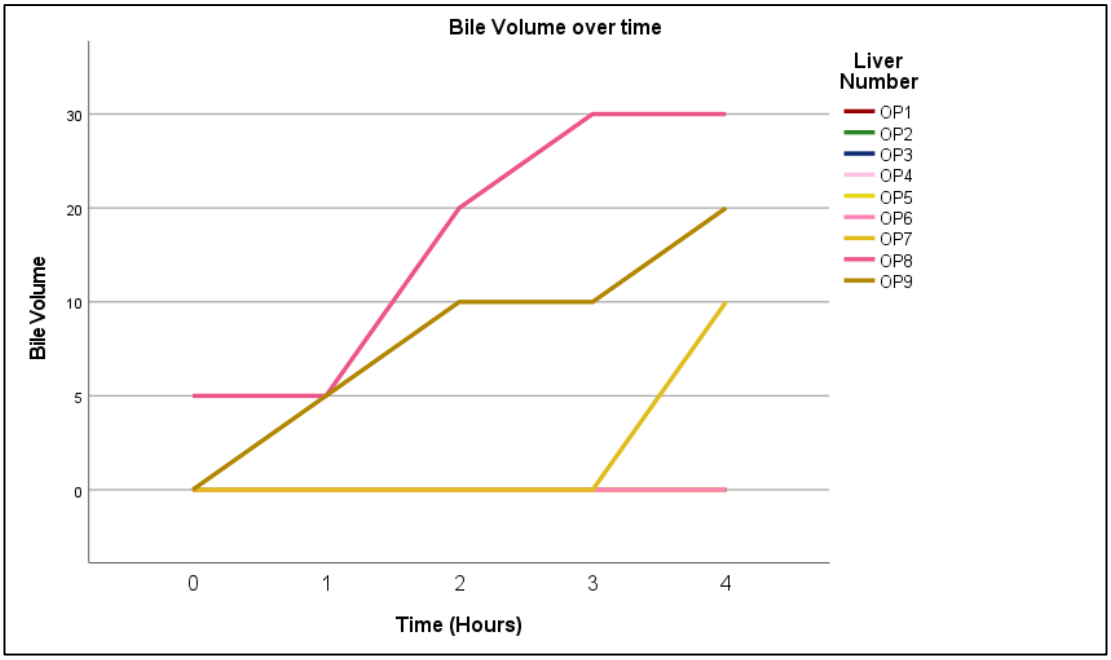

Figure 10: The above graph shows, how failing livers produce little to no bile whilst those considered viable can produce bile at varying rates.

As this study was to assess functionality of the perfusion circuit as a model, LFTs were taken and show comparable result to established literature. LDH was measured as a marker of tissue injury.

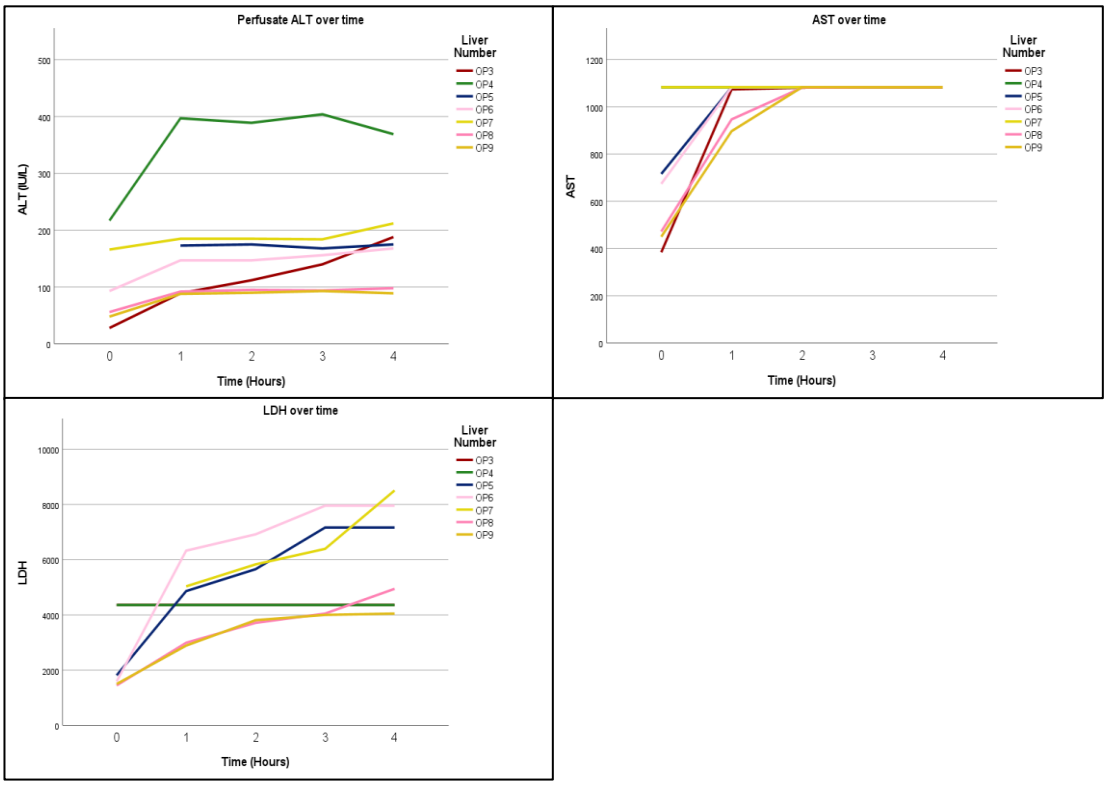

Figure 11: Graphs showing LFT changes during NMP in both successful and failed experiments.

2.9.4 Discussion

The results above show that the DBD model using circuit two was a viable test of function. In ideal conditions it produces livers that can clear lactate and produce bile, satisfying the major criteria set out in the literature[86].

The above graphs (Figs. 8-11) demonstrate the initial perfusion failures and although formal comparison with statistical testing has not been carried out, the curves serve as a baseline reading for a failing liver. The initial failures can be attributed to a faulty pump in the original circuit. This manifested as low portal vein flow at the set pressure of 10mmHg, it was not immediately apparent that any mechanical failure was responsible and as such alterations were made to the protocol with reduction of the intra-vascular resistance in mind. During these interventions, as can be seen in the above graph, the portal pressure was manipulated which in turn altered the flow and showed visibly better perfusion. None of the interventions listed in Table 6 made any difference to the bile production or lactate clearance aside from changing the circuit design. Once the faulty pump was replaced, physiological portal vein flows could be established, and the model behaved in keeping with other experimental groups. As a replacement a gravity dependant soft shell reservoir bag with manually controlled pressure clamps was used. This was adapted from work by the Leicester[65] and Organox groups[63].

The Cambridge research group show characteristic graphs of lactate production and clearance in their 2017 and 2018 papers, this graph shows a steep initial peak at varying intervals between 1 and 2 hours[64,110]. Our functioning livers did not recreate this exactly and there are two potential reasons for this: firstly, they measured blood gases more frequently in the first 90 minutes so it may just be that the graph's peak in this work represents a point on the downward slope. Secondly, the porcine livers in this study were procured to simulate the 'ideal' liver donor with minimal WIT and CIT, whilst the donors in the comparison papers were considered marginal with much larger WIT, CIT, or both. This may account for much larger initial lactate readings as the degree of I-RI will be considerably more. It must also be considered if there is some innate difference in porcine hepatic lactate

metabolism, a much older study looking at porcine ex-situ perfusion has documented similar lactate curves to that shown above using UW and a similar period of CIT[123]. As this group also took measurements hourly it is impossible to judge if this is a contributing factor but given what is known from other porcine liver studies, it seems that a point on the downward trajectory of the lactate level is the most likely outcome. There is evidence from a much older study that lactate clearance is possible with minimal liver flows, this study suggests that a minimum flow of 0.1L/min in the hepatic artery or portal vein is enough for the liver to clear lactate[124]. This has not been borne out in the above experiments and does not seem to represent the findings of more modern porcine or human studies[63–65,86,117].

The perfusate glucose in the unsuccessful perfusions mirrors the trajectory of the lactate climbing ever higher as the liver struggles to process the accumulating lactate. In the case of the successful perfusions the glucose also mirrors the clearance of the lactate climbing to a peak and slowly tapering down with the action of insulin. This is in keeping with the results of other groups and lends some support to the hypothesis that the DBD retrieval and short CIT leads to lower accumulated lactate and thus a reduced glucose peak, especially in comparison to the Cambridge groups findings in humans.

In terms of liver function tests the AST and ALT values for the successful perfusions were of a similar level as described in the literature when taking into account storage times[63,123]. The unsuccessful perfusions showed markedly elevated levels of ALT which is an indicator of extreme hepatic damage in a porcine model. Most groups did not see levels rising above 100 IU/L, even for their poorest livers, this adds more weight to the conclusion that these livers have failed. The AST in all livers quickly reached the limit of the analyser and so no meaningful conclusion can be drawn from this, for subsequent experiments a more robust analyser was used with larger reference ranges. LDH levels in the successful perfusions correlate well with those by other groups in the reperfusion period, again in unsuccessful perfusions they climbed well outside those seen in the literature.

2.9.5 Conclusion

The modifications made enabled the model to be translated into a DCD model as well as the established DBD model so any markers suggestive of increased IRI can be attributed to the severity of initial IRI and CIT (DCD) or the effects of the CIT (DBD).

Note:

Unfortunately, following the model development, the DBD nature of the experiment became cost prohibitive due to funding issues. In collaboration with colleagues at University of Leicester we adapted a DCD model following the same protocol with an altered retrieval process (detailed above). This was one of many challenges that presented itself: for further information to changes in experimental protocols due to adverse conditions please see the 'Barriers to completion' section in the discussion.

2.10 Hypothermic oxygenated machine perfusion (HOPE)

HOPE experiments have not been attempted in our laboratory previously, however there is currently a clinical trial based on HOPE at St James University Hospital. I was able to access training in this method through this clinical trial. HOPE follows similar principles to the NMP shown in this thesis, but there are a number of key differences. Primarily, it perfuses the organ in the temperature range of 8-12◦C. As stated in the introduction, there are two methods of delivering HOPE, either via portal perfusion or D-HOPE which uses both the hepatic artery and portal vein. There have been no significant differences found between the two methods and as such we decided to employ the simpler portal only perfusion. The perfusion pressure is variable with a maximum of 5mmHg with a target flow of 0.25L/min. The perfusion fluid is then oxygenated with a partial pressure of between 50-80KPa. The diagram below shows a schematic diagram of the perfusion circuit, in this case provided with a VITA Smart device (Bridge to life, Columbia, South Carolina, USA)

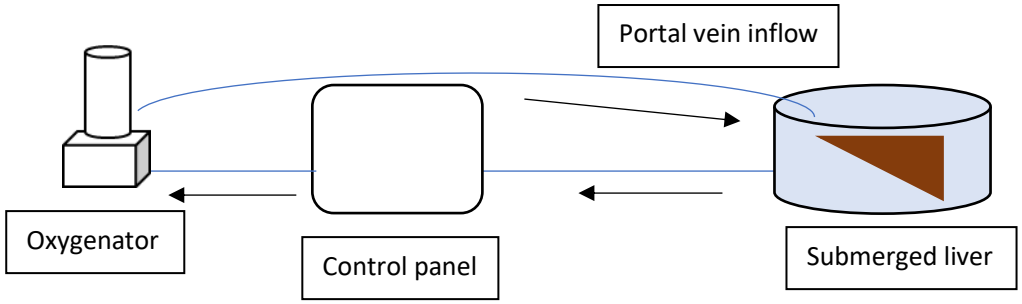

Figure 12: Circuit design for HOPE.

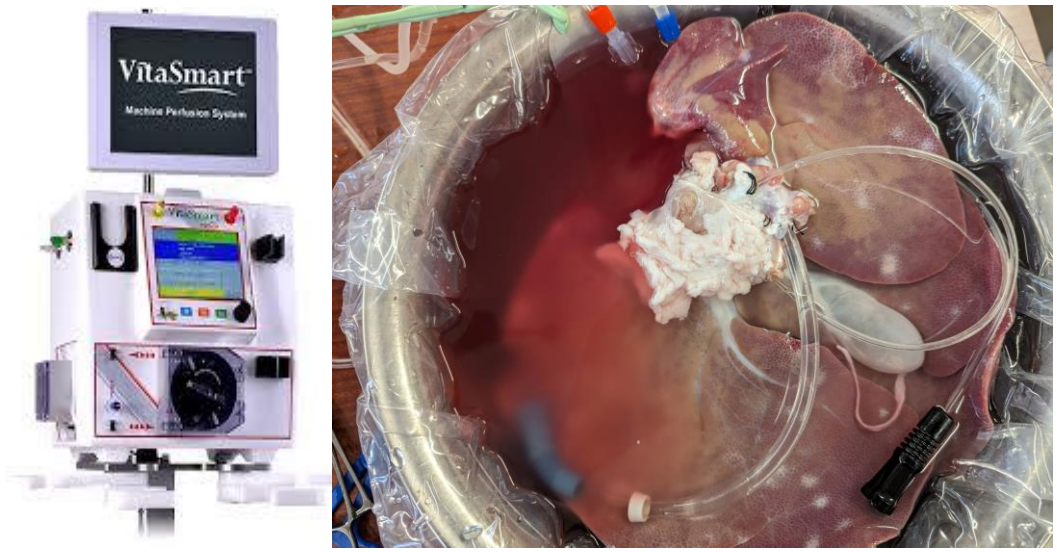

Figure 13: The VitaSmart device (Left, picture courtesy of Bridge-to-life) and a liver undergoing HOPE (right)

2.11 Biomarker examination

As mentioned in the introduction I have chosen to examine the role of FMN and its predictive role in liver viability if measure pre-NMP. In addition to this, I have examined levels of HMGB-1 Caspase-3 and Syndecan-1 in pre- and post-NMP perfusate. HMGB-1 is used as a marker of liver necrosis and makes up one of the key DAMP molecules discussed in a previous chapter it can be detected both in tissue homogenates and in serum/NMP perfusate[125]. Caspase-3 has is a marker of hepatocyte apoptosis and can be detected in both serum and tissue homogenates, it can be used to help differentiate necrosis and apoptosis in a liver I-RI model[77].

Syndecan-1 is a marker of glycocalyx dysfunction and is detectable in the serum, this marker is of great interest as it is believed that the protective action of PEG is via reduced glycocalyx disruption[21].

2.11.1 FMN: ELISA

FMN analysis was performed using the commercially available ELISA (Abbexa Ltd, Cambridge, England). Samples were taken during each experiment and centrifuged at 2500 RCF (relative centrifugal force) for 15 minutes they were then stored at -80◦C until analysis took place. All serum samples were then thawed and left to stand at room temperature for at least 1 hour.

A standard curve was produced with the following dilutions:

1000pg/ml	31.3pg/ml
500pg/ml	15.6pg/ml
250pg/ml	
125pg/ml	
62.5pg/ml	

Wash Buffer: The concentrated Wash Buffer was diluted 25-fold with distilled water (i.e. 30 ml of concentrated Wash Buffer was added into 720 ml of distilled water). It was warmed to room temperature and mixed gently to avoid crystal formation.

Detection Reagent A Working Solution Preparation: This was prepared no more than 1 hour before the experiment.

1. The total volume of working solution required was calculated.

2. Detection Reagent A was diluted 100-fold with Diluent A and mixed thoroughly.

Detection Reagent B Working Solution Preparation: This was prepared no more than 30 minutes before the experiment.

1. The total volume of working solution required was calculated.

2. Detection Reagent B was diluted 100-fold with Diluent B and mixed thoroughly.

Assay Protocol

All standards, samples and reagents were prepared as directed above. All data points were measured in duplicate with a standard curve produced for each 96 well plate.

1. Standard, test sample and control (zero) samples were aliquoted onto the pre-coated plate respectively, and their positions mapped. The solution was added to the bottom of each well without touching the side walls.

2. 50 µl of the diluted standards were pipetted into the standard wells.

3. 50 µl of Standard Diluent buffer were pipetted into the control (zero) well.

4. 50 µl of appropriately diluted serum was aliquoted into the test sample wells. The plate was gently agitated with a plate-shaker.

5. 50 µl of Detection Reagent A working solution was immediately aliquoted to each well and agitated with a microplate shaker. The solution turned purple. The plate was covered with a plate sealer and incubated for 45 mins at 37°C.

6. The cover was removed, and the solution discarded. The plate was washed three times with Wash Buffer, filling each well completely with Wash Buffer (350 µl) using a multi-channel Pipette allowing 1-2 mins soaking period. After the final wash, any remaining Wash Buffer was removed by aspirating or decanting. The plate was inverted and blotted against clean absorbent paper towels.

7. 100 µl of Detection Reagent B working solution was aliquoted to each well. The plate was sealed and incubated for 30 mins at 37°C.

8. The cover was removed, and the solution discarded. Then the wash process described above was repeated five times.

9. 90 µl of TMB Substrate was aliquoted into each well in a dark space to avoid light contamination, the solution turned from purple to yellow. The plate was covered with the plate sealer and mixed thoroughly with the plate-shaker. Then it was incubated at 37°C for 20 minutes.

10. 50 µl of Stop Solution was aliquoted into each well as per manufacturer's instructions this was done rapidly to ensure enzyme reaction cessation in all wells within a reasonable time.

11. The OD (True absorbance) was measured at 450 nm immediately using an ELISA reader.

The standard curve below is typical of the readouts given:

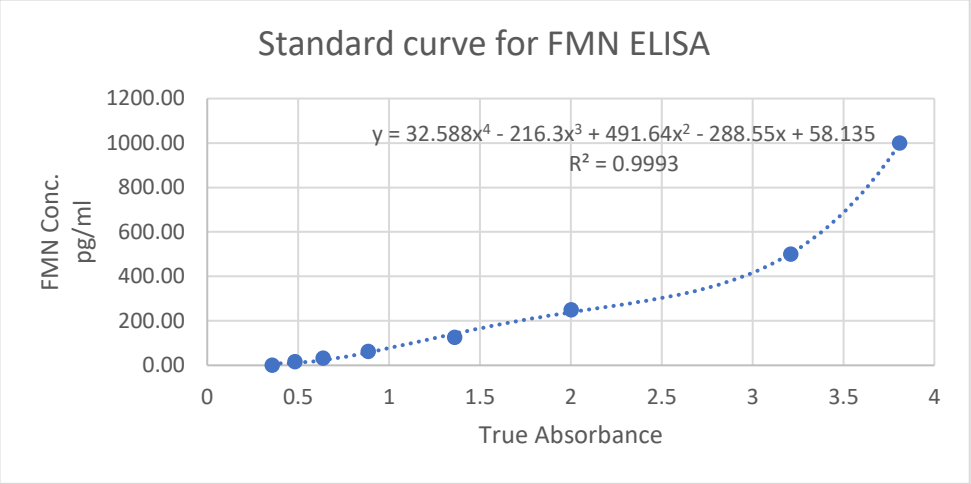

Figure 14: Standard curve used to convert absorbance data from digital calorimetry into a concentration.

The formula produced by the line of best fit is then used to convert the OD to the concentration detected. The above method is reproduced from Abbexa, Ltd, Cambridge, England[126].

2.11.2 Syndecan-1

Syndecan-1 analysis was performed using the commercially available ELISA (Abbexa Ltd, Cambridge, England). Samples were taken during each experiment and centrifuged at 2500 RCF for 15 minutes, they were then stored at -80◦C until

analysis took place. All serum samples were then thawed and left to stand at room temperature for at least 1 hour.

A standard curve was produced with the following dilutions:

10ng/ml	0.63ng/ml
5ng/ml	0.31ng/ml
2.5ng/ml	0.16ng/ml
1.25ng/ml	

Wash Buffer: The concentrated wash buffer was diluted 25-fold with distilled water (i.e. add 30 ml of concentrated wash buffer into 720 ml of distilled water). The wash buffer was warmed to room temperature to avoid crystal formation.

Detection Reagent A: Was prepared no more than 1 hr before the experiment. 1. Calculate the total volume of working solution required. 2. Dilute Detection Reagent A 100-fold with Diluent A and mix thoroughly. Pipette with a slow, smooth action to reduce volume errors.

Detection Reagent B: Prepare no more than 30 mins before the experiment. 1. Calculate the total volume of working solution required. 2. Dilute Detection Reagent B 100-fold with Diluent B and mix thoroughly. Pipette with a slow, smooth action to reduce volume errors.

Assay Protocol:

All standards, samples and reagents were prepared as directed above. All data points were measured in duplicate with a standard curve produced for each 96 well plate.

1. Standard, test sample and control (zero) were marked onto the pre-coated plate respectively, and their positions mapped. The solution was added to the bottom of each well without touching the side walls.
2. 100 µl of the diluted standard was aliquoted into the standard wells.
3. 100 µl of Standard Diluent buffer was aliquoted into the control (zero) well.

4. 100 µl of appropriately diluted sample was aliquoted into the test sample wells. A microplate shaker was used to mix together.
5. The plate was covered with a plate sealer and incubated for 90 mins at 37°C.
6. The cover was removed, and the liquid discarded.
7. 100 µl of Detection Reagent A was aliquoted to each well, turning the solution pale blue. The plate was covered with a plate sealer and incubated for 1 hr at 37°C.
8. The cover was removed, and the solution discarded. The plate was washed 3 times with 1X wash buffer. Each well was filled completely with wash buffer (350 µl) using a multi-channel Pipette. The plate was inverted and blotted against a clean absorbent paper towel.
9. 100 µl of Detection Reagent B was aliquoted to each well. The plate was sealed and incubated for 30 mins at 37°C.
10. The cover was removed, and the solution discarded. The wash process was repeated 5 times as described above.
11. 90 µl of TMB Substrate was aliquoted into each well, turning the solution yellow. The plate was covered with the plate sealer, mixed thoroughly, and incubated at 37°C for 10-20 minutes.
12. 50 µl of Stop Solution was aliquoted into each well.
13. The OD was measured at 450 nm immediately.

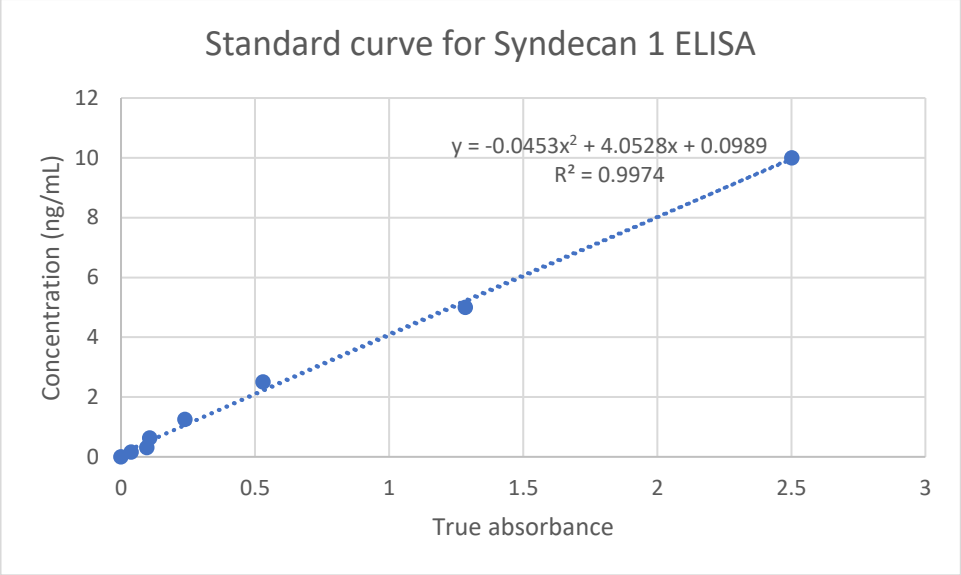

Figure 15: Standard Curve for Syndecan 1 ELISA

2.11.3 Caspase-3

Caspase-3 analysis was performed using the commercially available ELISA (Abbexa Ltd, Cambridge, England). Samples were taken during each experiment and centrifuged at 2500 RCF for 15 minutes they were then stored at -80◦C until analysis took place. All serum samples were then thawed and left to stand at room temperature for at least 1 hour.

A standard curve was produced with the following dilutions:

10ng/ml	0.63ng/ml
5ng/ml	0.31ng/ml
2.5ng/ml	0.16ng/ml
1.25ng/ml	

Wash Buffer: The concentrated wash buffer was diluted 25-fold with distilled water. It was warmed and mixed gently to avoid crystal formation.

Detection Reagent A: Was prepared no more than 1 hr before the experiment. Detection Reagent was diluted A 100-fold with Diluent A and mixed thoroughly.

Detection Reagent B: Was prepared no more than 30 mins before the experiment. Detection Reagent B was diluted 100-fold with Diluent B and mixed thoroughly.

Assay Protocol

All standards, samples and reagents were prepared as directed above. All data points were measured in duplicate with a standard curve produced for each 96 well plate.

