Temporary hepatic support by extra-corporeal liver cross-circulation



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A thesis submitted for the degree of

Doctor of Philosophy

Michaelmas 2025

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List of abbreviations

ACLF	Acute-on-chronic liver failure
ACT	Activated clotting time
AGC	Automatic gain control
ALF	Acute liver failure
ALT	Alanine transaminase
ANOVA	Analysis of variance
APACHE II	Acute Physiology And Chronic Health Evaluation II
ASGR-1	Asialoglycoprotein receptor-1
AST	Aspartate transaminase
ATG	Anti-thymocyte globulin
ATP	Adenosine triphosphate
AWERB	Animal Welfare and Ethical Review Body
A20	Tumour necrosis factor, alpha-induced protein 3 (protein)
BIS	Bi-spectral index
CA	California
CBD	Common bile duct
CDx	Cluster of differentiation x
CE-	Conformité Européenne
CHaRT	Centre for Healthcare Randomised Trials
CI	Confidence interval
CID	Collision-induced dissociation
CLIF-C ACLF	Chronic Liver Failure Consortium Acute-on-chronic Liver Failure score
CLIF-C OF	Chronic Liver Failure Consortium Organ Failure score
CLIF-SOFA	Chronic Liver Failure – Sequential Organ Failure Assessment
CMAH	Cytidine monophosphate-N-acetylneuraminic acid hydroxylase
CO	Cardiac output

COPE	Consortium for Organ Preservation in Europe
CRISPR-cas9	Clustered regularly interspaced short palindromic repeats – CRISPR-associated protein 9
CRRT	Continuous renal replacement therapy
CTU	Clinical trials unit
CVP	Central venous pressure
CYP450	Cytochrome P450
DAMPs	Damage-associated molecular patterns
DBD	Donation after brain death
DCD	Donation after circulatory death
DDA	Data-dependent acquisition
DNA	Deoxyribonucleic acid
DNACPR	Do not attempt cardio-pulmonary resuscitation
DPMAS	Dual Plasma Molecular Adsorption Exchange System
DSMC	Data Safety Monitoring Committee
EASL	European Association for the Study of the Liver
EASL-CLIF	European Association for the Study of the Liver – Chronic Liver Failure
ECMO	Extra-corporeal membrane oxygenation
EDTA	Ethylenediaminetetraacetic acid
ELAD	Extra-corporeal Liver Assist Device
ELC	Extra-corporeal liver cross-circulation
ELISA	Enzyme-linked immunosorbent assay
ELSO	Extracorporeal Life Support Organisation
EPCR	Extra-cellular protein C receptor (protein)
EQ-5D-5	EuroQol five-dimension, five-level questionnaire
FC	Fold change
FDA	Food and Drug Administration
FFP	Fresh frozen plasma

FVIII	Factor VIII
GHr	Growth hormone receptor
GP1bR	Glycoprotein 1b-IX-V receptor
GRIPP2	Guidance for reporting involvement of patients and the public, version 2
H&E	Haematoxylin and eosin
HCD	Higher energy collisional dissociation
HEK	Human embryonic kidney
HESI	Heated electrospray ionisation
HEV	Hepatitis E virus
HMGB1	High mobility group box-1
HMOX1	Haem oxygenase-1 (gene)
HO1	Haem oxygenase-1 (protein)
HPLC	High performance liquid chromatography
HR	Hazard ratio
HRA	Health Research Authority
IA	Indiana
IACUC	Institutional Animal Care and Use Committee
ICNARC	Intensive Care National Audit and Research Centre
ICU	Intensive care unit
IDEAL-D	Idea, Development, Exploration, Assessment and Long-term follow-up – Device
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Il-x	Interleukin-x
INR	International normalised ratio
IRI	Ischaemia-reperfusion injury
ISTH	International Society of Thrombosis and Haemostasis
IVC	Inferior vena cava

K^+	Potassium
KO	Knock-out
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography – tandem mass spectrometry
LC-MS	Liquid chromatography – mass spectrometry
LIMMA	Linear models for microarray data
MA	Massachusetts
MAC-1	Macrophage-1 antigen
MAP	Mean arterial pressure
MARS	Molecular Adsorbent Recirculating System
MEAF	Model for Early Allograft Function
MELD	Model for End-stage Liver Disease
MHRA	Medicines and Healthcare products Regulatory Agency
MN	Minnesota
MS	Mass spectrometry
Neu5Gc	n-glycolylneuraminic acid
NH	New Hampshire
NHS	National Health Service
NHSBT	National Health Service Blood and Transplant
NJ	New Jersey
NMP	Normothermic machine perfusion
NORS	National Organ Retrieval Service
OCS	Organ Care System
ODT	Organ Donation and Transplantation
ODTRN	Organ Donation and Transplantation Research Network
PA	Pennsylvania
PAMPs	Pathogen-associated molecular patterns
PaO_2/FiO_2	Partial pressure of oxygen / fractional inspired oxygen ratio