1. Standard, test sample and control (zero) were marked onto the pre-coated plate respectively, and their positions mapped. The solution was added to the bottom of each well without touching the side walls.
2. 100 µl of the diluted standard was aliquoted into the standard wells.
3. 100 µl of Standard Diluent buffer was aliquoted into the control (zero) well.
4. 100 µl of appropriately diluted sample was aliquoted into the test sample wells. A microplate shaker was used to mix together.
5. The plate was covered with a plate sealer and incubated for 90 mins at 37°C.
6. The cover was removed, and the liquid discarded.
7. 100 µl of Detection Reagent A was aliquoted to each well. The plate was covered with a plate sealer and incubated for 1 hr at 37°C.
8. The cover was removed, and the solution discarded. The plate was washed 3 times with 1X Wash Buffer. Filling each well completely with Wash buffer (350 µl) using a multi-channel pipette. After the final wash, any remaining Wash Buffer was removed by inverting the plate and blotting it against clean absorbent paper towels.
9. 100 µl of Detection Reagent B was aliquoted to each well, turning the solution pale blue. The plate was sealed and incubated for 30 mins at 37°C.
10. The cover was removed, and the solution discarded. The wash process was repeated 5 times.
11. 90 µl of TMB Substrate was aliquoted into each well. The plate was covered with the plate sealer and incubated at 37°C for 10-20 minutes.
12. 50 µl of Stop Solution was aliquoted into each well turning the solution yellow.
13. The OD was measured immediately at 450 nm.

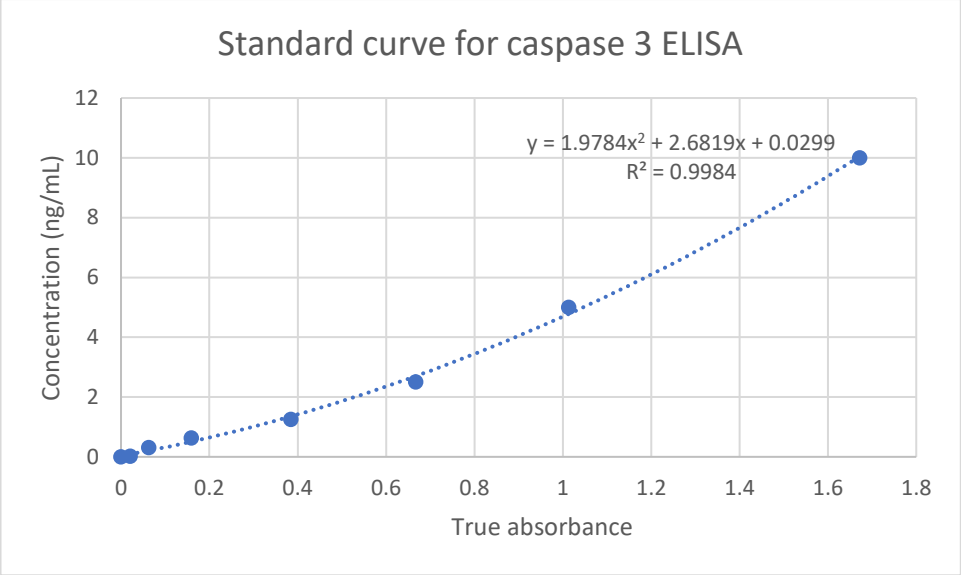

Figure 16: Standard curve for Caspase 3 ELISA

2.11.4 HMBG-1

HMBG-1 analysis was performed using the commercially available ELISA (Abbexa Ltd, Cambridge, England). Samples were taken during each experiment and centrifuged at 2500 RCF for 15 minutes they were then stored at -80◦C until analysis took place. All serum samples were then thawed and left to stand at room temperature for at least 1 hour.

A standard curve was produced with the following dilutions:

1000pg/ml	62.5pg/ml
500pg/ml	31.3pg/ml
250pg/ml	15.6pg/ml
125pg/ml	

Wash Buffer: The concentrated wash buffer was diluted 25-fold with distilled water (i.e. add 30 ml of concentrated wash buffer into 720 ml of distilled water). The wash buffer was warmed to room temperature to avoid crystal formation.

Detection Reagent A: Was prepared no more than 1 hr before the experiment. 1. Calculate the total volume of working solution required. 2. Dilute Detection Reagent A 100-fold with Diluent A and mix thoroughly. Pipette with a slow, smooth action to reduce volume errors.

Detection Reagent B: Prepare no more than 30 mins before the experiment. 1. Calculate the total volume of working solution required. 2. Dilute Detection Reagent B 100-fold with Diluent B and mix thoroughly. Pipette with a slow, smooth action to reduce volume errors.

Assay Protocol:

All standards, samples and reagents were prepared as directed above. All data points were measured in duplicate with a standard curve produced for each 96 well plate.

1. Standard, test sample and control (zero) were marked onto the pre-coated plate respectively, and their positions mapped. The solution was added to the bottom of each well without touching the side walls.
2. 100 µl of the diluted standard was aliquoted into the standard wells.
3. 100 µl of Standard Diluent buffer was aliquoted into the control (zero) well.
4. 100 µl of appropriately diluted sample was aliquoted into the test sample wells. A microplate shaker was used to mix together.
5. The plate was covered with a plate sealer and incubated for 1 hr at 37°C.
6. The cover was removed, and the liquid discarded.
7. 100 µl of Detection Reagent A was aliquoted to each well. The plate was covered with a plate sealer and incubated for 1 hr at 37°C.
8. The cover was removed, and the solution discarded. The plate was washed 3 times with wash buffer. Each well was filled completely with wash buffer (350 µl) using a multi-channel pipette after the final wash, any remaining wash buffer was removed by inversion of the plate and blotting it against clean absorbent paper towels.
9. 100 µl of Detection Reagent B was aliquoted to each well. The plate was sealed and incubated for 30 mins at 37°C.

10. The cover was removed, the solution discarded, and the wash process was repeated as described above, 5 times.
11. 90 µl of TMB Substrate was aliquoted into each well. The solution then turned from clear to pale blue. The plate was sealed with the plate sealer and mixed thoroughly. It was then incubated at 37°C for 10-20 minutes.
12. 50 µl of Stop Solution was aliquoted into each well, turning the solution yellow.
13. The OD was measured at 450nm immediately.

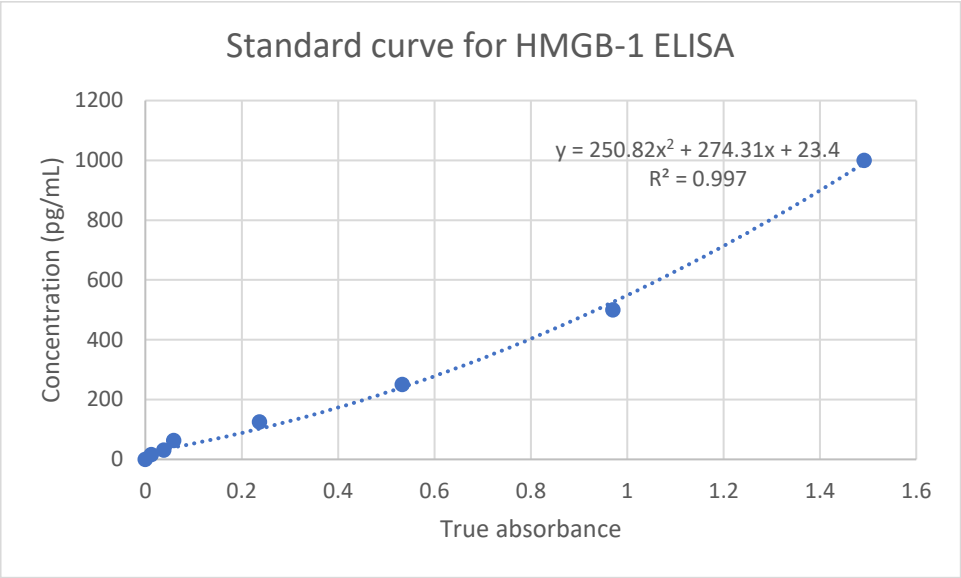

Figure 17: Standard curve for HMGB ELISA

2.12 Blood Gas analysis

Blood gas analysis was performed under standard conditions with paired samples taken from the simulated venous and arterial vasculature. Samples were processed within 5 minutes of collection using a table-top analyser (EPOC, Blood Analysis System, Epocal Inc. Wakely Canada). This was calibrated to veterinary values and blood samples were maintained at 39◦C.

2.13 Histology

From each organ a pre and post NMP biopsy was taken, randomly before perfusion and then in an area showing successful perfusion (visual evidence of blood flow) following NMP. A further biopsy was taken from those organs undergoing HOPE treatment after the HOPE procedure but prior to NMP. The staining was performed by pathology technicians at Leicester Royal Infirmary using the following protocols. In the interests of time and slide quality it was decided that the production of slides by a trained pathology technician would allow the primary researcher more time to train in their interpretation with less margin for error caused by sub-optimally produced slides.

2.13.1 Haemotoxylin and Eosin (H+E) livers

As is standard for liver biopsies all slides were stained for H+E this allowed us to identify cell types, (neutrophils, hepatocytes, endothelial cells and erythrocytes) in the biopsied sections. This stain also allowed us to assess for the degree of vacuolisation and coagulation necrosis (see histology results section for images).

Protocol

1. Slides were treated using a Leica stainer ST5010. The stainer was filled with reagents.
2. Stainer programme: Prog. 1 FFPE H&E stain was selected
3. The slide rack was placed into the loading drawer
4. The slide rack was removed and place into the xylene mount container
5. The slides were mounted with DPX under the extraction hood and allowed to air dry before re-use.

Stainer running order:

1. Oven
2. Station 1. Xylene

3. Station 2. Xylene
4. Station 3. 99% IMS
5. Station 4. 99% IMS
6. Station 5. 95% IMS
7. Wash Station 1
8. Station 12. Mayer's Haematoxylin
9. Wash Station 2
10. Station 11. Eosin (500ml + 300Acetic acid)
11. Wash Station 3
12. Station 14. 95% IMS
13. Station 15. 99% IMS
14. Station 16. 99% IMS
15. Station 17. Xylene
16. Station 18. Xylene

2.13.2 Periodic Acid-Schiff (PAS) and reticulin liver sections

PAS

The PAS stain allows quantification of tissue glycogen before and after NMP, the literature states that higher levels of glycogen depletion during SCS suggest increased severity of IRI [127]. Furthermore higher tissue levels of intra-cellular glycogen following NMP indicate better functional outcomes in an NMP model[128]. With this stain we aim to examine whether preservation with LS-A allows maintenance of glycogen levels in SCS or if stores accumulated during re-perfusion.

Protocol

1	Take slides to water. Rinse in distilled water	
2	Treat the Diastase section with saliva	30mins
3	Leave the PAS only section in distilled water	
4	Wash in running tap water	5mins
5	Rinse all slides in distilled water	

6	1% Periodic acid	10mins
7	Rinse in distilled water	
8	Schiff's Reagent	20mins
9	Wash in running tap water	10mins
10	Counterstain in Mayer's Haematoxylin	30secs
11	Wash in running tap water	5mins
12	Dehydrate, clear and mount in DPX	

Results

Neutral mucins	positive = pink staining
Glycogen	positive= pink staining in PAS only
Nuclei	blue

Reticulin

The reticulin special stain examines the reticulin skeleton of the hepatocyte plates, it is more commonly used in characterisation of hepatic tumours and areas of necrosis and fibrosis in other liver pathologies but shows characteristic breakdown in fulminant hepatitis of all aetiologies[129].

Protocol

1. Slides were racked and placed onto the Leica XL Stainer on programme 4.
2. Slides were treated with Potassium Permanganate solution (bottle A in Kit) for 5 minutes
3. Then washed off in water and Bleach with Oxalic Acid Solution (bottle B in Kit) for 1 minute.
4. Slides were washed well in running water.
5. Then rinse in distilled water and treated with Iron Alum (bottle C in Kit) for 15 minutes.

6. Slides were washed in several changes of distilled water.
7. Then treated with Ammoniacal Silver Stain (bottle D in Kit) for 2 minutes
8. Slides were washed well in several changes of distilled water.
9. Then reduced in 10% formalin and agitated for 2 minutes.
10. Slides were washed in water then, treated with Sodium Thiosulphate (bottle E in Kit) for 5 minutes.
11. Slides washed in water and counterstained with Van Gieson for 5 minutes.
12. Finally, they were rinsed in 99% IMS dehydrate, cleared and mounted in DPX.

Treated slides display a clear black cytoskeleton in healthy tissues but in necrosis/fulminant hepatitis go onto fracture and condense.

2.14 Bile analysis

A running total of bile volume was measured each hour by expressing the bile into a measuring cylinder and noting its volume at each hour measurement point. Cumulative bile volume was calculated at the end of the perfusion. Bile was inspected for blood staining and clarity. Bile pH was measured at room temperature using a digital pH meter (HI8520, HANNA Instruments LTD, Leighton-Buzzard, UK)

3.0 Statistical analysis, randomisation, and blinding

3.1 Sample size calculation.

The sample size calculation was undertaken to examine the link between perfusate lactate over the course of the perfusion in a DBD model. Other variables were examined as secondary outcomes such as perfusate potassium, bile flow and pH, LFTs and lactate dehydrogenase. This study was designed in a way expecting there to be some variation in lactate in the LS-A (intervention group) but that the mean should sit close to the viability value of 2.5mmol/L. Large effect sizes between intervention and control group were expected as from previous unpublished data livers preserved in UW or IGL-1 often fail under these conditions; as shown in the methods development sections failing livers produce climbing or static lactate levels.

3.1.1 Power Analysis Table

			Test Assumptions			
	N[b]	Actual Power	Power	Std. Dev.	Effect Size	Sig.
Overall Test[a]	18	.883	.8	3	1.110	.05

Table 7: Results of sample size calculation

3.1.2 Group Size Allocation for Overall Test

	N
LS-A	6
IGL-1	6
UW	6
Overall	18

Table 8: Group size to meet desired power.

3.1.3 Effects of the 3R's of animal research on sample size

Although the study eventually conducted is from a DCD model, the pre-collection analyses were done with the view of a DBD study. This meant that the 3R's (Replacement, Refinement and Reduction) were accounted for when estimating meaningful effect sizes and designing the study accordingly. I therefore purposefully aimed for large effect sizes with the expectation that most of the control group livers would show significant functional abnormalities i.e. would have perfusate lactates with means 1-2 standard deviation away the intervention group mean. When funding and logistical restrictions meant that the DBD study could no longer be performed the data collection was performed under the assumption that the severity of IRI in the DCD model would be of a similar or greater magnitude and thus similar levels of perfusate lactate would be detected across groups.

3.2 Statistical analysis

SPSS version 28. (Released 2021, IBM, Chicago, USA) was used for all statistical analysis. The Shapiro-Wilk test assessed for normal distribution in all datasets due to small sample sizes. Unless otherwise stated parametric data is displayed as means with standard error of the mean and non-parametric data is displayed as medians with inter-quartile ranges.

Individual organ curves were produced whilst developing the model, with the intention of having a baseline 'normal' function with which to compare subsequent comparative studies under harsher test conditions. The initial perfusions described in the method development section in fact served as a good baseline for what a failed perfusion looks like in terms of its functional parameters.

Grouped curves were produced for parameters of interest in comparison studies, this was done to give an idea about any potential relationship between individual time points and to see if 'threshold' values were present. All computed standard

error bars display +/- standard error of the mean and were calculated from individual time points, therefore any crossing of said bars does not discount the described statistical significance of the trend, although it does convincingly show how single time point measures in this model can introduce significant errors.

Multivariate analysis was undertaken using the MANOVA technique to account for repeated measures and comparing between group means. Those variables of interest displaying statistical significance in the MANOVA then underwent post hoc Tukey's honestly significant difference (HSD) pairwise testing. This was applied to ANOVAs with three or more groupings in the nominal independent variable. This gave significance readings based on dependent variable of interest and the effects of perfusion time, for more detailed comparison of independent variable effects. This technique is considered to have greater statistical power than the individual T-tests and as such their significance value is shown when describing the data.

Cumulative measures i.e. perfusion total bile pH or post-perfusion histological grading were performed using a standard ANOVA approach as the effect of time had already been controlled for within the sample. Histology was analysed semi-qualitatively through applications of a severity grading for ischaemia, presence of glycogen stores and degradation of the cytoskeleton.

3.3 Randomisation and blinding

Animals were randomised into a preservation solution allocation using a name drawn at random from a sealed container. This was done prospectively and drawn on the day of the retrieval by an independent member of the team. It was not possible to blind the operator to the solution choice during the retrieval or perfusion as this was all completed by a single researcher (PW) and each commercial solution has different packaging. During histological assessment, the examiners were blinded to the solution used in each sample at the time of analysis by using lab assigned sample numbers instead of the experimental labels, this could not be maintained throughout subsequent statistical analysis.

3.4 Timeline of work

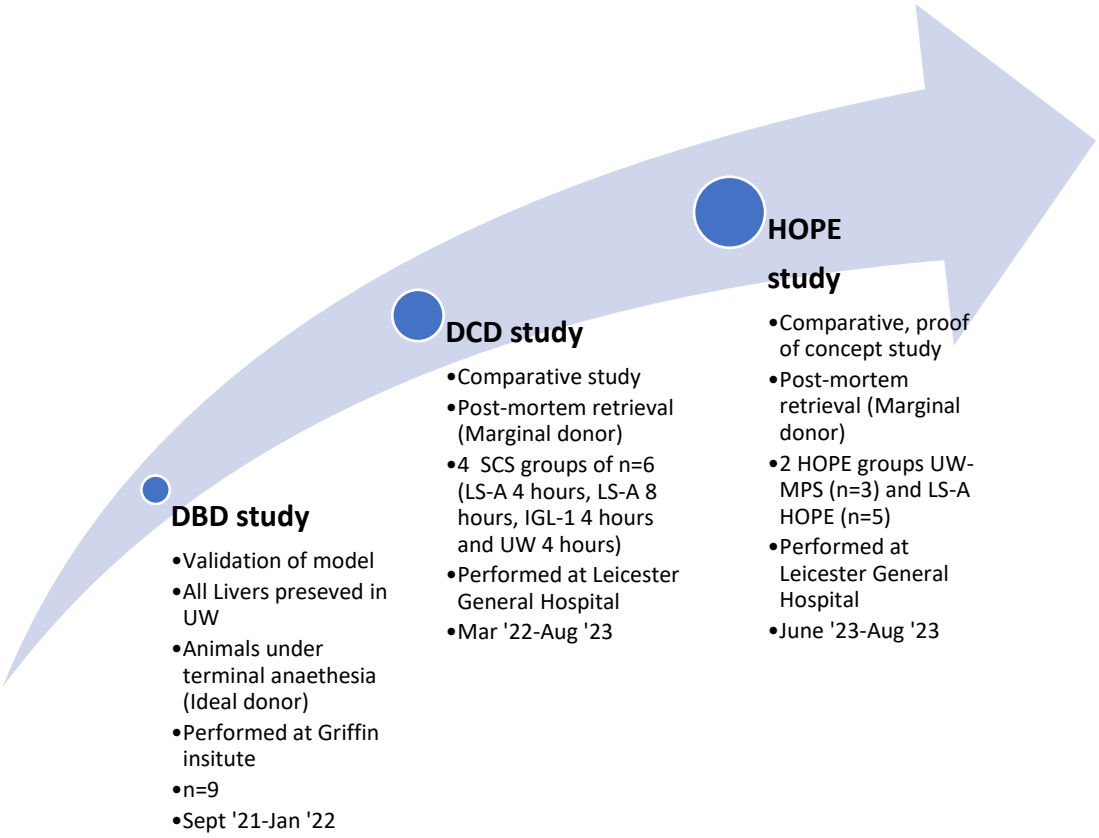

Figure 18: A time-line showing the schedule of work, where and when it was performed. The infographic also shows the groups and comparisons made.

4.0 Results

4.1 Excluded results

Results were excluded from comparative studies when they exhibited anatomical or circuit factors that were outside the role of the preservation method. These included: vascular anatomical variation precluding the perfusion of all lobes, oxygenator embolus or circuit failure.

4.1.1 Animal OP 11

Animal OP11 was randomised to the IGL-1 group, within the second hour of its perfusion there was a sudden yet partial reduction in blood flow into both the reservoir bag and the hepatic artery flow meter. Manual manometry confirmed a large pressure gradient across the oxygenator. With subsequent fall in the partial pressure of oxygen in the hepatic artery.

Several steps were taken to rectify this including pressure evacuation of the oxygenator and oxygenator exchange. This resulted in a lactate peak at 3 hours after an initial peak within the first hour, this is not a pattern that we have seen in other experiments. It is, however, often present in the clinical presentation of a pulmonary embolus and as such we could not wholly put the changes in this liver's performance down to the effect from the preservation solution and have excluded it from further analysis.

4.1.2 Animal OP13

OP 13 was randomised to LS-A. It was found to have variant anatomy during retrieval, namely a replaced right hepatic artery with origin from the SMA. In the DBD setting this can be rectified by taking an aortic patch with the feeding vessels. During the high-speed abattoir retrieval this is not a feasible option. The inflow vessel must be identified within seconds, then it is isolated and cannulated. Thus,

the liver received preservation fluid only through the coeliac part of the hepatic arterial system. On attempted reperfusion a standard protocol 10french catheter could be placed in the coeliac portion but the SMA portion would not and was cannulated with an IV canula instead. Both readings gave very reduced arterial flows at 0.07L/min and this liver was excluded.

4.1.3 Animal OP24

OP24 was assigned to the HOPE study. Working with the Leicester team I put together a cold perfusion circuit following the parameters presented in HOPE protocols[89,97,130]. As this was to provide data for a pilot study of 3 animals examining the function of LS-A during HOPE perfusion I had arranged to first complete a single perfusion using standard conditions. That is UW SCS period and UW-MPS HOPE delivery in order to confirm that this non-proprietary circuit could achieve the same results as the commercially available devices. In these papers portal only, perfusion is at 5mmHg with a flow rate of 0.25L/min. This liver did not behave in the predicted way[130,131] and had climbing lactates throughout as such we discarded it from our analysis and re-designed the HOPE study to use a commercially available device.

4.1.4 Animal OP 35

OP35 was the first animal in the HOPE pilot study to use the VITA Smart using UW SCS period and a UW-MPS HOPE period. The parameters dictated by the HOPE clinical protocol were observed and met during the hypothermic stage of the perfusion with no confounding factors. However, during the NMP stage of the experiment the oxygenation of the blood began to fall with eventual complete oxygenator failure after 2 hours. Although the oxygenator was rapidly changed, given the pattern of lactate clearance in HOPE followed by NMP experiments seen

with other groups I cannot discount the effect of the oxygenator failure as being responsible for the failure of this organ.

4.2 Comparison of LS-A with IGL-1 and UW at four hours preservation time in a DCD model

4.2.1 The schedule of work

After having established that the NMP model was able to differentiate between viable and non-viable livers in the laboratory in the Griffin Institute. The project was required to move to University Hospitals Leicester, this is detailed in 'Barriers to completion' below. This also included a change to the model from using live animals as a simulation of DBD to using immediately post-mortem animals to simulate DCD retrieval. The schedule set out 36 experiments overall to account for inevitable failures due to thrombosis or unsuitable tissues. Each experiment involved driving to Leicester, a full 18-hour day with 1-2 retrievals, storage period and NMP with clean-up afterwards. This was all performed by a single researcher, with assistance from those named in the acknowledgement section.

4.2.2 Summary of experimental conditions

This compared three groups (N=6) of DCD liver perfusions each preserved with different preservation solutions. The two comparison groups were IGL-1 and UW which are the gold standard in clinical practice in Europe and the UK/USA. The retrieval model is described as DCD as this is the closest clinical approximation to the procedure performed but the baseline quality of the livers is poorer due to severity of the IRI and the lack of systemically administered heparin. Due to the animals being used in the food chain, aortic perfusion with cold preservation solution was prohibited and organs were back-table flushed with heparinised

(12,500 units per 1L) cold preservation solution. The organs were then stored for a fixed period of 4 hours at between 0-4◦C. The NMP was performed as detailed above at a temperature of 39◦C for 4 hours.

4.2.3 Group characteristics.

		N	Mean	Std. Deviation	Significance
WIT (Mins)	LS-A	6	13:32	00:56	NS all comparisons
	UW	6	12:33	02:10	
	IGL-1	6	13:15	00:40	
Pre-perfusion weight (Kg)	LS-A	6	1.68	0.31	NS all comparisons
	UW	6	2.00	0.53	
	IGL-1	6	1.68	0.16	
Post-perfusion weight (Kg)	LS-A	6	2.37	0.47	NS all comparisons
	UW	6	2.58	0.47	
	IGL-1	6	2.15	0.12	
Change in weight. (Kg)	LS-A	6	.68	0.29	NS all comparisons
	UW	6	.58	0.15	
	IGL-1	6	.48	0.13	

Table 9:shows no baseline differences between Liver weights, before and after the perfusion and no significant difference in the mean WIT between groups.

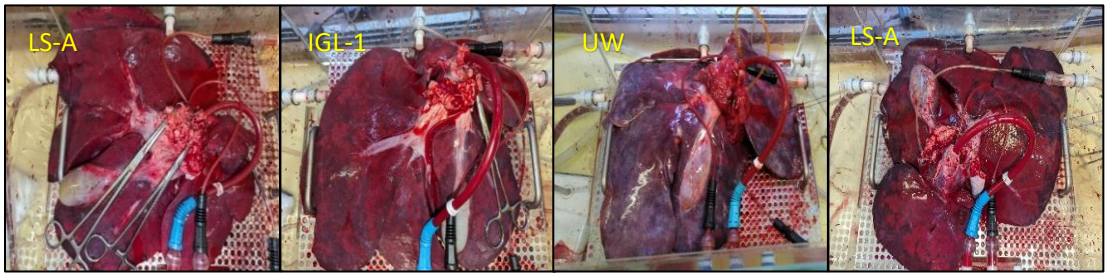

Figure 19: Typical appearance of livers within 30 minutes of starting NMP. The darker areas are areas of relative hypoperfusion.

There are macroscopic changes to the livers almost immediately evident on commencement of NMP, Figure 19 shows the typical pattern of perfusion seen with each preservation solution, with a greater degree of perfused tissue in the LS-A group compared to the others. These changes progress over time with differentiation of the darker and lighter areas.

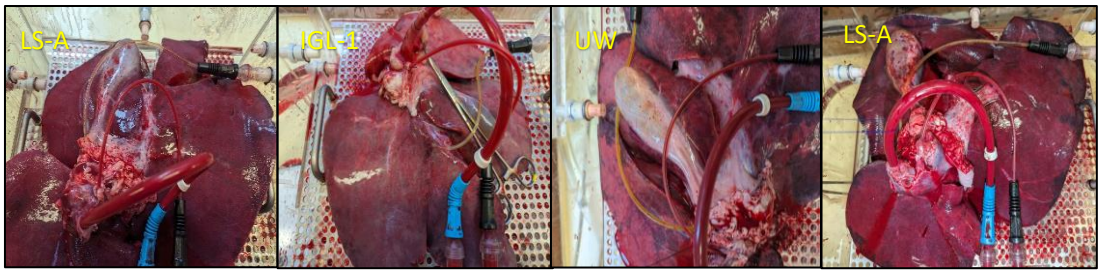

Figure 20: Livers with different preservation solutions, near the end of 4 Hours NMP (Please note the photographs are not of the same liver and have been chosen as the best quality for describing the phenomenon)

The figure above shows the progression with time of the darker areas in the liver that represent macroscopic non-perfusion, in keeping with the experimental results these are most marked in the UW preserved livers which show discrete patches of poor/non-perfusion.

4.2.4 Perfusion parameters

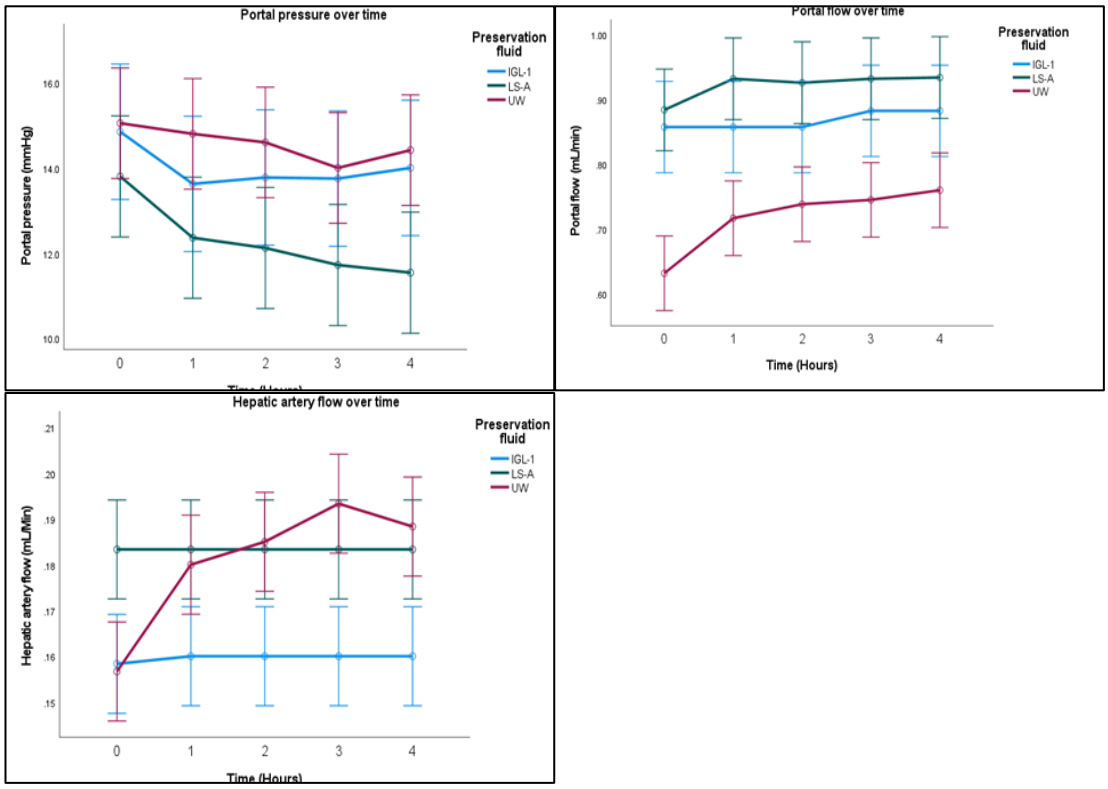

Figure 21: Graphs showing the angiodynamic changes during NMP, LS-A preserved livers show significantly higher portal flow at significantly lower pressure.

		Portal vein flow (L/min)	Sig.	Portal vein pressure (mmHg)	Sig.	Hepatic artery flow (L/min)	Sig.
LS-A 4 HOURS	UW	0.72 ± 0.08	<0.001	14.6 ± 1.1	0.003	0.181 ± 0.01	NS
	IGL-1	0.85 ±0.0.7	NS	14.0 ±1.3	NS	0.16 ± 0.01	0.003
	LS-A	0.92 ± 0.07	N/A	12.3 ±1.2	N/A	0.183 ± 0.01	N/A

Table 10: Grand means for portal flow and pressure and arterial flow.

The hepatic artery pressure was 90mmHg for all experiments. The two-way ANOVA performed looked at the effects of perfusion fluid and time for each comparison. The results shown indicate the effect attributed to perfusion fluid only and underwent Tukey's HSD post-hoc comparison. The graphs above show that there is no significant difference in hepatic artery flow between LS-A and UW groups. Portal pressure was significantly lower in LS-A group than in the UW. There were no significant differences found when comparing other groups for portal pressure. Portal flow was significantly higher in LS-A group than the UW group. There was no other significant difference in portal flows. The overall trend suggests livers preserved in LS-A had a higher portal flow at a lower pressure.

4.2.5 Perfusate blood gases

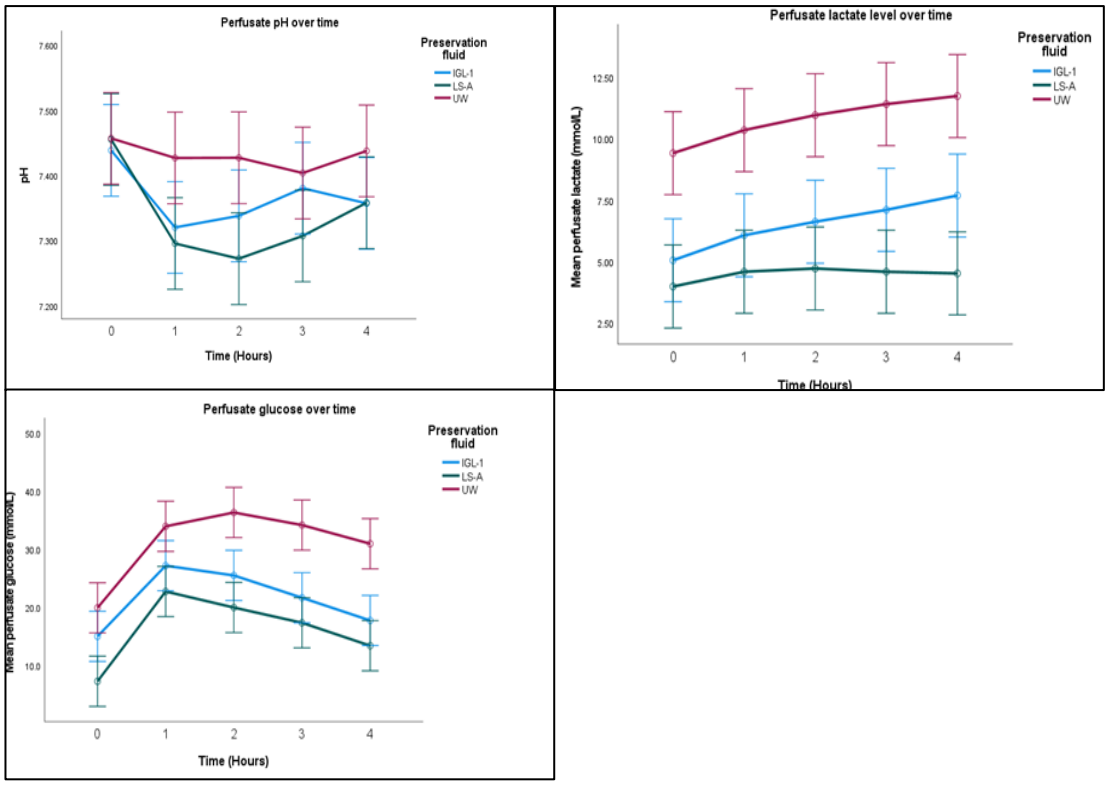

Figure 22: Graphs showing perfusate pH, lactate, and glucose. LS-A shows significantly lower lactate and glucose with no significant differences in pH.

		Perfusate pH	Sig.	Perfusate lactate (mmol/L)	Sig.	Perfusate glucose (mmol/L)	Sig.
LS-A 4 HOURS	UW	7.43 ± 0.07	NS	10.77 ± 0.4	<0.001	28.3 ±1.1	<0.001
	IGL-1	7.37 ± 0.06	NS	6.50 ±0.41	NS	20.9 ± 1.0	0.137
	LS-A	7.34 ± 0.08	N/A	4.48 ± 0.41	N/A	16.2 ± 1.0	N/A

Table 11: Grand means for perfusate pH, lactate, and glucose

The two-way ANOVA shows no significant differences between groups with regards to arterial pH during the perfusions. The perfusate lactate was significantly higher in the UW group than both the LS-A and IGL-1, there was no significant difference between LS-A and IGL-1. Figure 27 above breaks down the trend of perfusate lactate over time. The UW group had significantly higher glucose than both LS-A and IGL-1 but there were no significant differences between these two groups.

4.2.6 Perfusate Biochemistry

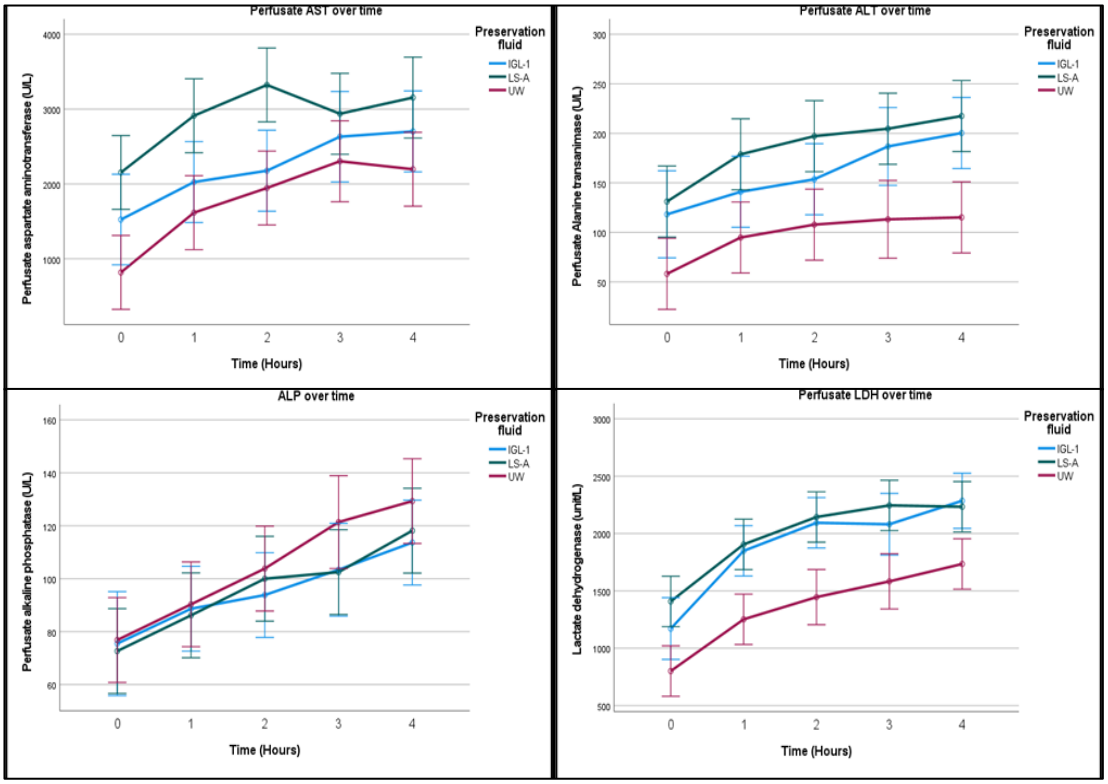

Figure 23: Graphs showing LFTs and LDH for preservation with different solutions after 4 hours SCS.

		Perfusate AST (IU/L)	Sig.	Perfusate ALT (IU/L)	Sig.	Perfusate ALP (U/L)	Sig.
LS-A 4 HOURS	UW	1754±582	<0.001	87.9±32.4	<0.001	118.9±15.9	0.01
	IGL-1	2212±561	NS	143.2±31.2	NS	91.3±14.5	NS
	LS-A	2896.5 ± 517	N/A	166±28.8	N/A	94.2±13.3	N/A

Table 12: Grand means for AST, ALT, and ALP

		Perfusate LDH (U/L)	Sig.
LS-A 4 HOURS	UW	1434.9±218	<0.001
	IGL-1	1764.3±210	NS
	LS-A	1935.7±194	N/A

Table 13: Grand mean for LDH

There is no significant difference in perfusate LFTs or LDH when comparing LS-A with IGL-1. UW has significantly lower levels of ALT, AST and LDH when compared with LS-A. ALP shows no significant differences. In the context of the Figure 19 above this is due to the volume of hepatocytes perfused rather than reflecting a true quantification of liver damage.

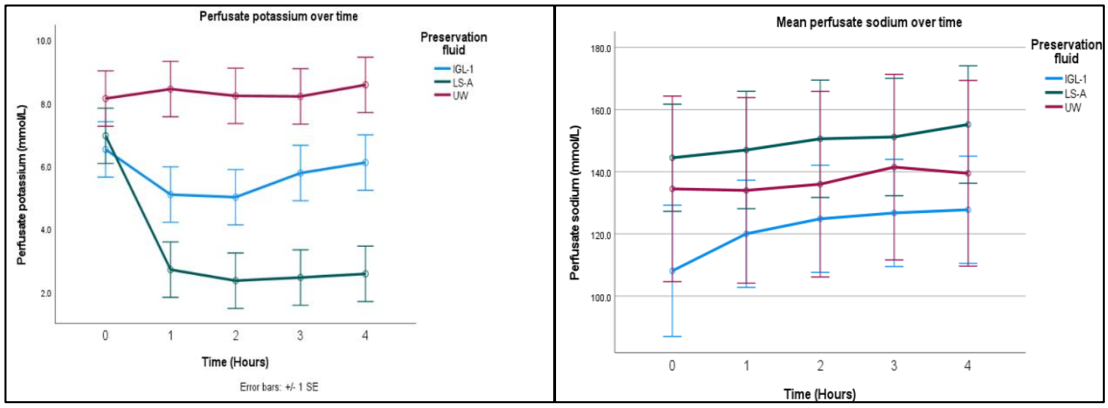

Figure 24: Graphs showing perfusate potassium and sodium concentrations for different preservation solution. LS-A shows significant hypokalaemia, this is surprising as liver NMP usually exhibits hyperkalaemia.

		Perfusate potassium (mmol/L)	Sig.	Perfusate sodium (mmol/L)	Sig.
LS-A 4 HOURS	UW	8.7±0.9	<0.001	139.4±3.2	<0.001
	IGL-1	5.0±0.8	0.009	147.6±3.1	NS
	LS-A	3.5±0.75	N/A	151.4±2.78	N/A

Table 14: Table showing grand means for perfusate potassium and sodium

LS-A shows significantly lower potassium levels than both UW and IGL-1, UW also has significantly lower sodium levels than LS-A.

4.2.7 Bile production and pH

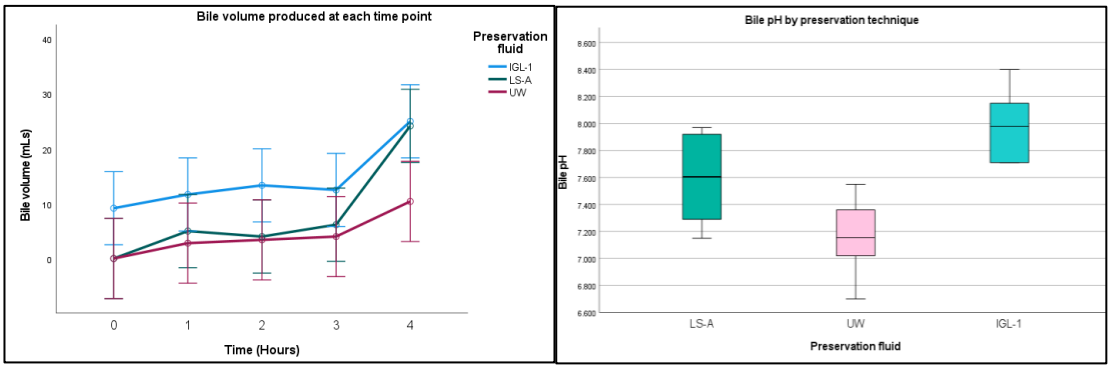

Figure 25: Graphs showing bile volume and pH.

		Bile volume (mL/hour)	Sig.	Bile pH	Sig.
LS-A 4 HOURS	UW	4.2±5.5	NS	7.162±0.24	NS
	IGL-1	17.01±5.4	NS	7.992±0.27	NS
	LS-A	7.1±2.8	N/A	7.59±0.24	N/A

Table 15: Mean bile production per hour and pH

There were no significant differences between volume of bile produced between any of the groups at any point during the perfusion. There were no significant differences between LS-A and IGL-1 or between LS-A and UW in terms of bile pH.

4.3 Comparison of LS-A extended preservation with standard UW and IGL-1 in a DCD model

As none of the gold standard groups reached the lactate threshold of <2.5mmol/L within the 4-hour perfusion there was little point in conducting further experimental long preservation studies in those groups therefore the LS-A long preservation group was compared to the pre-existing control groups. The labels LS-A 4hr and LS-A 8hr have been used to denote groups that underwent 4- or 8-hour SCS preservation with LS-A respectively.

4.3.1 Group characteristics

		N	Mean	Std. Deviation	Significance
WIT (Mins)	LS-A 4hr	6	13:32	00:56	All comparisons NS
	UW	6	12:33	02:10	
	IGL-1	6	13:15	00:40	
	LS-A 8hr	6	11:15	01:58	
Pre-perfusion weight (Kg)	LS-A 4hr	6	1.68	0.31	All comparisons NS
	UW	6	2.00	0.53	
	IGL-1	6	1.68	0.16	
	LS-A 8hr	6	1.75	0.22	
Post-perfusion weight (Kg)	LS-A 4hr	6	2.37	0.47	All comparisons NS
	UW	6	2.58	0.47	
	IGL-1	6	2.15	0.12	
	LS-A 8hr	6	2.27	0.16	
Change in weight. (Kg)	LS-A 4hr	6	0.68	0.29	All comparisons NS
	UW	6	0.58	0.15	
	IGL-1	6	0.48	0.13	
	LS-A 8hr	6	0.57	0.21	

Table 16: Group comparisons for extended preservation study. No significant differences seen.

The addition of the 8-hour preservation group did not change the comparisons between baseline characteristics, as previously stated there were no significant differences detected between groups.

4.3.2 Perfusion parameters

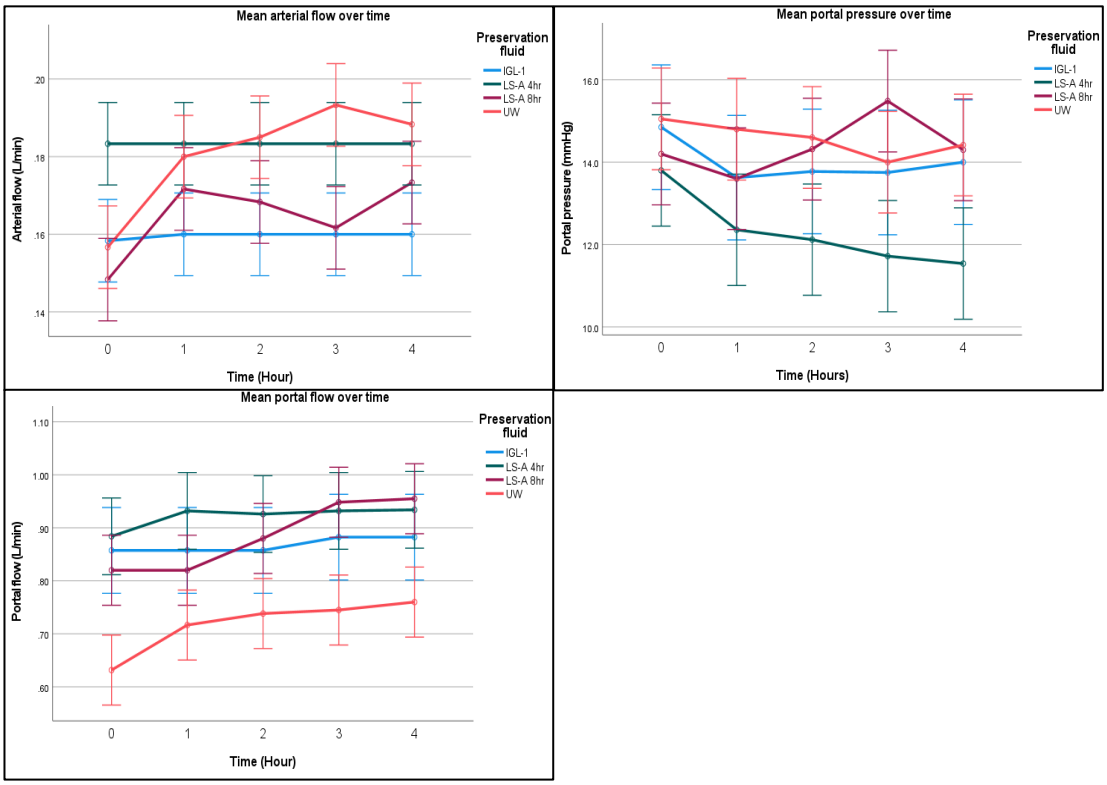

Figure 26: Graphs showing trends in angiodynamics between preservation solutions.

		Portal vein flow (L/min)	Sig.	Portal vein pressure (mmHg)	Sig.	Hepatic artery flow (L/min)	Sig.
LS-A 8 HOURS	UW	0.72 ± 0.08	<0.001	14.6 ± 1.1	NS	0.181 ± 0.01	NS
	IGL-1	0.85 ±0.0.7	NS	14.0 ±1.3	NS	0.16 ± 0.01	NS
	LS-A 4	0.92 ± 0.07	NS	12.3 ±1.2	0.03	0.183 ± 0.01	0.03
	LS-A 8	0.89±0.06	N/A	14.6 ± 1.1	N/A	0.165±0.01	N/A

Table 17: Grand means of portal pressure and flow and arterial pressure

With the addition of LS-A 8-hour preservation to the original experiments, it has shown significantly lower arterial flow than LS-A 4-hour preservation (p=0.03) but shows no other significant differences compared to the control groups. In terms of

portal flow and pressure there is significantly higher portal pressure in the LS-A 8-hour group compared to the LS-A -hour group.

4.3.3 Perfusate blood gas

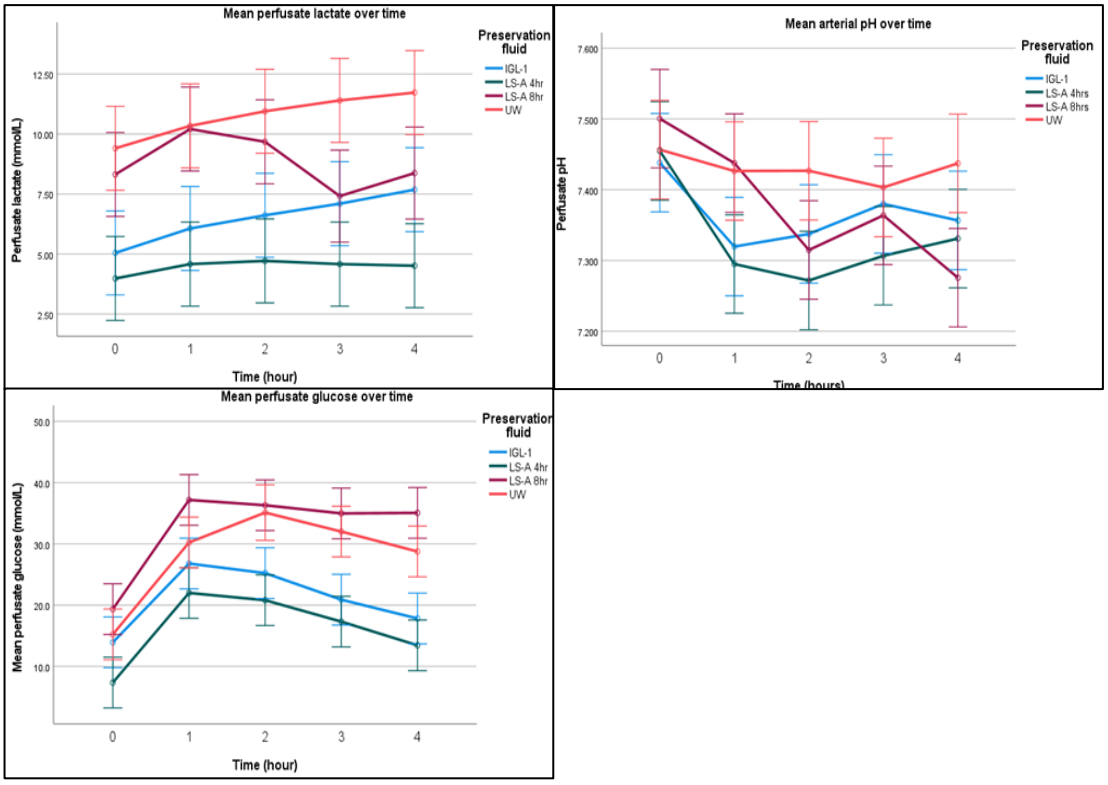

Figure 27: Graphs showing trends in perfusate lactate, arterial pH, and Glucose over time.