PAS	Periodic acid-schiff
pCMV	Porcine cytomegalovirus
pCO_2	Partial pressure of carbon dioxide
PCV3	Porcine circovirus 3
P_ECO_2	End-tidal cardon dioxide
PERV	Porcine endogenous retrovirus
PI	Principal investigator
PLHV	Porcine lymphotropic herpes virus
pO_2	Partial pressure of oxygen
PPI	Patient and public involvement
PROCR	Extra-cellular protein C receptor (gene)
PSMs	Peptide-spectrum matches
QALY	Quality-adjusted life year
QC	Quality control
RCT	Randomised controlled trial
REC	Research Ethics Committee
RNA	Ribonucleic acid
ROFG	Research Operational Feasibility Group
RR	Relative risk
RRT	Renal replacement therapy
SDa	Sialyl-dimeric antigen
SITU	Surgical Intervention Trials Unit
SLPI	Secretory leukocyte protease inhibitor
SMPDB	Small Molecule Pathway Database
sO2	Oxygen saturation
SOD1	Superoxide dismutase
SPAD	Single-Pass Albumin Dialysis
SST	Serum separator tube

TCEP	Tris(2-carboxyethyl)phosphine
TEAB	Triethylammonium bicarbonate
TGF-β	Transforming growth factor-β
THBD	Thrombomodulin (gene)
TM	Thrombomodulin (protein)
TMG	Trial Management Group
TMT	Tandem mass tag
TNFAIP3	Tumour necrosis factor, alpha-induced protein 3 (gene)
TNF-α	Tumour necrosis factor-α
TSC	Trial Steering Committee
TTP	Thrombotic thrombocytopenic purpura
TX	Texas
UK	United Kingdom
UKCA/CE	UK Conformity Assessed/Conformité Européenne
USA	United States of America
UW	University of Wisconsin
V_{Texp}	Expired tidal volume
vWF	von Willebrand factor
WATOK	Welfare of Animals at the Time of Killing
WI	Wisconsin
α1,3GT	α(1,3)galactosyltransferase
αGal	galactose-α-1,3-galactose
β4GalNT2	β-1,4-N-acetyl-galactosaminyltransferase 2
%CV	Coefficient of variance
10GE	10-gene edited
3KO.7TG.RI	Triple glycan knock-out; seven human transgene insertion; porcine endogenous retrovirus inactivation

Acknowledgements

I would like to thank my supervisors, Professor Peter Friend, Professor Constantin Coussios, and Sadr Shaheed for their insight, wisdom, dedication, and mentorship.

This work would not have been possible without the support, inspiration, and friendship of the transplant research fellows (John Fallon, Mohamed Elzawahry, Richard Dumbill, Hussain Abbas, Fungai Dengu, Hatem Sadik, Kazuyuki Gyoten, Georg Ebeling, Tamsyn Clark, Faysal Elgilani); the members of the Transplant Research Group (Professor Rutger Ploeg, Professor James Hunter, Letizia Lo Faro); Stephen Greenhalgh, Professor Eddie Clutton, and the staff at the Large Animal Research and Imaging Facility, University of Edinburgh; Professor Abraham Shaked, Professor Kim Olthoff, Professor Peter Reese (now at Vanderbilt University), and Professor Peter Abt at the University of Pennsylvania; the staff of the Gift of Life Donor Program; the OrganOx team (Chris Morris, Dan Fower, Jerome Hunckler (now at Spectrum Medical), Daniel Voyce, Andy Self, Craig Marshall); the eGenesis team (Susan Low, Leanne Lanieri, Mike Curtis, and the staff at eGenesis-WI and Accuro Farms); the ELC-1 co-investigators (Professor Will Bernal, Simon Knight, David Nasralla, Brian Hogan, Jess Scaife, in addition to Professor Peter Friend and Andy Self) and Patient and Public Involvement representatives; Tim James at the Department of Clinical Biochemistry, Oxford University Hospitals; the staff at the Long Compton Abattoir; and the staff at the Biomedical Sciences Building, University of Oxford.

I am deeply grateful to my family: my partner, Subin; my parents; my sister, Becky; and my brother, Andy. Their enthusiasm, understanding, and belief have been fundamental.

This thesis has been supported by a Clinical Research Training Fellowship provided by the Medical Research Council. The experiments have received additional funding from the Oxford Transplant Foundation; Oxfordshire Health Services Research Committee; OrganOx; and eGenesis.

Abstract

There is no temporary hepatic support system that can effectively bridge patients with acute or acute-on-chronic liver failure to either recovery of native liver function or allotransplantation. This has not been achieved by artificial and bio-artificial systems because they do not replicate the many, diverse, and deeply integrated roles of an intact liver. Extra-corporeal liver cross-circulation (ELC), in which a patient's blood is perfused through a donor liver maintained outside the body on a machine, may provide more comprehensive replacement of hepatic function, whilst avoiding the limitations of auxiliary transplantation. A novel ELC system ('*metra*-ELC') has been developed and evaluated.

The donor liver is initially perfused in isolation before connecting to the recipient. During this phase, the perfusate composition must be made safe for connection. Three techniques of *ex situ* renal replacement therapy during liver perfusion were assessed for this purpose: haemofiltration using a haemoconcentrator was selected for the *metra*-ELC protocol.