		Perfusate pH	Sig.	Perfusate e lactate (mmol/L)	Sig.	Perfusate glucose (mmol/L)	Sig.
LS-A 8 HOURS	UW	7.43 ± 0.07	NS	10.77 ± 0.4	NS	28.3 ±1.1	NS
	IGL-1	7.37 ± 0.06	NS	6.50 ±0.41	NS	20.9 ± 1.0	<0.001
	LS-A 4	7.34 ± 0.08	NS	4.48 ± 0.41	0.04	16.2 ± 1.0	<0.001
	LS-A 8	7.42±0.08	N/A	8.10±3.13	N/A	31.2±7.8	N/A

Table 18: Grand means of perfusate blood gases

4.3.4 Perfusate Biochemistry

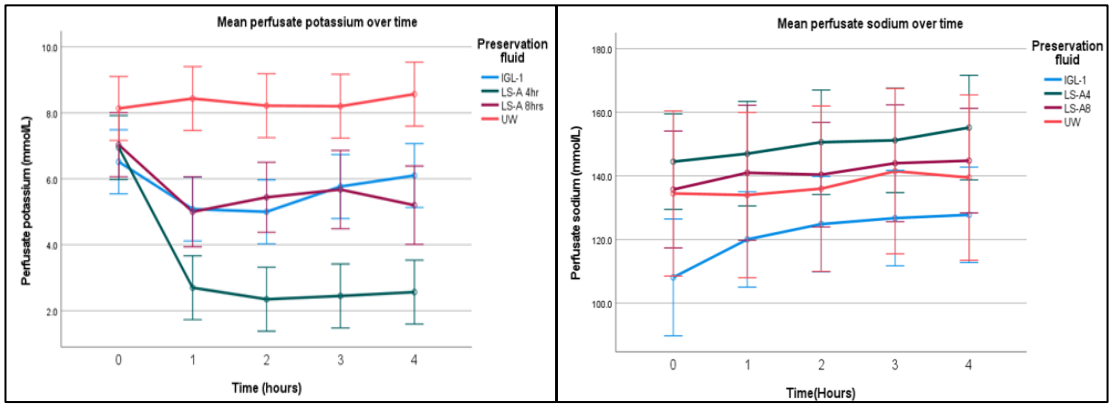

Figure 28: Graphs showing trends in sodium and potassium.

		Perfusate potassium (mmol/L)	Sig.	Perfusate sodium (mmol/L)	Sig.
LS-A 8 HOURS	UW	8.7±0.9	NS	139.4±3.2	<0.001
	IGL-1	5.0±0.8	0.002	147.6±3.1	<0.001
	LS-A 4	3.5±0.75	<0.001	151.4±2.78	NS
	LS-A 8	7.5±1.3	N/A	135.7±4.85	N/A

Table 19: Grand means of sodium and potassium

4.3.5 Perfusate LFT'S

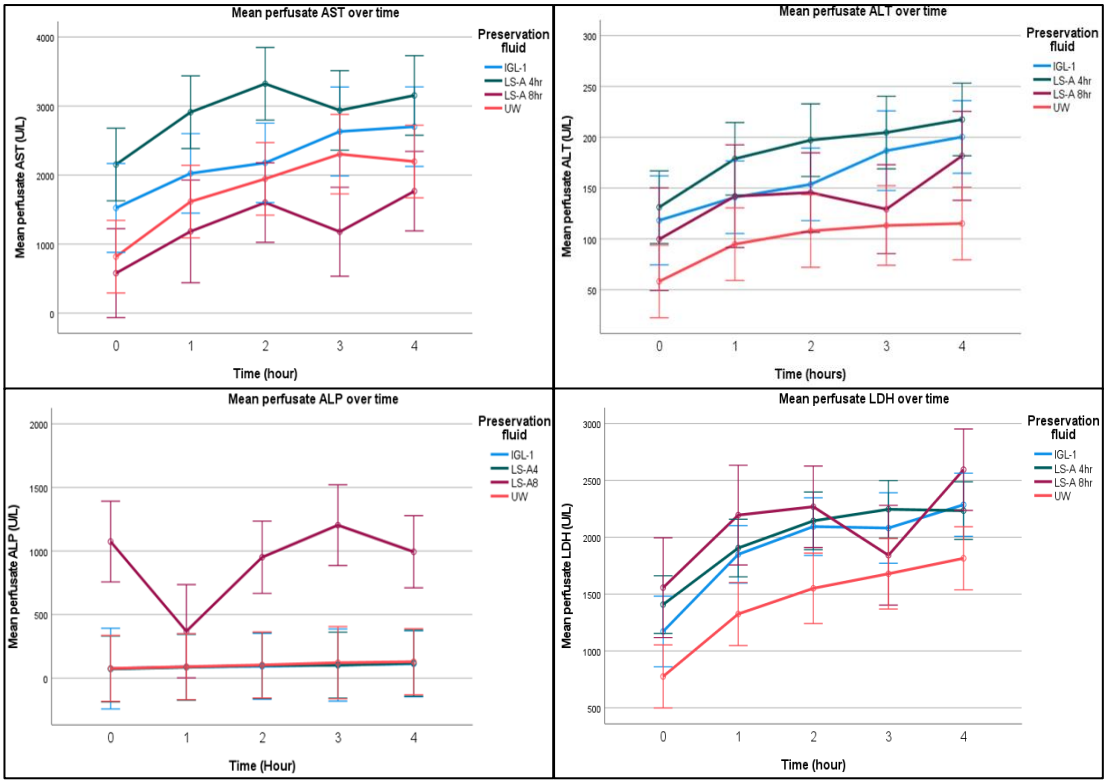

Figure 29: Graphs showing trends in perfusate LFT's over time.

		Perfusate AST (IU/L)	Sig.	Perfusate ALT (IU/L)	Sig.	Perfusate ALP (IU/L)	Sig.
LS-A 8 HOURS	UW	1754±582	NS	87.9±32.4	<0.001	118.9±15.9	0.01
	IGL-1	2212±561	NS	143.2±31.2	NS	91.3±14.5	NS
	LS-A	2896.5 ± 517	NS	166±28.8	NS	94.2±13.3	NS
	LS-A 8	1730±955	N/A	190±53	N/A	69.8±22.1	N/A

Table 20: Grand means of LFTS during perfusion

		Perfusate LDH (IU/L)	Sig.
LS-A 8 HOURS	UW	1434.9±218	0.001
	IGL-1	1764.3±210	0.13
	LS-A 4	1935.7±194	NS
	LS-A 8	2281±357	N/A

Table 21: Grand mean of LDH

4.3.6 Bile Volume and pH

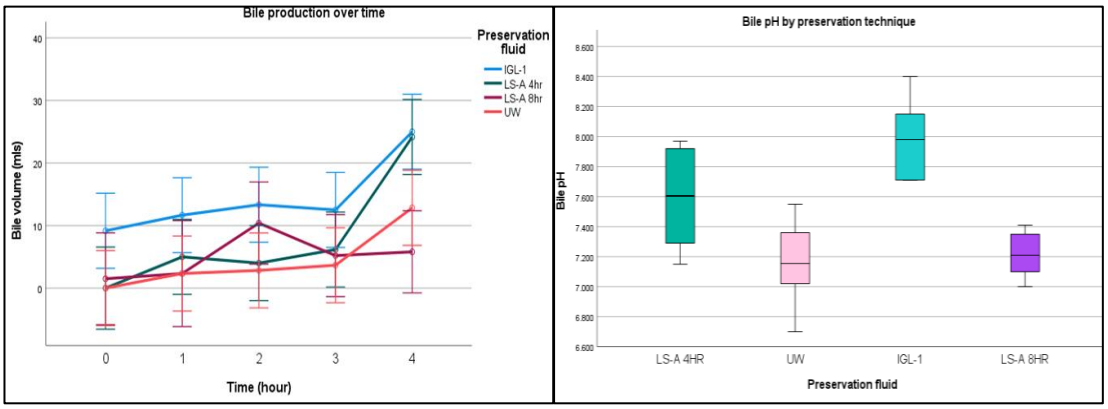

Figure 30; Graphs showing trends in bile volumes and pH.

		Perfusate bile volume(mLs)	Sig.	Perfusate bile pH	Sig.
LS-A 8 HOURS	UW	4.2±5.5	NS	7.162±0.24	NS
	IGL-1	17.01±5.4	NS	7.992±0.27	0.001
	LS-A 4	7.1±2.8	NS	7.59±0.24	NS
	LS-A 8	6.6±5.5	N/A	7.21±0.24	N/A

Table 22: Grand means of bile volume and pH

4.3.7 Comparison with VITTAL trial viability criteria

Criterion	LS-A 4hrs	IGL-1	LS-A 8hrs	UW
Lactate < 2.5mmol/l within 4 hrs	50%	0%	0% (1/6 reached 2.75)	0%
Bile production	100%	100%	100%	100%
pH ≥7.3	83.3%	50%	66.6%	100%
Glucose clearance	100%	66.6%	16.6%	50%
HA flow ≥150mL/min	100%	83.3%	83.3%	100%
PV flow ≥500mL/min	100%	100%	100%	100%

Homogenous perfusion	100%	70%	70%	0% (Large areas of hypoperfusion during NMP)
Meeting criteria set out in VITTAL trial	50%	0%	0%	0%

Table 23: Comparison of preservation solution performance with the viability criteria set out in the VITTAL trial.

The VITTAL trial set out validated viability criteria during NMP, although not universally accepted, they are the closest thing to consensus in the UK at present[113]. Here the NMP performance is compared to those criteria, to give an idea of how well the liver is functioning globally. By this definition there was no 'successful' perfusion carried out in the gold standard groups but 50% of the LS-A 4-hour livers met the viability criteria.

4.4 Proof of principle study using LS-A as HOPE perfusate.

4.4.1 Summary of experimental conditions

This compared two groups (UW-MPS n=3 and LS-A n=5) of DCD liver perfusions. The group sizes are small and unequal due to the lack of availability of UW SCS solution and the limited time frame in which to perform these experiments dictated by the rental agreement of the Vita smart device. The same retrieval procedure was followed as for the 4-hour and 8-hour preservation experiments. The SCS time was kept fixed at two hours to allow adequate time for organ transport and set up of the VitaSMART device. Subsequently organs underwent HOPE with a target portal flow of 250mls/min and max pressure of 5mmHg with dissolved oxygen content above 50Kpa at between 6-10◦C in keeping with the clinical protocol. This procedure was maintained for a further 2 hours at which point the liver was transferred directly to the NMP device following the conditions set out in the previous experiments.

4.4.2 Group characteristics.

		N	Mean	Std. Dev.	Sig.
WIT (Mins)	UW-MPS	3	12:31	00:02	NS
	LSA	5	11:53	01:45	
Post-perfusion weight (Kg)	UW-MPS	3	2.3667	.68069	NS
	LSA	5	1.8800	.32711	
Weight gain (Kg)	UW-MPS	3	.5000	.20000	NS
	LSA	5	.5600	.25100	
Pre-perfusion weight (Kg)	UW-MPS	3	1.8333	.64291	NS
	LSA	5	1.3200	.20494	
Post-HOPE weight (Kg)	UW-MPS	3	1.9333	.64291	NS
	LSA	5	1.3600	.25100	
HOPE portal pressure (mmHg)	UW-MPS	3	1.0000	.00000	P=0.038
	LSA	5	3.4000	1.51658	
HOPE Pa02	UW-MPS	3	65.6667	12.89703	NS
	LSA	5	61.0800	12.48327	
HOPE Flow (ml/min)	UW-MPS	3	250.0000	.00000	NS
	LSA	5	220.0000	67.08204	

Table 24: Group characteristics showing no significant differences in all categories aside from a higher-pressure during HOPE for the LS-A group.

There were no significant differences in all baseline characteristics during the HOPE perfusions aside from the portal pressure being significantly higher in the LS-A group(p=0.038). This reflects the small sample size as a single perfusion reaching the max acceptable perfusion pressure has skewed the data somewhat, this is not believed to have had a large effect on the results as at all times both the mean and the individual perfusion adhered to the HOPE perfusion protocol. In terms of LS-A function it is an important comparison that the dissolved oxygen concentration shows no significant difference with UW-MPS, supporting is use as a HOPE perfusate.

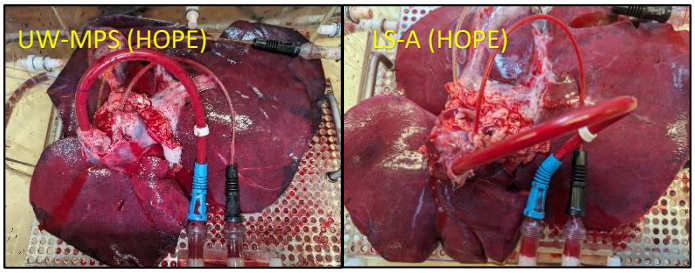

Figure 31: Photographs comparing tissue perfusion during NMP following HOPE with UW-MPS and LS-A

As in the previous experiment Figure 31 shows the overall perfusion of the liver within 30 minutes of starting NMP and figure 32 shows it after 4 hours. In comparison to the SCS images all livers have considerably more homogenous perfusion and display a greater extent of perfusion at the same stage in the experiment.

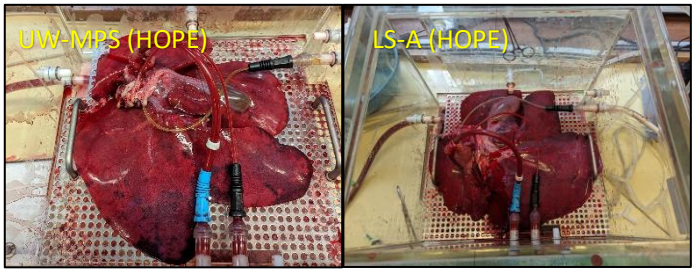

Figure 32:Photographs comparing livers near 4 hours of NMP (Please note the photographs are not of the same liver and have been chosen as the best quality for describing the phenomenon)

4.4.3 Perfusion parameters

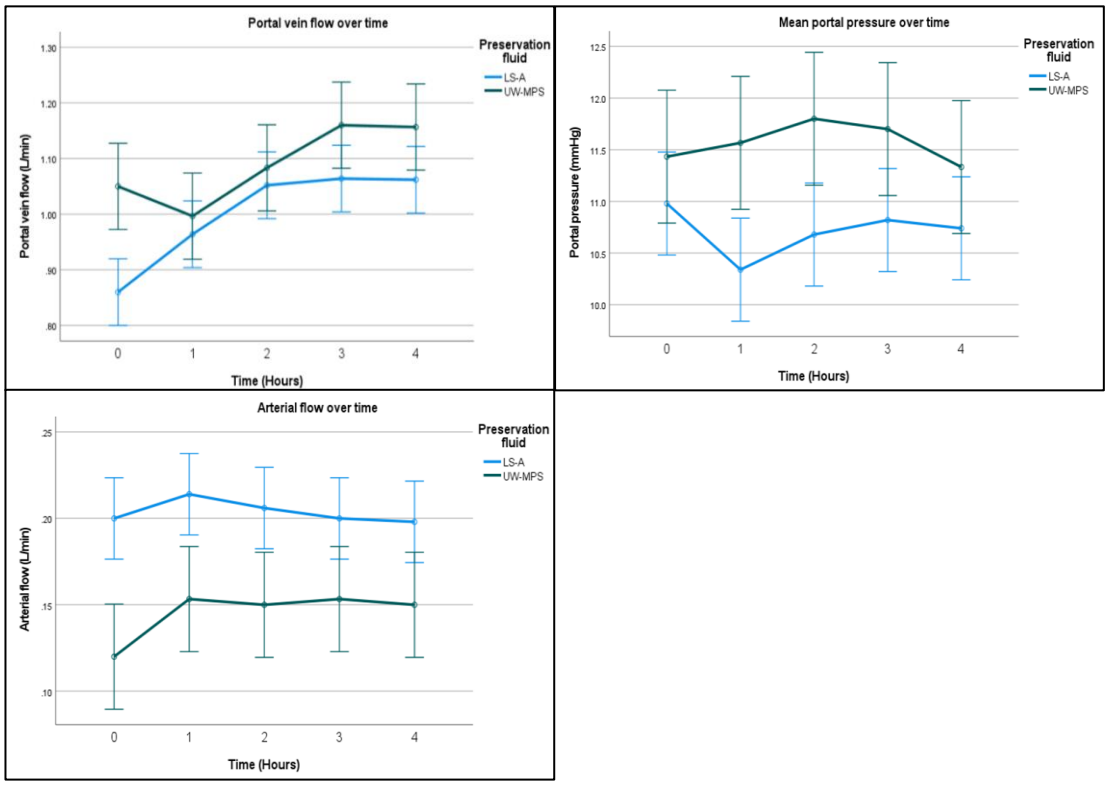

Figure 33: Graphs showing trends in angiodynamics during NMP following HOPE treatment.

	Portal vein flow (L/min)	Sig.	Portal vein pressure (mmHg)	Sig.	Hepatic artery flow (L/min)	Sig.
UW-MPS	1.1±0.14	NS	11.57±1.2	0.029	0.15±0.07	0.002
LS-A	0.90±0.18		10.97±1.7		0.20±0.1	

Table 25: Grand means of angiodynamics

In contrast to the SCS experiments, the portal flows showed no significant differences between groups (Grand mean LS-A 1±0.06L/min vs UW 1.09±0.07L/min). The portal pressure was again significantly lower in the LS-A group (Grand mean 10.72±0.45mmHg vs11.57±0.59mmHg (p=0.029)) however the overall pressures were considerably lower with higher flows than those seen in the SCS experiments. Arterial flow rates were significantly higher in the LS-A preserved group (Grand mean 0.20±0.02L/min vs 0.15±0.03L/min(p=0.002). The hepatic artery pressure was kept fixed at 90mmHg for these experiments.

4.4.4 Perfusate blood gas

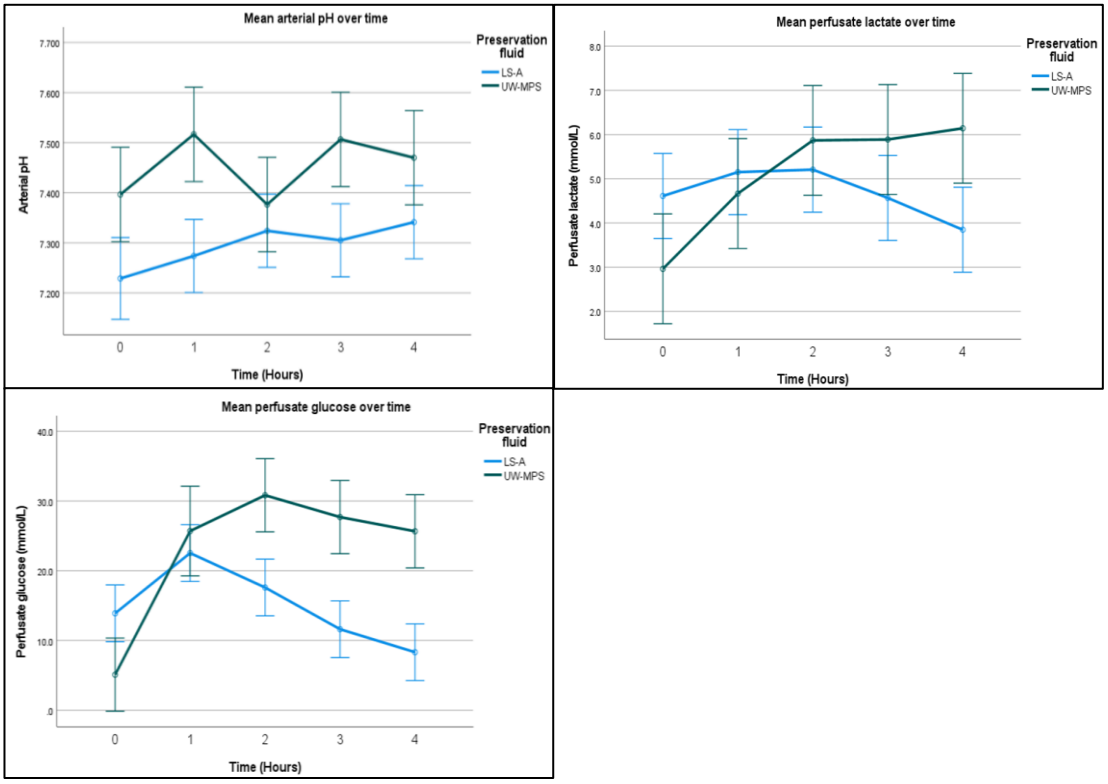

Figure 34: Graphs showing trends in arterial pH, Lactate, and glucose during NMP following the HOPE treatment.

	Perfusate pH	Sig.	Perfusate lactate (mmol/L)	Sig.	Perfusate glucose (mmol/L)	Sig.
UW-MPS	7.45±0.17	p=0.006	5.24±1.47	NS	31.8±3.2	<0.001
LS-A	7.30±0.07		5.36±2.03		17.2±4.54	

Table 26: Grand means of arterial blood gas results

The arterial pH is significantly lower in the LS-A group (Grand mean 7.30±0.07 vs 7.45±0.17(p=0.006). In comparison the SCS study the overall values are similar. There were no significant differences in perfusate lactate or glucose levels in this experiment, but the graph suggests a trend towards lower level in the LS-A group, given the small numbers in the control group this very well may represent a type 2 error. In comparison to the SCS values the perfusate lactate is considerably lower at 4 hours in both groups.

4.4.5 Perfusate Biochemistry

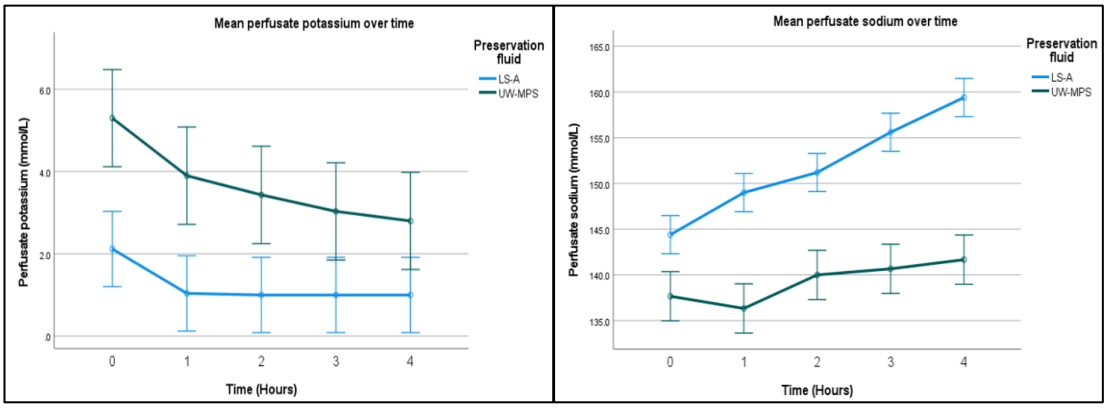

Figure 35: Graphs showing trends in sodium and potassium during NMP following HOPE.

	Perfusate Potassium (mmol/L)	Sig.	Perfusate sodium(mmol/L)	Sig.
UW-MPS	6.68±1.49	0.001	127±10.12	<0.001
LS-A	2.58±1.08		134±7.3	

Table 27: Grand means of sodium and potassium concentrations

The levels of perfusate potassium were noticeably lower in the HOPE experiments compared to the SCS series with significant levels of hypokalaemia in the LS-A group. Potassium levels were significantly lower in the LS-A group (Grand mean 1.2±0.8mmol/L vs 3.7±1.1mmol/L). The sodium levels for both groups show a mirroring of the potassium with significantly higher perfusate sodium in the LS-A group (Grand mean 151±0.9mmol/L vs 139±2.4(p=<0.001). As stated elsewhere in this thesis, hypokalaemia with hypernatremia in this type of experiment does not represent hypokalaemia per se only that little to no new potassium is being released from cells under the continuous, unopposed action of insulin.

4.4.6 Perfusate LFT's

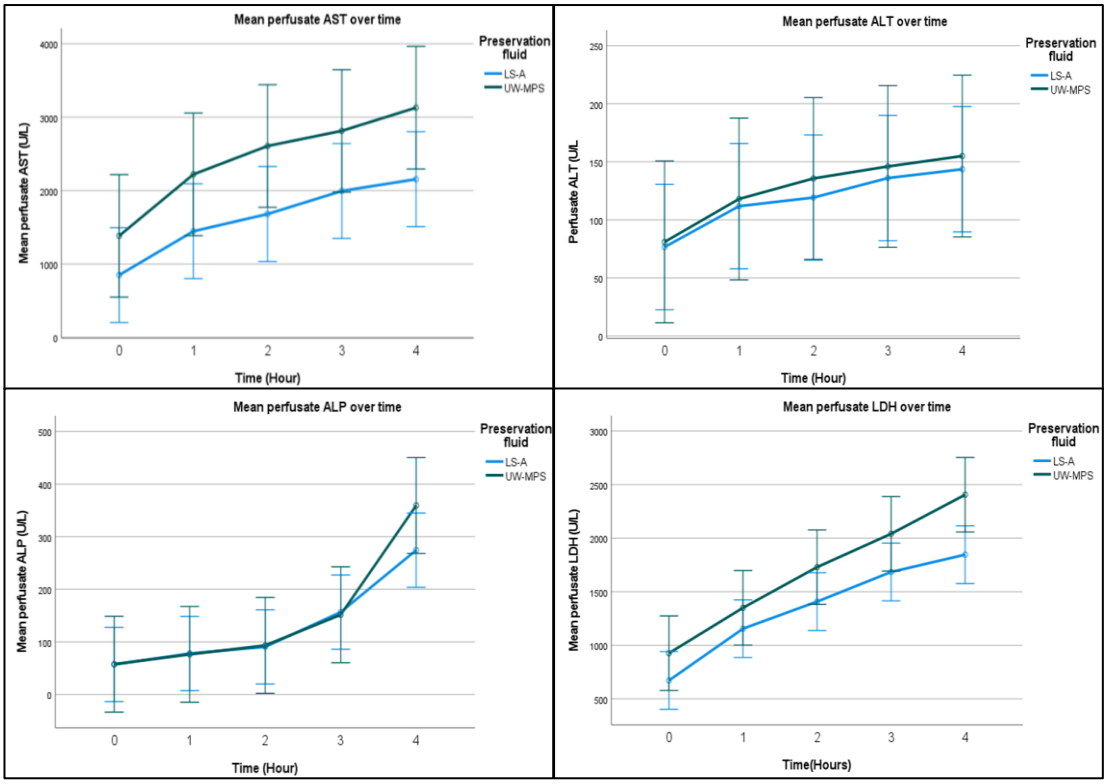

Figure 36: Graphs showing trends in LFT's and LDH over time, with no significant differences detected.

	Perfusate AST (IU/L)	Sig.	Perfusate ALT (IU/L)	Sig.	Perfusate ALP (IU/L)	Sig.
UW-MPS	2044.4±1746	NS	104.0±148.8	NS	99.7±26.2	0.04
LS-A	1129.7±1259		136.8±9.07		51.7±36.6	

Table 28: Grand means of LFT'S

	Perfusate LDH (U/L)	Sig.
UW-MPS	1765.7±3.29	0.04
LS-A	1333.7±249	

Table 29: Grand mean of LDH

There was no significant difference seen in any of the LFT'S or LDH during the perfusion, the overall all levels in all comparisons are similar to those seen in the SCS experiments. As evidenced by Figure 31 the organ perfusion is much more uniform following HOPE than those seen in Figure 19 from the SCS model and so I do not believe there is any 'empty liver' artifact effecting these results.

4.4.7 Bile Volume and pH

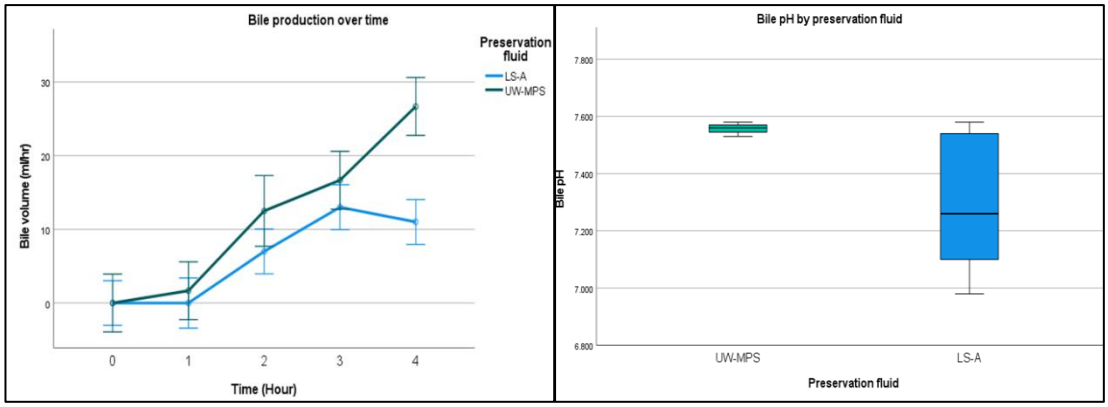

Figure 37: Graphs showing trends in bile volume and pH during NMP following HOPE.

	Bile volume (ml)	Sig.	Bile pH	Sig.
UW-MPS	10.67±3.6	NS	7.56±0.05	NS
LS-A	6.2 ±2.79		7.29±0.33	

Table 30: Grand mean of bile volume and pH

There is no significant difference in the bile volume or pH when comparing LS-A and UW-MPS in HOPE.

4.4.8 Comparison with VITTAL trial viability criteria

Criterion	LS-A	UW-MPS
Lactate < 2.5mmo/l within 4 hrs	40%	0%
Bile production	100%	100%
pH ≥7.3 at 4 hrs	83.3%	50%
Glucose clearance	100%	33%
HA flow ≥150mL/min	100%	66.6%
PV flow ≥500mL/min	100%	100%
Homogenous perfusion	100%	100%
Meeting criteria set out in VITTAL trial	40%	0%

Table 31: Summary characteristics showing comparison with viability criteria set out in VITTAL trial.

As in the SCS experiments, none of the control group subjects met the viability criteria for the VITTAL trial whereas 40% of livers receiving LS-A based HOPE met the criteria during NMP. Although the overall 'success' rate is lower for this experiment I believe this to be a consequence of the lower number of perfusions performed.

4.5 Biomarkers of liver injury

Shapiro-wilk test showed that all biomarker results followed a normal distribution and as such all statistical tests compared parametric data. Univariate analyses were performed comparing pre and post NMP samples (hour 0 and hour 4) across the different preservation solutions. The only exception to this was the FMN level as our preliminary work had shown peak levels to be found pre NMP and in the first hour of perfusion with slight change thereafter. In keeping with this 0 and 1 hours FMN levels were not compared against each other within preservation solution group as this comparison was of no scientific value.

4.5.1 FMN

SCS

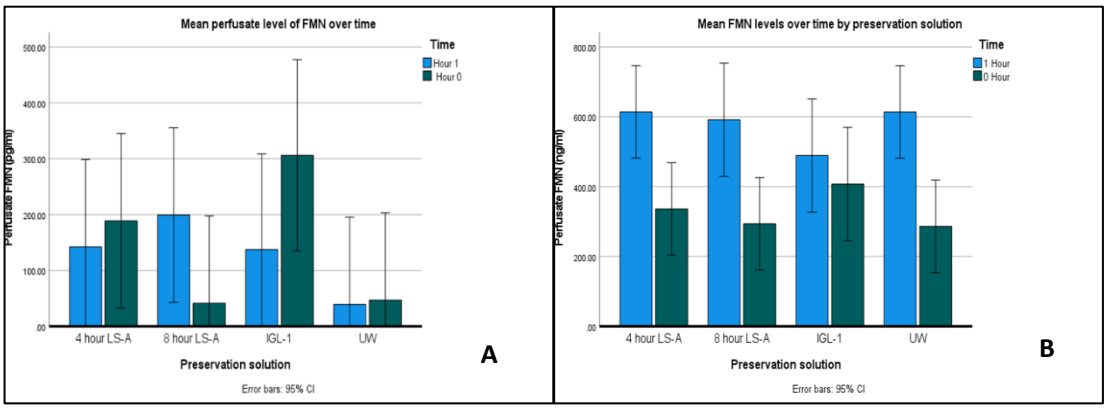

Figure 38: SCS experiments, graphs showing levels of FMN detection by ELISA (A) and Fluorescence spectrometry (B)

		Mean FMN levels 0-hour (ELISA)	Sig.	Mean FMN levels 1-hour (ELISA)	Sig.	Mean FMN levels 0-hour (FLUOR)	Sig.	Mean FMN levels 1-hour (FLUOR)	Sig.
LS-A 4 HOURS	UW	46.9±156.4	NS	39.1±156.37	NS	286.3±132.5	NS	614.1±132.5	NS
	IGL-1	306.334±171.3	NS	137.5±171.3	NS	407.7±162.2	NS	489.4±162.2	NS
	LS-A 4	188.9±156.4	N/A	142.4±156.4	N/A	336.3±132.5	N/A	614.3±132.5	N/A
	LS-A 8	41.4±156.4	NS	199.3±156.359	NS	293.7±132.5	NS	591.7±162.2	NS

Table 32: Summary of FMN results

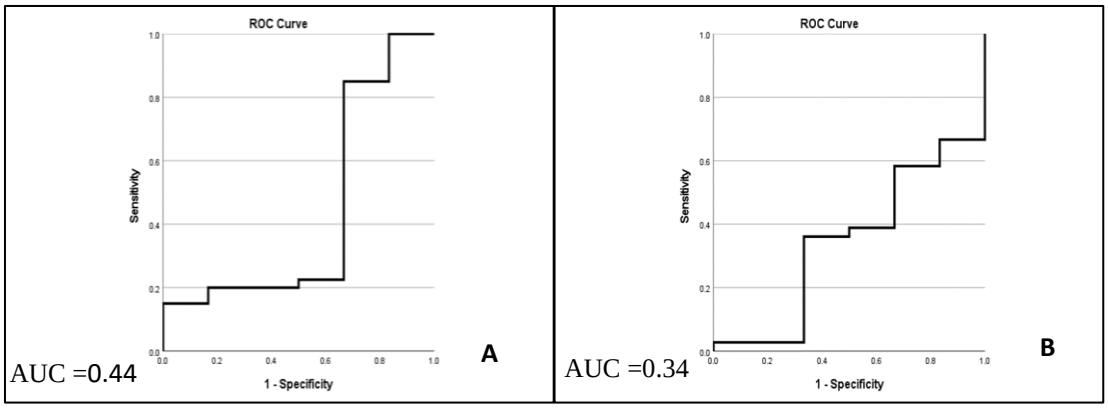

Figure 39: SCS experiments, Receiver operator characteristic (ROC) curves for FMN levels at one hour for ELISA (A) and flurescences spectrometry (B)

The above ROC curves examine the relationship between expressed FMN levels and the Livers meeting the criteria for the VITTAL trial in the SCS portion of the trial. The area under the curve (AUC) shows that in this study FMN levels are a very poor indicator of meeting the viability criteria.

HOPE

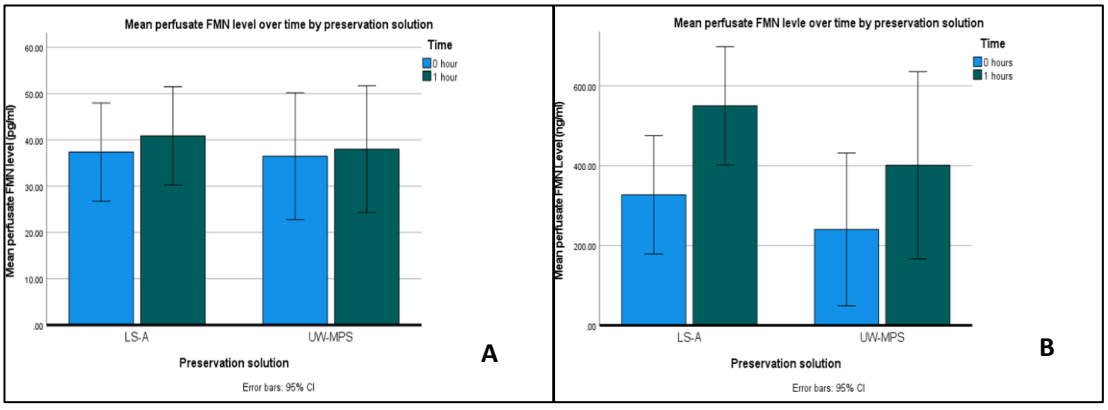

Figure 40: HOPE experiments graphs showing FMN detection by ELISA (A) and Fluorescence spectrometry (B)

	Mean FMN levels at 0-hours (ELISA, pg/ml)	Sig.	Mean FMN levels at 1-hours (ELISA pg/ml)	Sig.	Mean FMN levels at 0-hours (Fluor. Spec. ng/ml)	Sig.	Mean FMN levels at 1-hours (Fluor. Spec.ng/ml)	Sig.
UW-MPS	37.4±12.7	NS	38.1±13.9	NS	242.01±330.05	NS	181.12±330.05	NS
LS-A	38.2±10.4		40.5±11.1		2.88.84±255.66		503.41±255.66	

Table 33: Mean FMN levels at 0- and 1-hour using ELIZA and fluorescence spectrometry

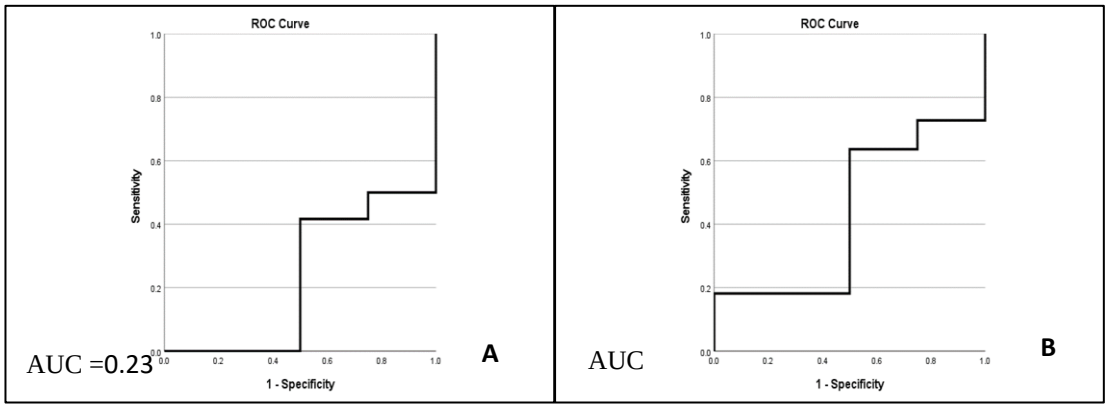

Figure 41: HOPE experiments, ROC curves for FMN levels at one hour for ELISA (A) and fluorescence spectrometry (B)

The above ROC curves examine the relationship between expressed FMN levels and the Livers meeting the criteria for the VITTAL trial in the HOPE portion of the trial. The AUC shows, in this study, FMN levels are a very poor indicator of meeting the viability criteria.

4.5.2 SYNDECAN-1

SCS

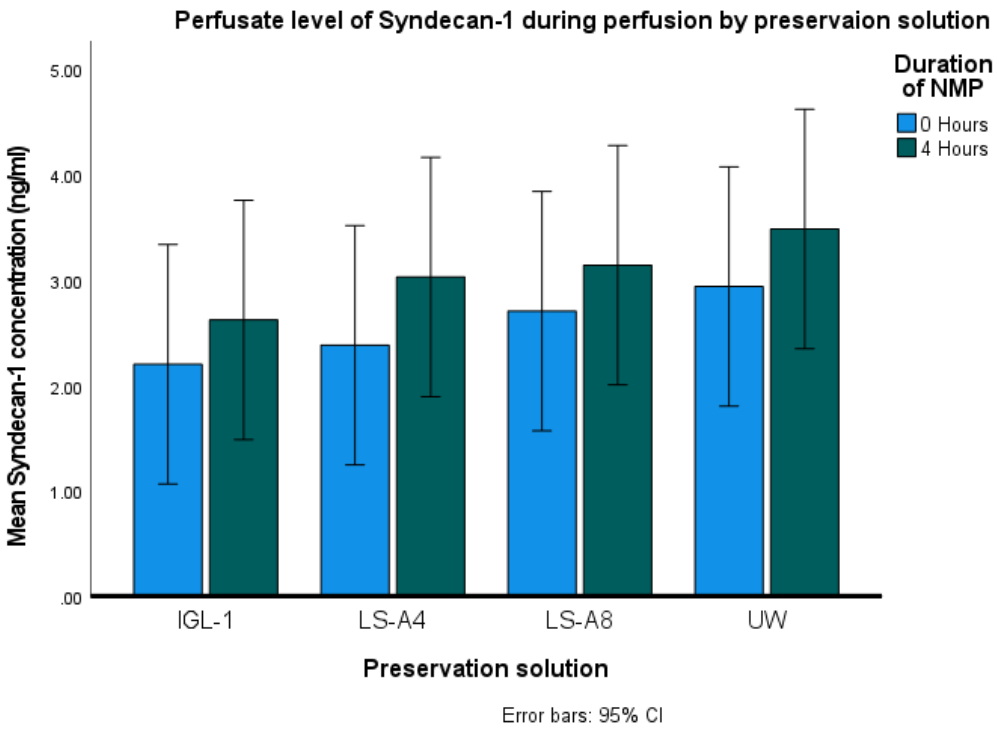

Figure 42: SCS experiments, graph showing perfusate levels of Syndecan 1 at start and end of NMP.

		Mean Syndecan-1 levels at 0-hours (ng/ml)	Sig.	Mean Syndecan-1 levels at 4-hours (ng/ml)	Sig.
LS-A 4 HOURS	UW	2.94±1.2	NS	3.48±1.2	NS
	IGL-1	2.2±1.2	NS	2.62±1.2	NS
	LS-A 4	2.68±2.8	N/A	3.03±2.8	N/A
	LS-A 8	2.70±1.2	NS	3.14±1.3	NS

Table 34: Mean Syndecan levels before and after perfusion

In the SCS experiments all groups showed an increase in perfusate levels over the perfusion period. There were no significant differences found within preservation solution groups when comparing hour 0 to hour 4 of NMP. There were no significant differences found between preservation solutions at either 0 or 4 hours for the presences of Syndecan-1 in the perfusate.

HOPE

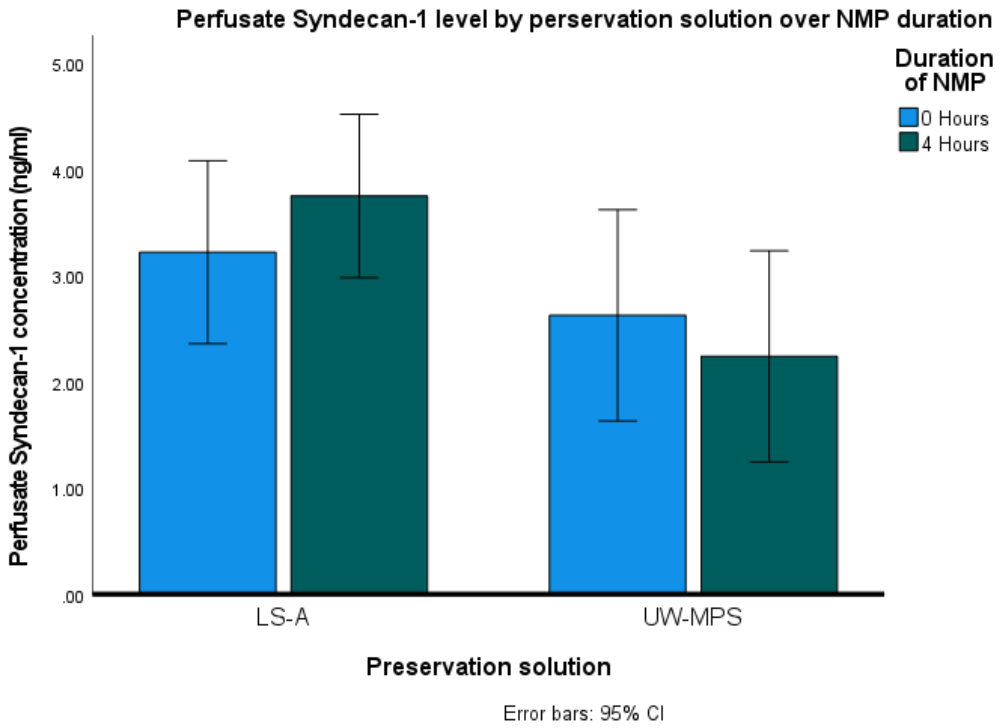

Figure 43: HOPE experiments, graph showing perfusate levels of Syndecan 1 at start and end of NMP.

	Mean Syndecan-1 levels at 0-hours (ng/ml)	Sig.	Mean Syndecan-1 levels at 4-hours (ng/ml)	Sig.
UW-MPS	2.627±1.02	NS	3.75±0.79	NS
LS-A	2.99±0.79		2.24±1.02	

Table 35: Mean syndecan-1 levels before and after NMP (HOPE)

In the HOPE experiment, only the LS-A group shows an increase in Syndecan -1 over time, there are no significant differences found within the UW-MPS group. The 0-hour level does not differ significantly with preservation solution. The 4-hour level however is significantly lower in the UW-MPS group compared with LS-A.

4.5.3 HMBG-1

SCS

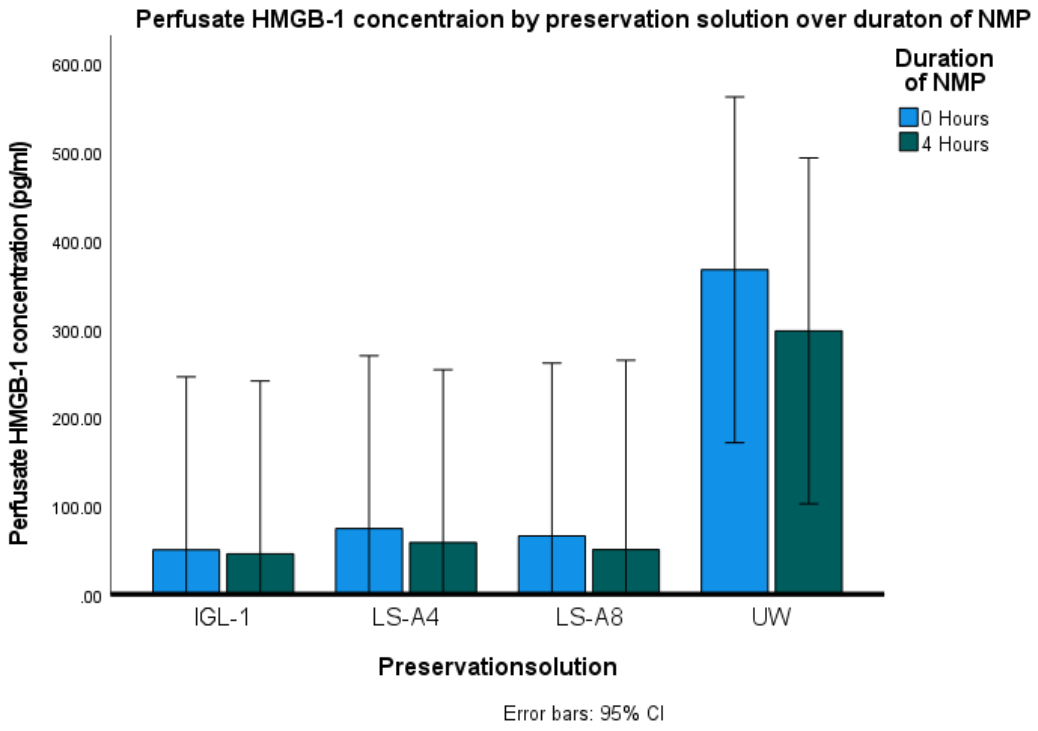

Figure 44: SCS experiments, graph showing perfusate HMGB-1 level at beginning and end of NMP.

		Mean HMGB-1 levels 0-hour (pg/ml)	Sig.	Mean HMGB-1 levels 4-hour (pg/ml)	Sig.
LS-A 4 HOURS	UW	366.5±200.7	0.04	297.5±200.7	NS
	IGL-1	50.2±200.7	NS	45.6±200.7	NS
	LS-A 4	74.1±200.7	N/A	58.4±200.7	N/A
	LS-A 8	65.8±219.9	NS	50.3±245.8	NS

Table 36: Mean HMGB-1 level before and after perfusion

In the SCS experiments, all groups showed a decrease in perfusate HMGB-1 over the course of NMP. There were no significant differences found when comparing 0-hour and 4-hour levels within preservation solution groups. There was significantly lower HMGB-1 perfusate levels at 0-hour found in the LS-A4 and LS-A8 compared with the UW group). There were no significant differences found between groups at the end of NMP.

HOPE

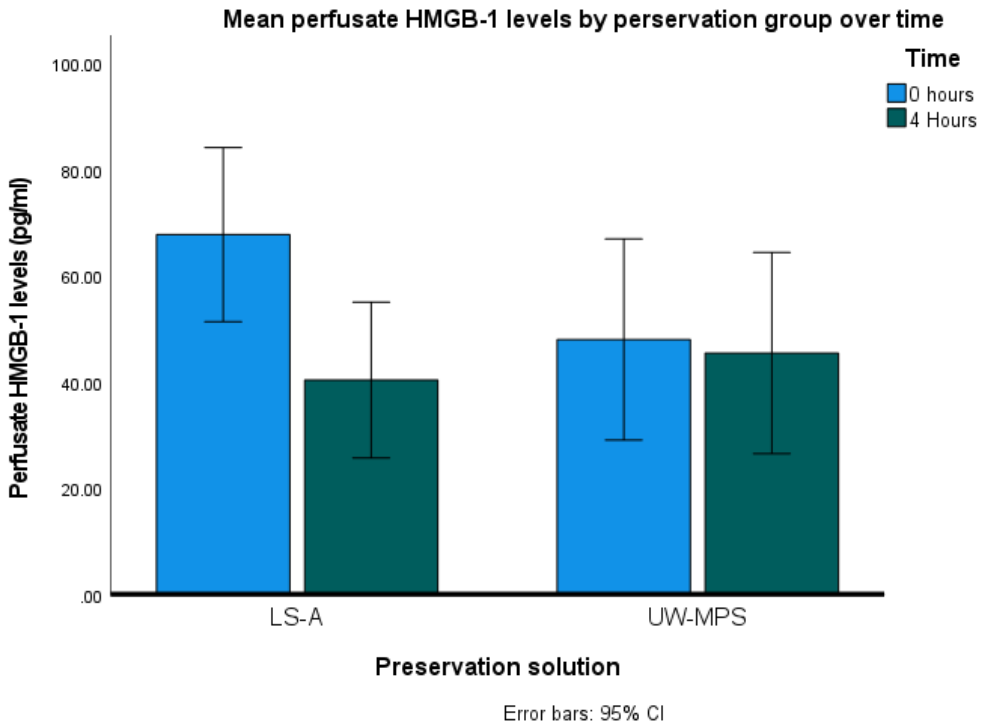

Figure 45: HOPE experiments, graph showing perfusate HMBG-1 levels at beginning and end of NMP.

	Mean HMGB-1 levels at 0-hours (ng/ml)	Sig.	Mean HMGB-1 levels at 4-hours (ng/ml)	Sig.
UW-MPS	47.97±19.25	NS	45.42±19.25	NS
LS-A	63.43±14.91		40.34±14.91	

Table 37: Mean HMGB-1 levels before and after NMP (HOPE)

There were no, differences between groups at 0 or 4 hours when considering the HOPE experiments. There was however a significant reduction in HMGB-1 levels during NMP in the LS-A group).

4.5.4 CASPASE-3

SCS

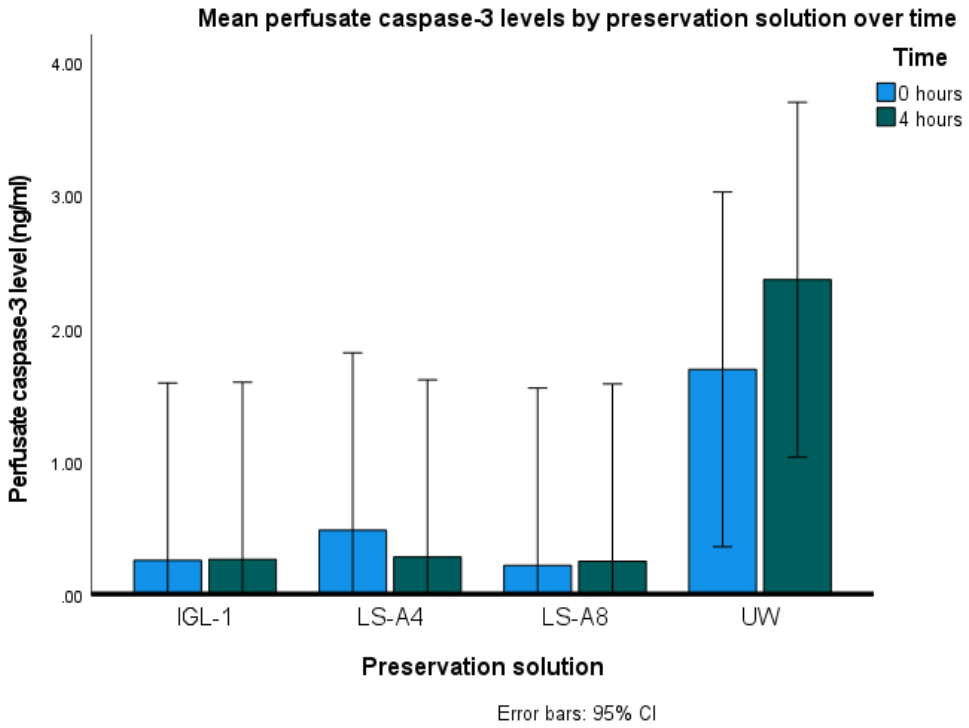

Figure 46: SCS experiments, graph showing perfusate caspa-3 levels at beginning and end of NMP.

		Mean Caspase-3 levels 0-hour (ng/ml)	Sig.	Mean Caspase-3 levels 4-hour (ng/ml)	Sig.
LS-A 4 HOURS	UW	1.69±1.4	NS	2.37±1.4	0.03
	IGL-1	0.25±1.4	NS	0.26±1.4	NS
	LS-A 4	0.48±3.4	N/A	0.28±3.4	N/A
	LS-A 8	0.22±0.93	NS	0.22±1.5	NS

Table 38: Mean caspase 3 levels before and after NMP

There were no significant differences found between groups at the start of NMP and no differences within groups across the duration of NMP. At 4 hours there were significantly higher levels of perfusate caspase-3 in the UW group when compared with LS-A 4 hours.

HOPE

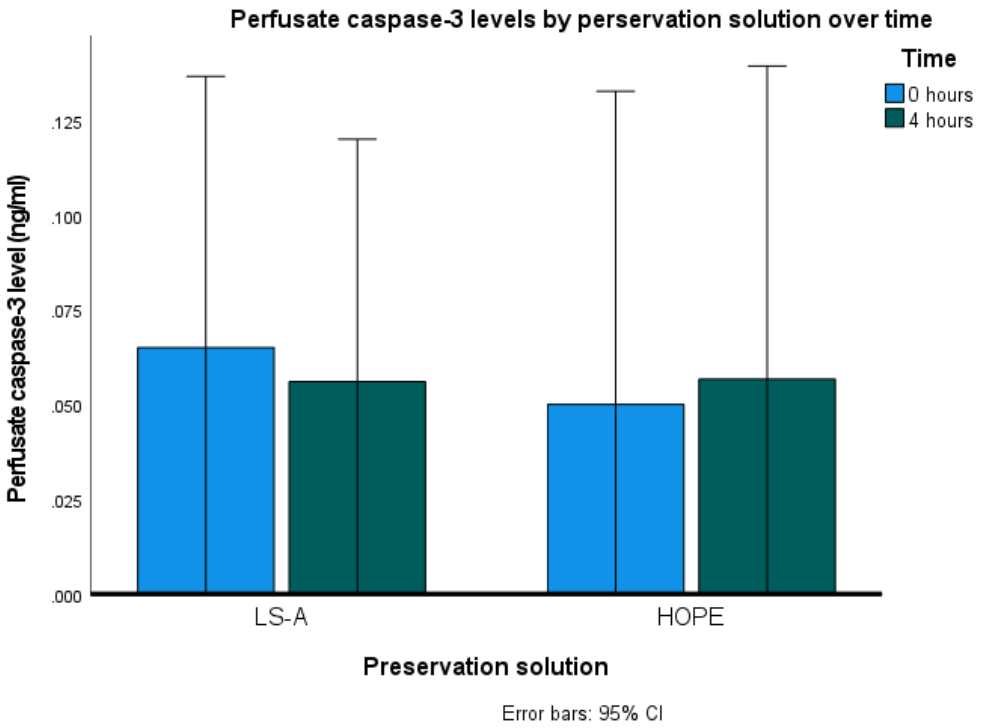

Figure 47: HOPE experiments, graph showing perfusate levels of caspase-3 at beginning and end of NMP.

	Mean Caspase-3 levels 0-hour (ng/ml)	Sig.	Mean Caspase-3 levels 4-hour (ng/ml)	Sig.
UW-MPS	0.05±0.08	NS	0.06±0.08	NS
LS-A	0.06±0.06		0.06±0.06	

Table 39: Mean caspase 3 levels before and after NMP (HOPE)

There was no significant difference within or between preservation solutions with regards to caspase-3 levels during the HOPE experiments.

4.6 Histology

4.6.1 Severity scoring

Histology was reviewed by two examiners; a 10% sample was compared for concordance in severity ratings to reduce inter-rater variability. The raters were the primary researcher (Paul Williams), and Dr Claire Corps. Mr Williams had received training from Dr Corps in histology interpretation as well as from Professor Kevin West in Leicester university whilst Dr Corps holds a PhD in histological examination of preserved livers. Three different staining methods were used, and each type of staining was rated using mild, moderate, or severe depending on the element under examination. The H+E stain looked at the degree of vacuolisation along with neutrophil infiltration following the severity score set out by Ali et al[132]. The reticulin stain looked at loss of reticulin network or 'clumping' of fibres consistent with necrosis whilst the PAS stain looked at the presence of intra-cellular glycogen evidenced by the number of magenta hued bodies within each cell. Cells were examined under 16x magnification. It is important to note that severity in one stain does not necessarily follow into the other two.

H+E

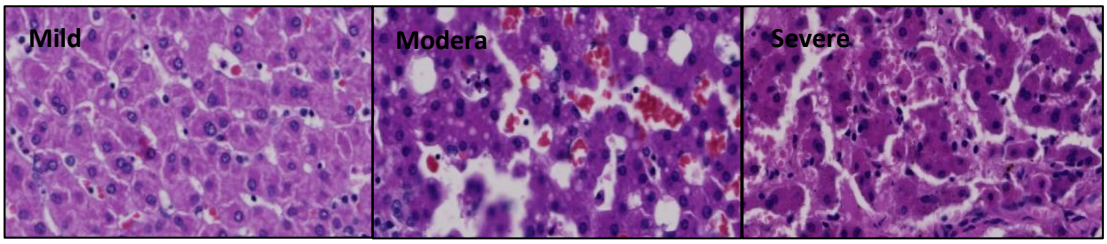

Figure 48: H+E-stained slides of liver tissue, displaying mild, moderate, and severe ischaemic insult as laide out in below table.

	Mild	Moderate	Severe
Neutrophil infiltration	0-1 Visible	1-5	5< or clusters of neutrophils

Vacuolisation	Normal sinusoidal pattern	Widening sinusoids/ intra-cellular vacuoles	Loss of normal sinusoidal pattern
Large areas of intraparenchymal haemorrhage	Absent	Absent	Present (not shown in picture)

Table 40: Criteria for ischaemia severity score

Reticulin

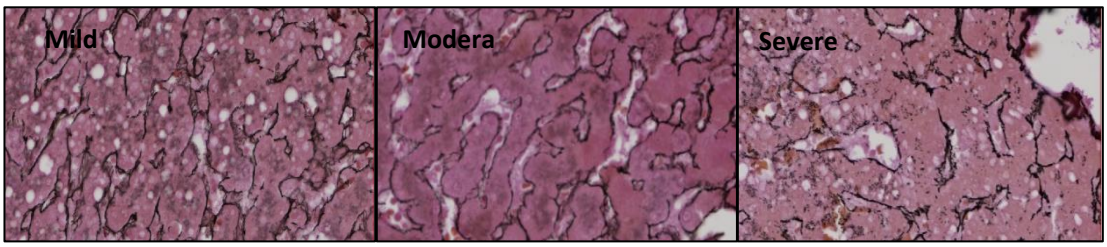

Figure 49: Reticulin stain liver sections showing mild, moderate, and severe degradation of reticulin structure as laid out in below table.

	Mild	Moderate	Severe
Reticulin pattern	Normal sinusoidal outline	Reduced sinusoidal outline, small amount of intra-cellular debris	Loss of sinusoidal pattern, clumped reticulin around vessels, widespread intracellular debris

Table 41: Criteria for reticulin severity score

Periodic Acid-Schiff stain

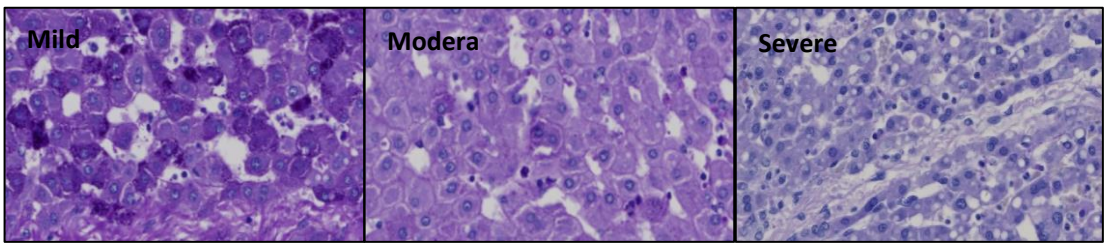

Figure 50: PAS-stained slides showing mild, moderate, and severe glycogen depletion as laid out in table below.

	Mild	Moderate	Severe
Presence of magenta hued intracellular bodies	Presence in >50% of cells or large intra cellular clusters	Presence in 20-50% of cells	Presence in less than 20% of cells or scanty

Table 42: Criteria used to define mild, moderate, and severe glycogen depletion.

4.6.2 Static cold storage experiments

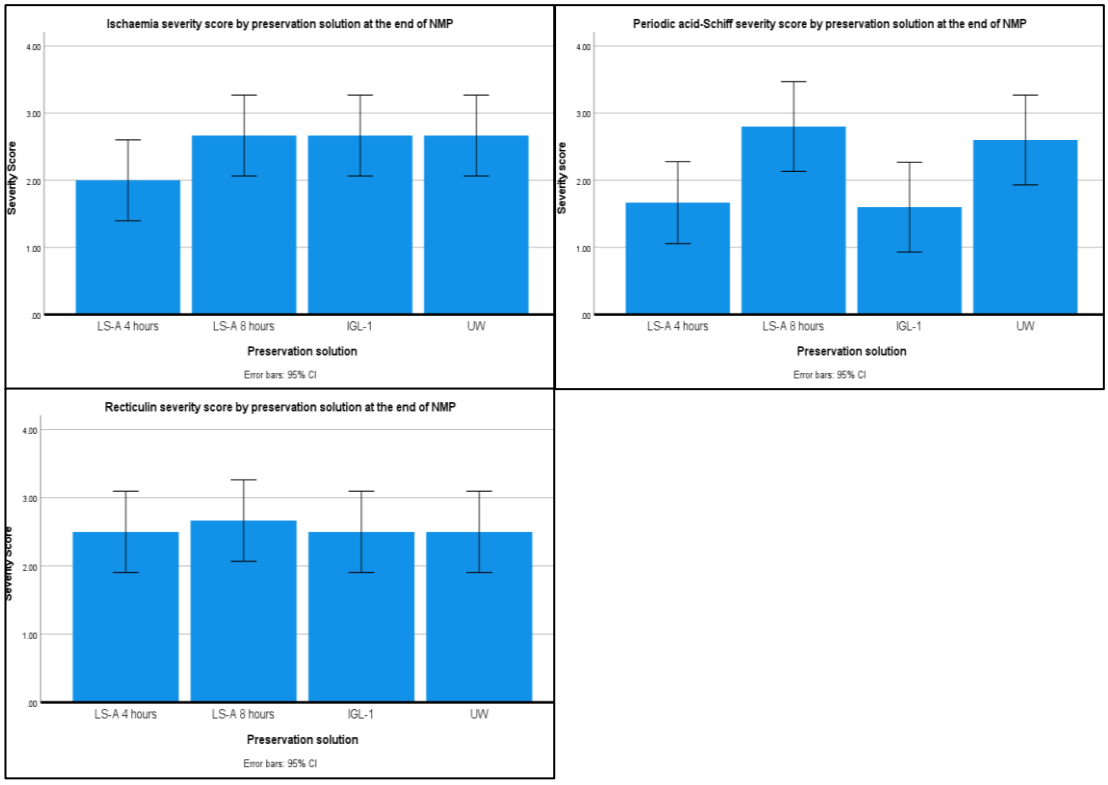

Figure 51:SCS experiments, graphs showing severity score for Ischaemia, reticulin degradation and glycogen depletion at the end of NMP.

	Preservation solution	Mean Ischaemia score	Sig.	Mean Reticulin score	Sig.	Mean PAS score	Sig.
LSA-4	UW	2.67±0.61	NS	2.5±0.60	NS	2.60±0.70	0.04
	IGL-1	2.67±0.61	NS	2.5±0.60	NS	1.61±070	NS
	LS-A 4	2.0±0.60	N/A	2.5±0.60	N/A	1.6±0.61	N/A
	LSA-8	2.67±0.61	NS	2.67±0.60	NS	2.80±0.67	0.017

Table 43: Mean histology grading scores for SCS experiments

4.6.3 Features of ischaemia

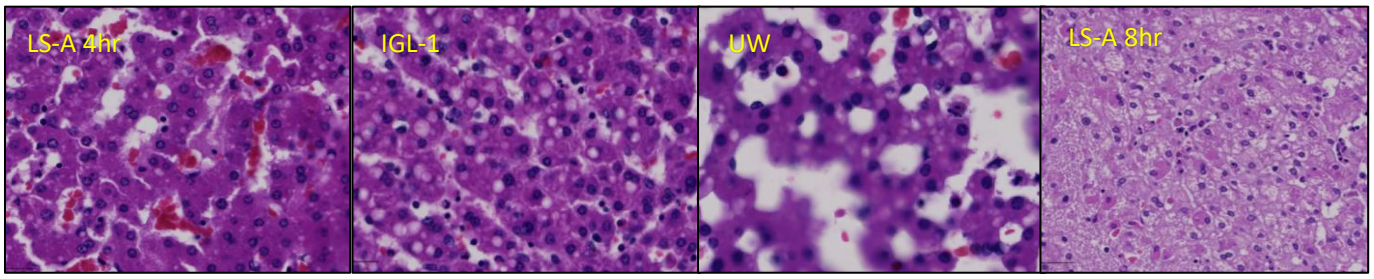

Figure 52: (from right to left, all 16x mag) Histology showing moderate vacuolisation in a LS-A 4hr preserved liver, whilst all other samples show severe vacuolisation albeit with different patterns. The large intra-cellular vacuoles are seen in the IGL-1 sample as evidence by the circular lack of stain, whilst in the UW and LS-A 8hrs large vacuoles and widening of sinusoids can be appreciated

4.6.4 Special Phenomena

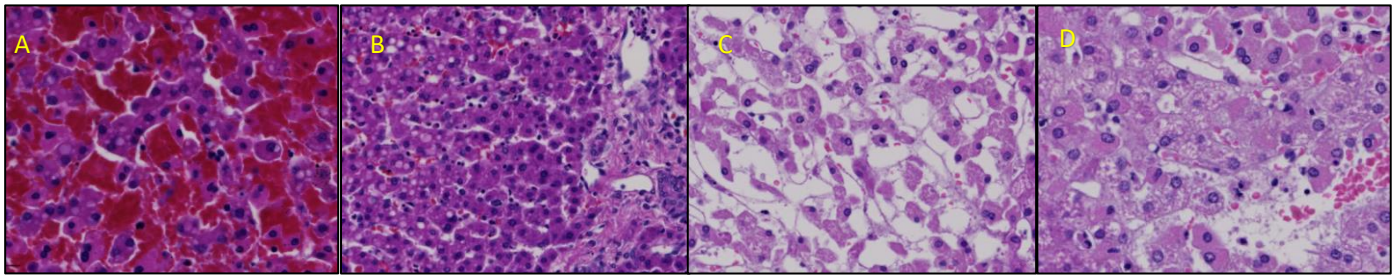

Figure 53: Phenomena given special mention. Photograph A (16x mag) shows an area of intraparenchymal haemorrhage and sinusoidal bleeding this is thought to signify significant ischaemia. B (10x mag) shows peri-portal sparing with worsening vacuolisation more remote from the portal triad. C (10x mag) areas of coagulation necrosis occurred only rarely but usually in the worst performing livers. D loss of nuclear staining intensity, usually in areas of cell lysis

4.6.5 Features of reticulin loss

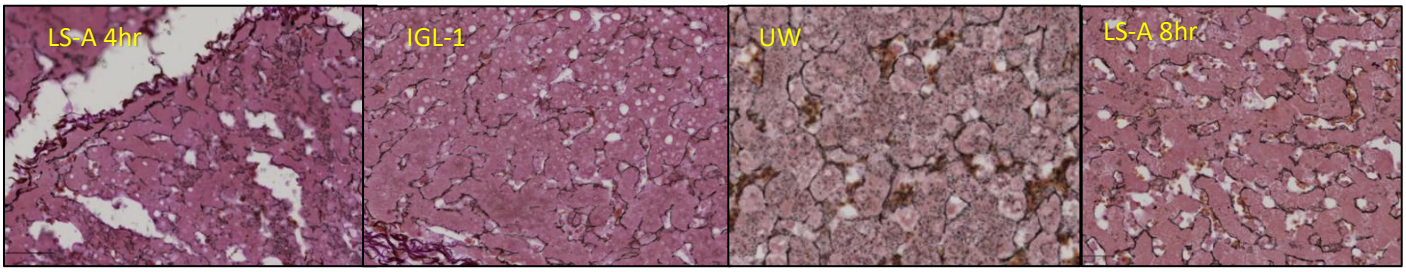

Figure 54: Photographs showing reticulin patterns with each preservation solution. There is better preservation of the reticulin skeleton in the IGL-1, LS-A 4 and 8 hr groups as evidenced by less reticulin deposition in the cytosol

4.6.6 Features of glycogen depletion

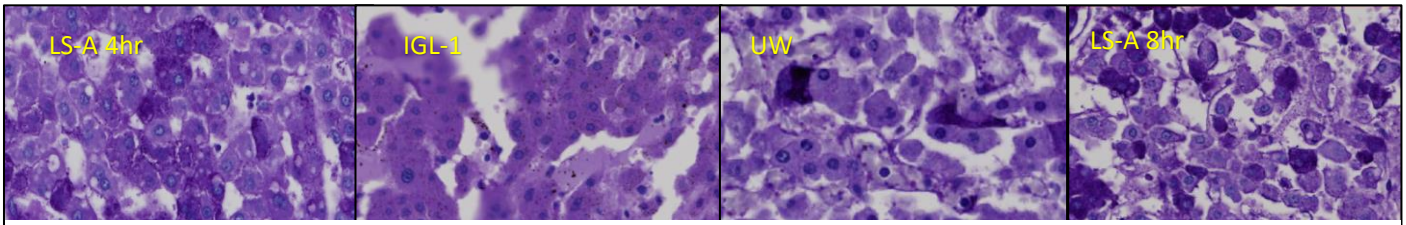

Figure 55:Liver tissues showing glycogen deposition by preservation fluid. The small magenta globules that can be seen intra-cellularly represent glycogen stores. These are most numerous in the LS-A 4hr, and IGL-1 sample. The UW and LS-A 8hr sample show considerably less glycogen

4.6.7 HOPE experiments.

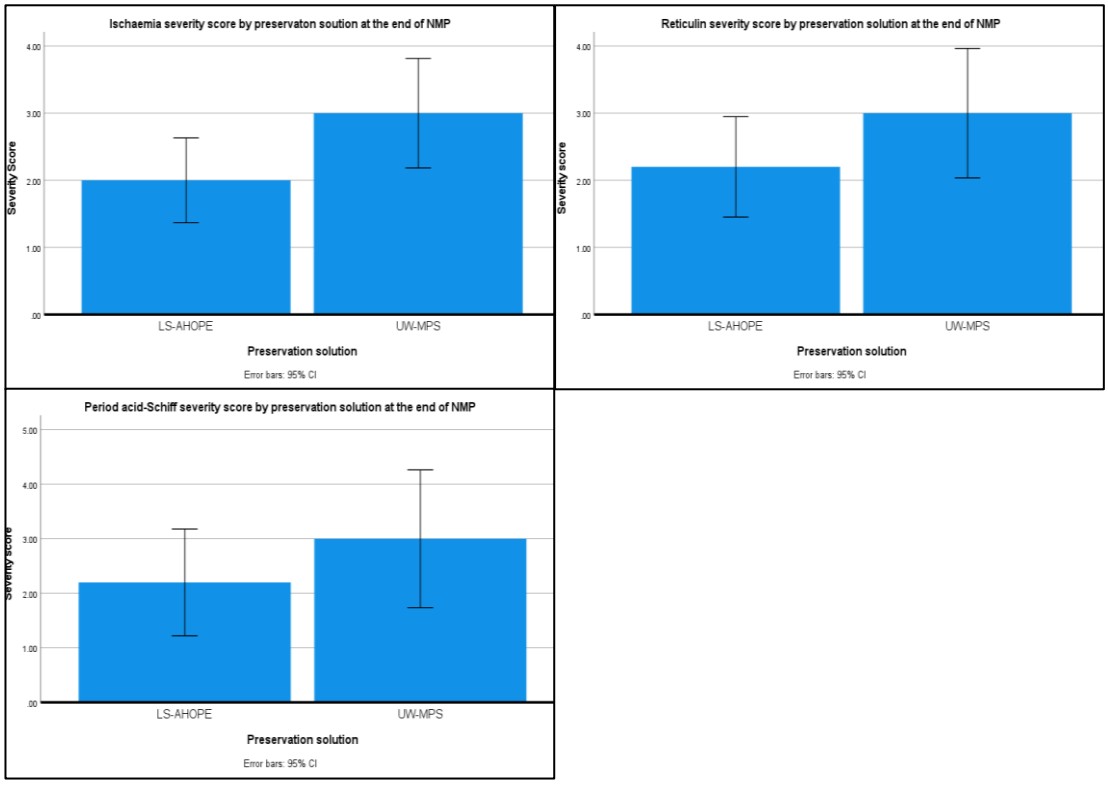

Figure 56: HOPE experiments, graphs showing severity scores for ischaemia, reticulin degradation and glycogen depletion at the end of NMP.

	Mean Ischaemia score	Sig.	Mean Reticulin score	Sig.	Mean PAS score	Sig.
UW-MPS	3.0±0.9	0.03	3±1.0	NS	3.0±1.2	NS
LS-A	2.0±0.7		2.2±0.8		2.2±1.0	

Table 44: Mean histology grading scores for HOPE experiments

5.0 Discussion

5.1 Use of LSA in the DCD setting at clinically applicable and extended preservation times

In this *ex-vivo* isolated porcine liver study, the novel preservation solution LS-A has shown some advantages over both gold standard solutions, which are in current clinical use. Although levels of ALT, AST and LDH are significantly lower in the UW group, we believe this is due to areas of absent perfusion during NMP in the UW

experiments and have concluded that LS-A performs at least as well if not better than both solutions in the DCD setting. This is the first study that shows this difference outside of rodent models and so represents a significant step in the development of LS-A towards a first in man trial. Most importantly 50% of the LS-A preserved livers fulfilled the transplant viability for NMP criteria set out by Mergental et al[86109]. None of the gold standard organs reached these levels. This is especially significant as the criteria were developed using a packed red cell model, whereas our model used whole autologous blood to simulate the post-transplant milieu more closely where one would expect more organ dysfunction.

Significantly lower perfusate lactate levels over the course of the perfusion are consistent with literature findings of improved post-transplant function and favourable histology. Although there is no consensus as to a threshold value for lactate for a liver to be considered transplantable, return to lactate concentration below 2.5mmol/L over the course of the perfusion correlates with acceptable post-transplant outcomes[64,86,110]. Although the ability to clear lactate per se does not indicate viability post-transplant, all the control group livers in this study did not reach the criteria suggested as viable in clinical trials of NMP[109]. The importance of this lies in how lactate is metabolised by the liver, most of it is cleared in peri-portal hepatocytes or zone 1 of the liver lobule which are the last cells to be affected by hypoxia or poor perfusion. Both zone 2 and 3 are more susceptible to hypoxia and hence failure to clear lactate during NMP is a sign of established panlobular injury[111]. Both control groups were comprised of solutions currently in clinical use for SCS with decades of outcomes following clinical liver transplant, the prevention of pan-lobular injury by LS-A in these exceptionally marginal livers should translate to reduced overall injury in less marginal organs.

Significantly lower perfusate potassium levels have two interpretations in the context of this comparison. The first is due to the difference in ion concentrations in the preservation solutions themselves; IGL-1 contains 20mmols/L and UW 125mmol/L of potassium whereas in LS-A there is only 5mmol/L, importantly there is 120mmol/L of sodium in IGL-1 but only 45mmol/L in LS-A. The second explanation for this trend is that overall tissue preservation is better resulting in

less cell lysis. The circuit is fixed so there is no other route for potassium to enter the circulation other than by cell lysis. This hypothesis is supported by higher perfusate sodium concentrations in the LS-A groups which does not follow the pattern of the pre-existing ion concentrations and fits better with the prolonged unopposed action of insulin throughout the perfusion. The high levels of insulin given, in the context of a fixed potassium concentration, will drive potassium into the cells at the expense of sodium release. If the trend seen in the graph was due to pre-existing concentrations, after preservation you would expect both potassium curves to trend downward under the gradual influence of insulin, but this does not occur, in fact the IGL-1 begins to climb again near the end of the perfusion. In addition to this IGl-1 and UW livers underwent pre-perfusion 'washing out' with 0.9% saline as in clinical practice whilst the LS-A livers did not undergo any washout. These concepts represent a significant advantage of LS-A over the controls, better tissue preservation and reduced cell lysis is self-evident in its advantage. Removing the time burden of a pre-implantation flush has a two-fold advantage. First it removes a step in what is one of the most complex and time pressured operative procedures. Secondly it prevents the dilution effect of washing out the preservation solution, meaning that the active components are functioning up until the restoration of circulation to the organ in both the NMP and re-implantation. As longer implantation times are associated with poorer outcomes especially with consideration of EAD and graft loss, reducing the absolute time by removing a step and reducing the functional implantation time could have a significant impact[133,134]. This would need an appropriately powered clinical study to explore its significance further.

Although the UW group shows significantly lower levels of ALT, AST and LDH than those livers preserved in LS-A these results need to be interpreted in the context of NMP. Figures 19 and 20 above show the macroscopic difference in tissues preserved in each fluid, as can be clearly seen there is a much greater volume of tissue perfused during NMP following storage in LS-A. This fits with the hypothesis that the level of perfusate liver enzymes are lower as the hypo-perfused tissues are never able to release their cellular contents into the circulating perfusate otherwise

known as the ‘empty liver’ phenomenon. This is especially apparent when considering the LDH, AST and ALT levels found when developing this model, when compared to these baseline experiments with UW preservation (see developing the model), livers with minimal WIT and 1 hour CIT showed higher levels of LDH throughout the perfusion with similar ALT levels and much higher 2-hour AST levels. These livers, preserved in UW and with minimal comparative warm or cold ischaemic insult, should show reduced perfusate enzyme levels if this relationship was that of severity-response. The fact that these livers showed much more homogenous perfusion with NMP is highly suggestive that the results seen during the comparative phase are due to sequestered enzymes in non-perfused tissue. Furthermore, although ALT has been used as a surrogate marker in clinical liver transplant, those NMP studies performed have not found levels during NMP to be predictive of post-transplant outcome [111]. ALT and AST levels across all groups are consistent with those reported by other researchers but a large proportion of the NMP literature does not report LFT change over time[63,64,110]. The HMGB-1 and Casapse-3 levels in the UW group represent significant necrotic and apoptotic cell markers entering perfusate which are not present in the other groups even with LS-A at 8 hours. This is in keeping with the other results shown here and shows a similar pattern to other groups work although levels cannot be directly compare due to species difference [135]. It would seem contradictory that the empty liver phenomenon would affect the liver enzymes differently to the cellular markers of necrosis measured in this study. With regard to AST and ALT leakage of these enzymes into the blood stream requires hepatocyte cell death to occur and to be in contact with the blood stream[136]. Contrary to this several studies have now shown that HMBG-1, despite being an intra-nuclear enzyme is actively secreted by cells in response to oxidative stress, further to this ALT and AST are produced predominantly by hepatocytes whilst HMGB-1 is also be secreted by the vascular endothelium[137].

Histology shows considerable IRI throughout the samples and as such I have used an objective scoring system to try and reduce bias in this semi-qualitative analysis. At 4 hours SCS preservation time there is no significant difference in histological

scoring in any parameter between LS-A and the gold standard solutions. This is largely representative of the severity of the marginality of the livers in this study. With the addition of the 8-hour LS-A preservation group significantly less glycogen storage can be seen, this is in keeping with the natural history of preservation injury with depletion of intracellular glycogen stores as time goes on. The HOPE study histology shows no significant difference between the LS-A and the UW- MPS group in any of the examined parameters. This supports the other findings of this study that LS-A can deliver HOPE to a similar standard as UW-MPS

It is important that the retrieval technique is considered in interpretation of these results. The retrieval in the DCD comparative experiments was immediately post-mortem with no circulating heparin administered, the mean WIT is 12:29 which is within the limits for a clinical transplant but at the higher end of acceptability[138].The animals have a period of haemodynamic instability that is not adequately accounted for in the measured WIT but all animals undergo the same procedure and so it is unlikely to have affected between groups function. During humane slaughter animals undergo electrical stunning before exsanguination which leads to high circulating levels of catecholamines, which will then be present, at least initially in the perfusate on reperfusion[139]. The abattoir retrieval differs from clinical retrieval in that the organ is removed en-bloc with its feeding vessels and then flushed with heparinised preservation solution on the back bench whilst in clinical practice, intravenous heparin is administered, then the aorta is canulated and flushed. This leads to a short period of blood stasis with inevitable microthrombi production which is then compounded by the fact that pig blood is hypercoagulable in comparison to humans[140–142]. Overall, when considering the whole study population, the baseline quality of the retrieved livers is significantly worse than a clinical DCD retrieval. Which makes it even more significant that 50% of the LS-A livers met the transplant viability criteria within 4 hours.

The vast majority of NMP organ studies use either leucocyte and or plasma deplete whole blood or the clinical equivalent, packed red cells, as the major constituent of the perfusate. Very few studies use autologous whole blood. This is due to the propagation of the IRI being more severe when immune and thrombogenic cells

and cytokines are present from the retrieval process. The exclusion of these blood cells fits well with the majority of these study designs which looks to improve organ function using NMP as a treatment and so removing as many detrimental effects prior to implantation as possible is logical. In our study we have chosen to use autologous whole blood when comparing SCS with various solutions. This is to allow the NMP to simulate the transplant process, the high circulating level of vasoactive substances present in the donor blood simulates, at least in part, the reaction that would occur during implantation. This is especially important when comparing LS-A to other solutions as one of its active ingredients PEG 20 has a stabilising effect on the glycocalyx in the organ vasculature giving better preservation through reduced leucocyte and platelet adhesion. Along with the factors mentioned in the previous paragraph the use of whole unaltered blood contributes to our overall function rate being less than other study groups given that the CIT was controlled at four hours. Interestingly, despite the proposed mechanism of action of PEG based preservation solutions, through protection of the glycocalyx, the marker we have used in this study (Syndecan-1) has shown no significant changes in perfusate levels, this is unusual as Syndecan-1 has been well documented across multiple species, including pigs, to be have measurable serum and urine levels in response to glycocalyx dysfunction of various aetiologies [135,143]. Therefore, further study is warranted looking at the expression of this biomarker along with others such as heparan sulphate, using serum and tissue homogenate samples to examine if this is a true result or simply due to low expressed perfusate levels. The levels of caspase 3 and HMBG-1 were significantly higher in the UW group, this fits with the large areas of necrosis seen macroscopically. There was no difference with regards to the other preservation solutions.

5.2 Use of LSA as a HOPE perfusate

The data from this pilot study is the first study to support LS-A as an MPS solution showing that its dissolved oxygen concentration is enough to support the HOPE

procedure. The sample size here is small but no significant differences were elicited in the primary outcome measure, perfusate lactate, although the propensity for type 2 error is high. Overall, the livers performed better than those preserved with SCS with visibly better perfusion and lower mean perfusate lactates across control and intervention groups.

LS-A displays several characteristics during HOPE that are comparable to UW-MPS, it allows the same rate of portal flow with similar levels of oxygen delivery. The significantly higher-pressure during HOPE, seen in this study is likely to be an artifact given the small number of study subjects, in fact from the raw data, it can be seen to arise from a solitary case. The preservation period was purposefully kept short with 2 hours of SCS followed by 2 hours of HOPE to give the best chance of functional livers given the poor baseline quality from the abattoir.

During NMP LS-A demonstrated significantly lower portal vein pressure, which is accompanied by a significantly higher arterial flow throughout the perfusion, this would suggest a better perfusion overall either from reduced intra-vascular obstruction or compression from oedema. Although LS-A has shown a significantly lower arterial pH of 7.30 in this study, the significance of this is not entirely clear, the literature regards a pH of 7.30 and above as one of the markers of good performance during NMP but there has not be greater discrimination between normal pH values[113]. The trend of a significantly lower perfusate potassium is again seen during HOPE with LS-A, as previously discussed this should offer intra-operative benefits in managing haemodynamic instability during implant.

Although there are no significant differences between absolute perfusate glucose and lactate found during the multivariate analysis, the summary measures shown in Table 31 show that none of the organs in the UW-MPS group met the viability criteria set out in the VITTAL trial whilst those in the LS-A group met considerably more of the outcomes. Thus, considerably less weight can be taken from the minor differences seen in the HOPE study as compared to the much larger differences and failure patterns seen in lactate and glucose metabolism in the SCS studies. This study helps to highlight the need for a consensus approach to defining liver graft

viability during NMP so that comparable livers can be followed up when undergoing different procedures.

There was no significant difference in LFT's and LDH between both groups, this is a more meaningful comparison than in the SCS trial as the macroscopic pattern of perfusion seen was more comparable between LS-A and UW-MPS, so the results are less likely to be affected by confounding factors. HMGB-1, caspase-3 and syndecan-1 all showed no significant differences between groups and support the hypothesis that LS-A HOPE can offer similar if not better outcomes than UW-MPS. The examined histology has shown little difference between groups in both SCS and HOPE experiments, likely due to the severity of the initial IRI.

The key limitations of this study are again the sample size and the severity of the ischaemia reperfusion injury at explant. The effects detected within this experiment are large and so the small sample size is justified, however it is not clear if these effects would translate into clinical practice given the pre-existing strategies in place to reduce the initial IRI. Overall, these results suggest that LS-A works at least as well as UW-MPS in a HOPE model of severely marginal liver grafts. There are areas, namely perfusate potassium levels in which LS-A provided some advantage over UW-MPS which should translate into a clinical benefit.

5.3 Predictive value of FMN

The results of this study do not support the use of FMN as predictive biomarker following SCS or HOPE preservation methods. It does not correlate with other parameters used to determine viability following NMP. This study was conducted in conjunction with another group that have previously found positive correlations between HOPE perfusate FMN and subsequent transplant outcomes in humans, even using their methodologies there is still no demonstration of predictive value. What does require further investigation is the discrepancy between the ELISA detection and the fluorescence spectrometry detection of FMN, there appears to be a 1000-fold higher level in the amounts detected by fluorescent spectrometry

which is not in keeping with the natural history of the FMN protein. If anything, the fluorescence spectrometry should have shown lowered levels as the test was performed 1-2 months after the ELISA, furthermore if the spectrometry results are to be believed, it should follow that the ELISA displayed upper limit values which was not the case. This could be down to a more accurate reading from spectrometry but could also mean the molecule detected by spectrometry was not available for binding during the ELISA due to species or oxidative state. If further investigations into the usefulness of FMN are to be conducted then a third method, for example gas chromatography should be used to ensure that the conversion between the arbitrary units of the spectrometer and the absolute concentration are confirmed. ROC curves were performed to contrast the one-hour value of perfusate FMN with the number of livers meeting the viability criteria from the VITTAL trial. As otherwise shown from this data there appears to be no correlation between FMN perfusate levels and livers meeting viability criteria.

5.4 Barriers to completion of this study

5.4.1 Funding withdrawal

This study received partial funding from an industrial partner who helped to finance the initial DBD experiments at an animal facility in London. During early 2022 the industrial partner had part of its funding removed by its parent company and thus the scale of the DBD studies had to be curtailed. We adapted the protocol with colleagues at the University of Leicester to facilitate a much more economic DCD model to ensure it was within the Leeds Hospitals Charity grant that provided the other funding stream.

5.4.2 COVID-19

A good portion of this work was completed under COVID restrictions this led to a whole plethora of logistical issues that had to be overcome. Firstly, at the conception of the project the large animal labs in Leeds university could not facilitate the work due to closures, this led to the research group having to source other premises in the Griffin institute. Subsequently this led to various instances of cancelled experiments due to staff sickness/COVID outbreaks within the Griffin's team. Managing the logistics of a laboratory, with such a wide array of equipment and consumables 200 miles away was challenging at the best of times but resulted in experimental cancellations on only one occasion due to a broken pump head.

5.4.3 Changing geographical location

As mentioned above the animal facility in London was funded by the industrial partner. This also included the provision of lab space for the re-perfusion studies. As such when the contract for the study could no longer be supported, the lab equipment had to be relocated to a new facility. Colleagues in Leicester were kind enough to provide lab space to collaborate with us in this perfusion model. They also went on to provide invaluable help and expertise in the DCD retrieval model.

5.4.4 Experimental failure

Despite preparing a detailed protocol in line with evidence from the literature this project suffered several experimental failures at the outset. This was due to a faulty pump which eventually failed completely. Once this pump was replaced with a gravity fed bag the experiments functioned in line with the established literature. In an effort to investigate why the experiments were failing I went through a process of altering various parameters, drugs and circuits which took 2-3 months to adequately explore.

5.4.5 Scarcity of organs and loss of renal work

The model that provided most of the organs perfused in this study, referred to as DCD, was entirely reliant on the availability of food chain animals in a local abattoir. This supply came weekly on a Thursday and was limited to two animals. Simultaneous perfusion of livers was successfully conducted on several occasions, but its use was limited by the availability of assistants as constant circuit supervision was required. The retrieval time was mandated between 10 and 11:30AM at the abattoir. In order to make this project workable by a lone operator and given the poor baseline quality of the organs, more detailed studies looking at optimal WIT and CIT and the subsequent comparisons could not be performed. The process of retrieval was further curtailed by the need for an assistant during the retrieval procedure, this was altruistically provided by a combination of spouse, friends, medical students, and mostly other M.D students at the university of Leicester. Across the study period this greatly reduced the practical retrieval of livers due to availability.

Having to abandon the renal portion of this work has significantly reduced the amount of data produced for analysis. In the context of the experiments performed, it does not impact the results in a material way. However, it does affect the overall conclusions as to use of LS-A in a clinical study and this is down to how organs are retrieved. It is rarely the case that kidneys are declined following retrieval when livers are not due to relatively higher resistance to IRI. Even clinical scenarios of liver failure without kidney derangement are rare in organ donors. Thus, having been unable to gather meaningful data on renal performance means that any human multi-organ retrieval study does so with little kidney data which will raise important ethical issues. That being said it does not preclude this altogether as other researchers in our laboratory have looked at this analysis in rat model giving favourable results.

5.4.6 Home office/AWERB delays

As there was no availability at Leeds to perform this study and there was now no option to perform it at the Griffin institute where the model development work had been completed, the study team sought to amend the project licence to allow the work to be done in Nottingham university's animal house which had links with Leicester hospital research group. This simple amendment to the project licence took between 3 and 5 months to be fully implemented. These delays were a combination of waiting for home office approval and the arduous AWERB process at the university of Nottingham.

5.4.7 Renovation works at animal facility in Nottingham.

As stated in previous documentation I am a surgical trainee with a national training number, this limits the availability of 'time out of training' periods. I had to my knowledge, negotiated with the animal facility at the university of Nottingham to perform the proposed DBD study at a price within our budget in a manageable time scale during the M.D period. Notwithstanding the long AWERB delays at this facility detailed above. Following the AWERB approval we had planned to start the study in April/May 2023. During our initial site visit, in April 2023 the Nottingham team highlighted the intensity of the study and the difficulties in providing staff adequately trained in retrieval and large animal anaesthesia. They later provided re-assurances this work could be done. Unfortunately, the facility at Nottingham is older and in need of repair. This led to them informing us that this study could not begin until the end of July 2023 and then further dates would be subject to staff availability during the summer break. This would not have been compatible with the study period and as such I decided in conjunction with my supervisors to extend the scope of the initial DCD study adding a third comparison group, looking at extended preservation and looking at the application of LS-A in HOPE.

5.4.8 Supply issues with machine perfusion fluid.

Towards the end of 2022 Bridge to Life, the company responsible for the UK's supply of both UW SCS solution and UW-MPS issued a field safety notice for its products that was escalated to the MHRA. This arose due to faulty packaging to some batches of UW solution which had allowed degradation of the product in storage and contamination with fungal organisms[144]. By this point the SCS solution needed for the study had already been procured and was fortunately not identified as one of the contaminated batches. The subsequent process review and quality improvement process at Bridge-to-Life did impact the study, however, as there was no availability of UW-MPS to establish a baseline in the HOPE study until end of July 2023, delaying this work by at least one month.

6.0 Conclusion

In this DCD, *ex-vivo* porcine model LS-A performed better when compared against gold standard solutions using externally validated viability criteria. Although UW does appear to show lower levels of traditional marker of hepatocyte damage, evidenced by Liver enzymes and LDH, this is caused by the areas of non-homogenous perfusion meaning the functional liver in contact with the perfusate was considerably reduced. Despite its promise in the literature FMN levels have not shown any prognostic value in this study regardless of detection technique. Even with the use of fluorescence spectrometry, the key to real-time measurement, the levels detected do not correlate with eventual outcomes.

This warrants further investigation in both the clinical and pre-clinical settings. In the pre-clinical setting, I have only examined DCD livers at the limits of marginality and as such a study looking at DBD livers either using discarded human livers or protocols initially proposed in this thesis using porcine *ex-vivo* perfusion could be considered. This would examine whether the large effect size seen is still applicable

in livers with a lesser degree of IRI. A significant foreseeable issue with using discarded human livers would be the confounding effect of using another solution for organ flushing. As almost all livers are retrieved in the multi-organ retrieval setting, any initial studies would have to account for the lack of kidney data by using an alternative solution for aortic flushing with subsequent storage in LS-A. The next step would require live animal transplants in a DBD model, and only then would it be appropriate to proceed to clinical studies. In the first instance, a single centre pilot study should be undertaken with the view to rolling out the protocol as a multicentre national or international trial, a large sample size would be needed due to the relative rarity of short-term graft complications such as EAD and PNF. This protocol could also capture discarded livers to continue the NMP investigation.

The ability of LS-A to extend acceptable CIT for livers has not been adequately defined by this study, as although it has shown no significant differences between UW and IGL-1 at 4-hours and LS-A at 8 hours, the baseline quality of the organs in this study creates significant confounders when compared to similar literature. Future studies should aim to eliminate the initial large warm ischaemic insult by following the originally set out DBD protocol; in this way the individual factors contributing to an organ's marginality can be better defined.

LS-A also functions in HOPE using currently available protocols. In general, it produced better quality livers during the NMP stage of the experiment. There were no significant differences found when examining perfusate lactate but 40% of LS-A preserved livers met viability criteria whilst no UW-MPS livers reached this threshold. The trend for lower liver enzymes using UW does not follow into the HOPE experiment, as these livers displayed much more homogenous perfusion than their SCS counterparts giving weight to the 'empty liver' hypothesis causing spurious low LFT results in the SCS experiments. Further study is again needed here in the form of live animal transplants; it is more feasible to see how studies using LS-A as a HOPE perfusate could move more quickly into the clinical sphere than its use during SCS as its application can be limited to a single organ after retrieval with a traditional solution. The regulatory requirements for introducing a new SCS solution are currently prohibitive for economic reasons.

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8.0 Appendix 1: Stability report LS-A

Stability analyses looking at levels of active ingredients in LS-A and their decomposition over time and with different storage variables. This work was performed by the team producing LS-A at HPS in Huddersfield. This work is included as quality control for the LS-A solution as the shelf life was not known when work began and producing small batches throughout the work was not economically feasible. The stability data suggests that all batches would be stable up to 24 months after production.

NHS	Calderdale & Huddersfield NHS Foundation Trust Huddersfield Pharmacy Specials Acre Mill Site	Development Template
QC3.34F1	QC Development Work	Version 2

Stability Report for Leeds Solutions Abdominal Organ Preservation Solution, Batch 410930X (24M)

Author: Philip Williams
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Table of Contents

1. Introduction

1.1. Background

A batch of Leeds Solution - Abdominal Organ Preservation Solution (LS-A) was manufactured for sterility validation and for use in an ICH stability study for shelf-life justification for the product. The batch was manufactured under GMP in the sterile manufacturing unit at Huddersfield Pharmacy Specials according to BMR LSA006S_410930X, with the formulation as listed below in Table 1.1.

Table 1.1 Formulation of Leeds Solution – Abdominal Organ Preservation Solution, batch 410930X

Component	Concentration (mmol/L)
Lactobionic acid	50
Potassium hydroxide	5
Sodium hydroxide	45
Glutathione (reduced)	3
Disodium hydrogen orthophosphate (dodecahydrate)	26.45
Sodium dihydrogen orthophosphate (dihydrate)	16.66
Glutamic Acid	3
Sucrose	100
Salicylic acid	0.25
Allopurinol	0.4
Diltiazem Hydrochloride	0.0221
PEG 20,000	1
Water	q.s.

Nitrogen purging was employed to remove dissolved oxygen from the solution before the batch was aseptically filled into 1000 mL EVA bags (manufactured by Haemotronic) that had previously been sterilised by gamma irradiation. Each filled bag of LS-A solution was placed into a gas impermeable foil pouch containing a sachet of oxygen scavenger (O2zero 200CC) and sealed under vacuum.

The batch was stored at 2-8°C and 25°C/60%RH for stability testing at intervals up to 24 months. The test methods used for the analysis performed in this stability study can be found in the finished product specification LSA006S V3.0. The purpose of this report is to describe the stability data obtained for Examination, Odour, pH, Total Diltiazem, Allopurinol, Salicylic Acid, Glutamic Acid, Total Glutathione, Osmolality and Particle Counts in Leeds Solution Abdominal Organ Preservation Solution, batch number 410930X.

2. Test Products

Drug Product	Description	Batch Number
Leeds Solution Abdominal Organ Preservation Solution	1L Aseptically filled production batch presented in oxygen resistant packaging (EVA plastic/ oxygen scavenger/ gas impermeable overwrap) manufactured 30SEP2021	410930X

3. Stability Protocol

For the full details of the stability protocol, refer to SLJ_LSA006S_004.

3.1. Testing Requirements

Test	Description	Limits (Over shelf-life)
(a)	Examination	Secondary Packaging – A vacuum sealed foil pouch containing a filled and sealed bag of LA-A solution and a sachet of oxygen scavenger Primary Packaging – A filled and sealed bag of LS-A solution free from leaks and visual defects. Ports may show slight yellow/ brown colouration from irradiation treatment Solution – A clear to light straw-coloured solution free from particulate matter
(b)	Extractable Volume	950 – 1050 mL
(c)	Odour	Odourless to faint sulphurous odour
(d)	pH	6.5 – 7.5
(e)	Lactobionic Acid (In-process)	95.0 – 105.0 %Nominal
(f)	Sucrose	3.249 – 3.591 %w/v
(g)	Total Diltiazem	0.00090 – 0.00110 %w/v
(h)	Allopurinol	0.0050 – 0.0060 %w/v
(i)	Salicylic Acid	0.0031 – 0.0038 %w/v
(j)	Glutamic Acid	0.0396 – 0.0528 %w/v
(k)	Total Glutathione	0.0553 – 0.0968 %w/v
(l)	Osmolality	323 – 357 milliosmol/Kg
(m)	Particle Counts	10µm ≤ 50/mL 25µm ≤ 5/mL
(n)	Sterility Test	Sterile
(o)	Bacterial Endotoxin	< 0.5 IU/mL

3.2. Testing Schedule

	T=0	1M	2M	3M	6M	9M	12M	15M	18M	24M
Pull date	01Oct21	01Nov21	01Dec21	01Jan22	01Apr22	01Jul22	01Oct22	01Jan23	01Apr23	01Oct23
2-8°C	10 (a-o)	2 (a,c-d,g-m)	2 (a,c-d,g-m)	2 (a,c-d,g-m)	2 (a,c-d,g-m)	2 (a,c-d,g-m)	2 (a,c-d,g-m)	2 (a,c-d,g-m)	2 (a,c-d,g-m)	10 (a-d,g-o)
25°C/60%RH		2 (a,c-d,g-m)	2 (a,c-d,g-m)	2 (a,c-d,g-m)	3 (a,c-d,g-m)					

4. Stability Data

4.1. Physicals

Examination of Solution – The examination of the solution for batch 410930X was unchanged at all time points during the 24M stability study.

Examination of Primary Packaging – The examination of the primary packaging for batch 410930X was unchanged at all time points during the 24M stability study.

Examination of Secondary Packaging – The examination of the secondary packaging for batch 410930X was unchanged at all time points during the 24M stability study.

Extractable Volume – The extractable volume of batch 410930X was stable when tested at time points T=0 and T=24M of the stability study.

Table 4.1.1 Extractable volume of Leeds Solution Abdominal Organ Preservation Solution, batch 410930X when tested at T=0 and T=24M

Condition	T=0	24M
2-8°C	1008	985

Odour – The odour of batch 410930X was unchanged at all time points during the 24M stability study.

pH – The pH of batch 410930X was stable at all time points during the 24M stability study

Table 4.1.2 pH of Leeds Solution Abdominal Organ Preservation Solution, batch 410930X during the 24M stability study.

Condition	T=0	1M	2M	3M	6M	9M	12M	15M	18M	24M
2-8°C	6.8	6.7	6.8	6.7	6.7	6.7	6.7	6.7	6.7	6.8
25°C/60%RH		6.7	6.7	6.7	6.7					

Osmolality – The osmolality of batch 410930X was stable at all time points during the 24M stability study

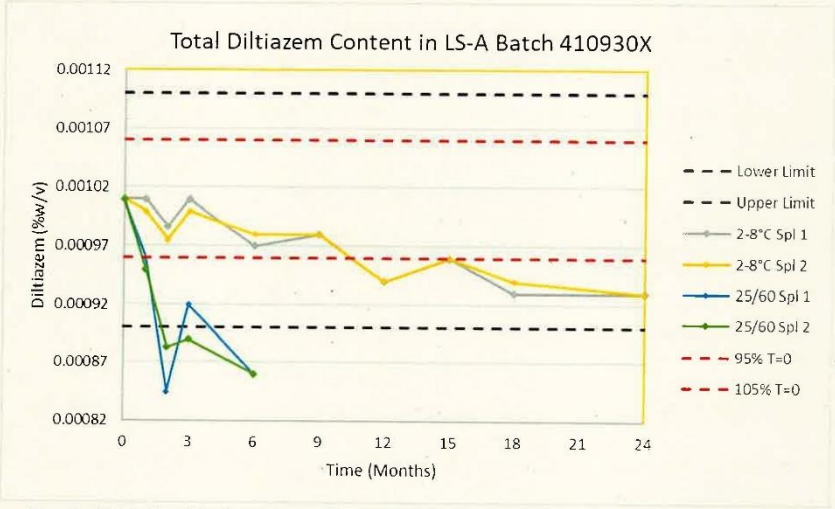
Table 4.1.3 Osmolality of Leeds Solution Abdominal Organ Preservation Solution, batch 410930X during the 24M stability study.

Condition	T0	1M	2M	3M	6M	9M	12M	15M	18M	24M
2-8°C	341	343	343	341	342	341	342	341	343	341
25°C/60%RH		341	344	344	344					

4.2. Total Diltiazem

Table 4.2.1 Total Diltiazem content of Leeds Solution Abdominal Organ Preservation Solution, batch 410930X during the 24M stability study.

Condition	T0	1M	2M	3M	6M	9M	12M	15M	18M	24M
2-8°C	0.00101	0.00101	0.00099	0.00101	0.00098	0.00098	0.00094	0.00096	0.00094	0.00093
25°C/60%RH		0.00096	0.00087	0.00091	0.00086					



Graph 4.2.1 Graphical representation of the Total Diltiazem content of Leeds Solution Abdominal Organ Preservation Solution, batch 410930X during the 24M stability study.

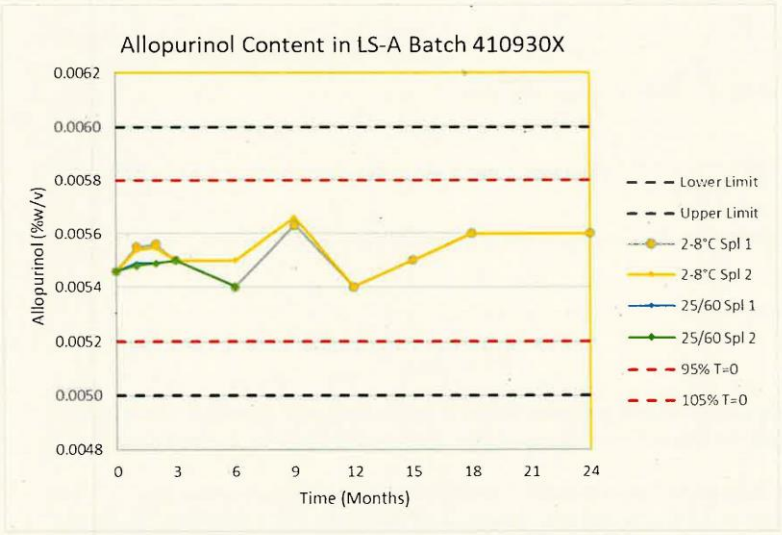
There is a downward trend in the results during storage at 2-8°C with the 12 month result breaching the ±5%Nominal warning limit, however all results are still within the specification limits at 24 months.

A similar downward trend is observed for the samples stored at 25°C/60%RH. After 2 months storage, all results (with the exception of one replicate at the 3 month timepoint) are out of specification.
Investigations were performed which concluded there was no assignable cause for the OOS and they were true results in trend with other timepoints, see OOS/2806, OOS/3292 and OOS/3468 for further details.

4.3. Allopurinol

Table 4.3.1 Allopurinol content of Leeds Solution Abdominal Organ Preservation Solution, batch 410930X during the 24M stability study.

Condition	T0	1M	2M	3M	6M	9M	12M	15M	18M	24M
2-8°C	0.0055	0.0056	0.0056	0.0055	0.0055	0.0056	0.0054	0.0055	0.0056	0.0056
25°C/60%RH		0.0055	0.0055	0.0055	0.0054					



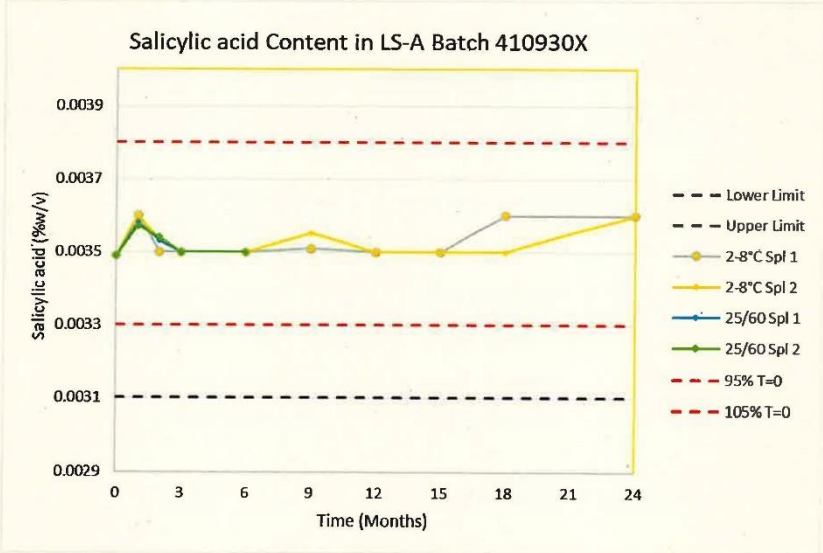
Graph 4.3.1 Graphical representation of the Allopurinol content of Leeds Solution Abdominal Organ Preservation Solution, batch 410930X during the 24M stability study.

The Allopurinol content remains stable and within specification limits after 24 months storage at 2-8°C and 6 months storage at 25°C/60%RH and is unaffected by storage temperature.

4.4. Salicylic Acid

Table 4.4.1 Salicylic Acid content of Leeds Solution Abdominal Organ Preservation Solution, batch 410930X during the 24M stability study.

Condition	T0	1M	2M	3M	6M	9M	12M	15M	18M	24M
2-8°C	0.0035	0.0036	0.0035	0.0035	0.0035	0.0035	0.0035	0.0035	0.0036	0.0036
25°C/60%RH		0.0036	0.0035	0.0035	0.0035					



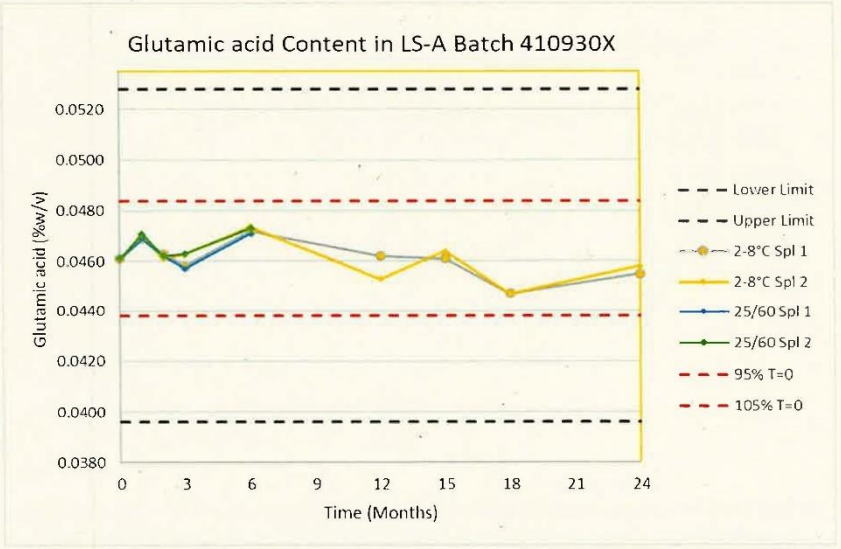
Graph 4.4.1 Graphical representation of the Salicylic Acid content of Leeds Solution Abdominal Organ Preservation Solution, batch 410930X during the 24M stability study.

The Salicylic Acid content remains stable and within specification limits after 24 months storage at 2-8°C and 6 months storage at 25°C/60%RH and is unaffected by storage temperature.

4.5. Glutamic Acid

Table 4.5.1 Glutamic Acid content of Leeds Solution Abdominal Organ Preservation Solution, batch 410930X during the 24M stability study.

Condition	T0	1M	2M	3M	6M	9M	12M	15M	18M	24M
2-8°C	0.0461	0.0470	0.0462	0.0461	0.0473	-	0.0458	0.0463	0.0447	0.0457
25°C/60%RH		0.0470	0.0462	0.0460	0.0472					



Graph 4.5.1 Graphical representation of the Glutamic Acid content of Leeds Solution Abdominal Organ Preservation Solution, batch 410930X during the 24M stability study.

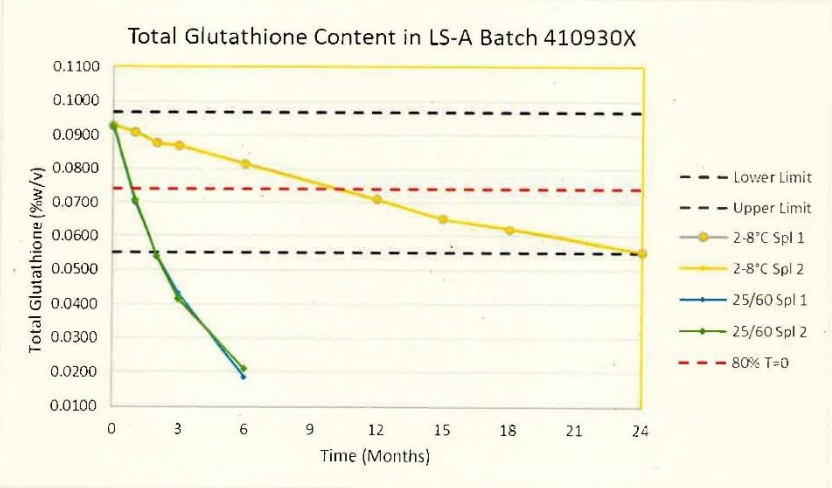
The 9 month timepoint was not analysed for glutamic acid content due to the HPLC column required for the analysis being unsuitable for use. A new column was ordered however, due to the specialist nature of the column and global shortages, there was a significant delay which made analysis impossible.

The Glutamic Acid content remains within specification limits after 24 months storage at 2-8°C and 6 months storage at 25°C/60%RH and is unaffected by storage temperature.

4.6. Total Glutathione

Table 4.6.1 Total Glutathione content of Leeds Solution Abdominal Organ Preservation Solution, batch 410930X during the 24M stability study.

Condition	T0	1M	2M	3M	6M	9M	12M	15M	18M	24M
2-8°C	0.0927	0.0909	0.0877	0.0871	0.0816	-	0.0711	0.0651	0.0621	0.0554
25°C/60%RH		0.0706	0.0541	0.0423	0.0198					



Graph 4.6.1 Graphical representation of the Total Glutathione content of Leeds Solution Abdominal Organ Preservation Solution, batch 410930X during the 24M stability study.

The 9 month timepoint was not analysed for total glutathione content due to the HPLC column required for the analysis being unsuitable for use. A new column was ordered however due to the specialist nature of the column and global shortages, there was a significant delay which made analysis impossible.

The 2-8°C Total Glutathione content is within the specification limits over the course of the study. A downward trend is observed with the results, with the 80%Nominal warning limit being breached at approximately 10 months. This is expected and has been seen during previous stability studies, see SLJ_LSA006S_002 and LSA006S_003 for more details.

The data reported here could be used to support a shelf life extension to 24 months with recommended storage conditions of 2-8°C.

The Total Glutathione content after 2 months storage at 25°C/60%RH dropped to well below the proposed specification limit. This was expected and has been seen in previous batches of similar composition. Leeds Solution – Abdominal (LS-A) is not suitable for storage at ambient temperatures to ensure stability of Total Glutathione content.

4.7. Particle Counts

Table 4.7.1 10µm Particle Counts of Leeds Solution Abdominal Organ Preservation Solution, batch 410930X during the 24M stability study.

Condition	T0	1M	2M	3M	6M	9M	12M	15M	18M	24M
2-8°C	1.6	0.44	5.47	5.84	1.57	4.04	1.60	1.77	15.93	8.00
25°C/60%RH		2.10	3.47	1.27	10.57					

Table 4.7.2 25µm Particle Counts of Leeds Solution Abdominal Organ Preservation Solution, batch 410930X during the 24M stability study.

Condition	T0	1M	2M	3M	6M	9M	12M	15M	18M	24M
2-8°C	0.1	0.04	0.20	0.10	0.24	0.30	0.70	0.64	1.44	0.14
25°C/60%RH		0.04	0.00	0.04	1.13					

The number of particles is below the limits for 24 months when stored at 2-8°C and 6 months when stored at 25°C/60%RH.

4.8. Sterility

Table 4.8.1 Sterility of Leeds Solution Abdominal Organ Preservation Solution, batch 410930X at both T=0 and T=24M

Condition	T0	24M
2-8°C	Complies	Complies

The sterility of the solution remained compliant for the duration of the 24 month stability study when stored at 2-8°C.

4.9. Bacterial Endotoxins

Table 4.9.1 Bacterial Endotoxins testing of Leeds Solution Abdominal Organ Preservation Solution, batch 410930X at both T=0 and T=24M

Condition	T0	24M
2-8°C	Complies	Complies

The bacterial endotoxins remained compliant for the duration of the 24 month stability study when stored at 2-8°C.

5. Conclusion

After a comprehensive review of the data reported here and comparison to the specification LSA006S V3.0, it can be recommended that the shelf life of the product be extended to 18 months with recommended storage conditions of 2 – 8°C.

The data suggests that the product is stable for 24 months, however, the Total Glutathione result at the 24 month timepoint was right on the lower limit of the specification. Combined with the clear downward trend in the results across the stability study period. Further studies would be recommended to prove the product is stable for 24 months.

Investigations have been performed to identify the degradation products of Reduced Glutathione in order to assess the impact they will have on the product, see QC Job 2021.010. It is recommended that the toxicity of the identified degradants is determined before the shelf life is extended.

The product is unstable when stored at 25°C/60%RH.

6. Appendix 1 – Stability Data 2-8°C

Calderdale and Huddersfield NHS

Product Code	LSA0065	Batch number	410930X	DOM	30-Sep-21
Product Name	Leeds Solutions - Abdominal Organ Preservation Solution				
Strength	N/A		Pack size	1L	
Stability Protocol Reference	SU_LSA0065_004				
Duration of Stability Study	24M				
Timepoints required	0, 1, 2, 3, 6, 9, 12, 15, 18, 24M				
Storage Condition(s)	2-8°C				

			Timepoint																			
Test	Specification	T=0	1M		2M		3M		6M		9M		12M		15M		18M		24M	
			Spl 1	Spl 2	Spl 1	Spl 2	Spl 1	Spl 2	Spl 1	Spl 2	Spl 1	Spl 2	Spl 1	Spl 2	Spl 1	Spl 2	Spl 1	Spl 2	Spl 1	Spl 2
Examination	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies
Extractable Volume	950 - 1050 mL	1008																	980	990
Odour	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies
pH	6.5 - 7.5	6.77	6.75	6.71	6.75	6.76	6.72	6.73	6.70	6.75	6.74	6.75	6.72	6.72	6.68	6.70	6.69	6.68	6.72	6.81
Lactobionic acid (In-process)	95.0 - 105.0 %Nominal	102.36																		
Sucrose	3.249 - 3.591 %w/v %w/v (95.0 – 105.0 %Nominal)	3.267																		
Total Diltiazem	0.00090 – 0.00110 %w/v (90.0 – 110.0 %Nominal)	0.00101	0.00101	0.00100	0.00099	0.00098	0.00101	0.00100	0.00097	0.00098	0.00098	0.00098	0.00094	0.00094	0.00096	0.00096	0.00093	0.00094	0.00093	0.00093
Allopurinol	0.0050 – 0.0060 %w/v (90.0 – 110.0 %Nominal)	0.0055	0.0056	0.0055	0.0056	0.0056	0.0055	0.0055	0.0054	0.0055	0.00563	0.00566	0.0054	0.0054	0.0055	0.0055	0.0056	0.0056	0.0056	0.0056
Salicylic Acid	0.0031 – 0.0038 %w/v (90.0 – 110.0 %Nominal)	0.0035	0.0036	0.0036	0.0035	0.00353	0.0035	0.0035	0.0035	0.0035	0.00351	0.00355	0.0035	0.0035	0.0035	0.0035	0.0036	0.0035	0.0036	0.0036
Glutamic Acid	0.0396 – 0.0528 %w/v (90.0 – 120.0 %Nominal)	0.0461	0.0469	0.0470	0.0463	0.0461	0.0458	0.0463	0.0472	0.0474			0.0462	0.0453	0.0461	0.0464	0.0447	0.0447	0.0455	0.0458
Total Glutathione	0.0553 – 0.0968 %w/v (60.0 – 105.0 %Nominal)	0.0927	0.0909	0.0908	0.0877	0.0877	0.0869	0.0872	0.0816	0.0815			0.0711	0.0711	0.0651	0.065	0.0619	0.0622	0.0554	0.0554
Osmolality	323 – 357 mosm/kg	341	342	343	342	343	342	341	341	342	341	341	340	343	341	341	342	348	340	341
Particle Counts	10µ≤50/mL	1.6	0.40	0.47	6.01	4.93	8.07	3.6	0.93	2.20	4.07	4.00	2.47	0.73	0.67	2.87	12.73	19.13	4.20	11.80
	25µ≤5/mL	0.1	0.00	0.07	0.33	0.07	0.13	0.07	0.20	0.27	0.47	0.13	1.40	0.00	0.07	1.20	2	0.87	0.07	0.20
Sterility Test	Sterile	Complies																	Complies	
Bacterial endotoxins	< 0.5 IU/mL	Complies																	Complies	
Method reference	LSA0065	version	3																	

7. Appendix 2 – Stability Data 25°C/60%RH

HPS Calderdale and Huddersfield NHS

Product Code	LSA006S	Batch number	410930X	DOM	30-Sep-21
Product Name	Leeds Solutions - Abdominal Organ Preservation Solution				
Strength	N/A		Pack size	1L	
Stability Protocol Reference	SLJ_LSA006S_004				
Duration of Stability Study	6M				
Timepoints required	0, 1, 2, 3, 6M				
Storage Condition(s)	25°C/60%RH				

Test	Specification	T=0	Timepoint 1M Spl 1	1M Spl 2	2M Spl 1	2M Spl 2	3M Spl 1	3M Spl 2	6M Spl 1	6M Spl 2
Examination	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies
Extractable Volume	950 - 1050 mL	1008								
Odour	Slightly Sulphurous	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies
pH	6.5 - 7.5	6.77	6.71	6.71	6.73	6.72	6.70	6.70	6.69	6.69
Lactobionic acid (in-process)	95.0 - 105.0 %Nominal	102.36								
Sucrose	%w/v (95.0 – 105.0 %Nominal)	3.267								
Total Diltiazem	0.00090 – 0.00110 %w/v (90.0 – 110.0 %Nominal)	0.00101	0.00096	0.00095	0.00085	0.00088	0.00092	0.00089	0.00086	0.00089
Allopurinol	0.0050 – 0.0060 %w/v (90.0 – 110.0 %Nominal)	0.0055	0.0055	0.0055	0.0055	0.0055	0.0055	0.0055	0.0054	0.0054
Salicylic Acid	0.0031 – 0.0038 %w/v (90.0 – 110.0 %Nominal)	0.0035	0.0036	0.0036	0.00353	0.00354	0.0035	0.0035	0.0035	0.0035
Glutamic Acid	0.0396 – 0.0528 %w/v (90.0 – 120.0 %Nominal)	0.0461	0.0469	0.0471	0.0462	0.0482	0.0457	0.0463	0.0471	0.0473
Total Glutathione	0.0553 – 0.0968 %w/v (60.0 – 105.0 %Nominal)	0.0827	0.0702	0.0710	0.0541	0.054	0.0431	0.0415	0.0186	0.021
Osmolality	323 – 357 mosm/kg	341	342	340	344	343	342	345	344	344
Particle Counts	10µ≤50/mL	1.6	0.73	3.47	3.4	3.53	0.93	1.60	12.47	8.57
	25µ≤5/mL	0.1	0.07	0.00	0.00	0.00	0.00	0.07	0.33	1.33
Sterility Test	Sterile	Complies								
Bacterial endotoxins	<0.5 IU/mL	Complies								

Method reference LSA006S version 2

9.0 Appendix 2: Ethical Approval

The Griffin Institute
For Surgical Training & Research

6th August 2024

To whom it may concern,

This is to confirm that the research work carried out by Dr Paul Williams at The Griffin Institute (TGI) from September 2021 was performed in line with Home Office standards and the TGI's Good Laboratory Practice standards, after having received ethical approval at the local AWERB (AWERB Reference: TGI_021_001).

Yours Faithfully,

Professor Jia Hua