The *metra*-ELC system was tested with healthy pigs for 3 hours, confirming feasibility *in vivo*. It was then evaluated for 24 hours using allografts to treat pigs with acute liver failure. ELC effectively replaced lost native hepatic detoxification, excretory, synthetic, metabolic regulatory, and immunologic functions.

Xenografts are a potential source of livers for ELC. Transgenic pig livers (triple glycan knock-out; seven human transgene insertion; porcine endogenous retrovirus inactivation) were perfused *ex situ* for 48 hours with pooled human whole blood, demonstrating preserved graft viability. The mechanism of liver xenograft-associated thrombocytopenia was investigated during short-duration *ex situ* isolated xenoperfusion.

ELC using transgenic pig livers was examined in the decedent model. Decedents 1 – 3 underwent ELC with their native livers intact for 72 – 84 hours. The xenografts consistently produced bile and augmented hepatocellular function. Decedent 4 underwent native hepatectomy, followed by 48 hours of ELC with maintenance of normal lactate, pH, international normalised ratio, ammonia, and minimal vasopressor requirement. Thrombocytopenia developed within one hour of starting ELC in all decedents.

Phase-1 clinical trials will determine the safety and feasibility of the *metra*-ELC system using (i) deceased donor human livers declined for transplantation and (ii) transgenic pig livers to support patients with acute-on-chronic liver failure.

1 Introduction

1.1 Background

Liver failure is distinguished from failure of other organs by the absence of a therapy that can replace the role of the failing native organ, even temporarily. Whilst patients with renal, cardiac, and pulmonary failure may be supported with dialysis, ventricular assist devices, and extra-corporeal membrane oxygenation (ECMO)[7-9], no equivalent exists for patients with liver failure. An effective temporary liver support system could bridge patients with acute or acute-on-chronic liver failure (ALF, ACLF) to either recovery of native liver function or allotransplantation. These conditions carry very high early mortality rates, with few liver-directed treatment options short of transplantation.

The fundamental difference between these other organs and the liver is that the organ functions that sustain life can be replicated by an artificial system, at least in the short term. The earliest renal dialysis systems could filter the blood of a patient with renal failure across a semi-permeable membrane to correct life-threatening hyperkalaemia. Such primitive systems could keep patients with renal failure alive temporarily. Subsequent innovations have resulted in modern dialysis machines that can modulate electrolytes, acid-base balance, and fluid status, and thereby keep patients alive for years[7].

Artificial liver support systems, typically based on albumin dialysis ± damage/pathogen-associated marker adsorption, cannot maintain patients with liver failure even for a matter of days. Whilst they can improve select biochemical parameters (ammonia, bilirubin, international normalised ratio (INR)) and inflammatory markers (cytokines, endotoxin), this does not translate into improved survival in ALF or ACLF[10-14]. A corollary is that the life-sustaining functions of the liver extend beyond the classically measured biochemical hallmarks.

The functions of the liver are performed across multiple cell types (*table 1*) and comprise an array of biochemical pathways. They can be broadly categorised as: metabolic, detoxification, synthetic, excretion, reticulo-endothelial, immunologic, and vitamin/nutrient storage. These are deeply embedded within the wider physiology of the organism; often the role of the liver is central

regulation[15]. These factors (multiple life-sustaining functions across varied domains that must be dynamically integrated with the rest of the body) explain why short-term artificial liver support systems have failed. This also explains why standard biochemical markers of liver failure (e.g. ammonia, bilirubin, INR) have not correlated with therapeutic effect: many of the life-sustaining functions of the liver are not captured by these measures. That the life-sustaining roles of a liver are undertaken by multiple cell types explains why bio-artificial liver support systems (based on cultures of hepatocytes) have failed to improve survival in ALF and ACLF[16-18].

This leads to the hypothesis that effective liver support requires a whole liver. This is seen in the striking impact of liver transplantation on patients with ALF or ACLF[19,20]. This concept underlay initial attempts at cross-circulation between a patient with ALF and a blood type-matched healthy volunteer in the 1960s[21,22]. Liver transplantation is frequently not possible due to a combination of disease severity, acuity, and co-morbidity, compounded by the shortage of suitable donor organs[1,20,23,24]. Cross-circulation with a healthy volunteer is neither practical nor, due to the significant risks incurred by the volunteer, ethically acceptable.

Auxiliary liver transplantation, in which the native liver remains *in situ*, offers a potential temporary solution for patients with ALF. The aim is to support patients long enough for their native liver to recover. Since only temporary graft function is required, this could, theoretically, enable use of donor allografts that would not achieve acceptable graft survival if used for standard transplantation or, potentially, xenografts. However, auxiliary heterotopic implantation is a major surgical procedure for an unwell patient that carries excessive risk if the plan is to subsequently remove the graft[25-28].

Extra-corporeal liver cross-circulation (ELC) is a technique in which blood from the systemic circulation of a patient is treated by perfusion through a donor liver maintained under close-to physiological conditions on a machine outside the body. In the context of temporary hepatic support, this has a number of advantages over auxiliary transplantation: (i) ELC is minimally invasive (requiring only venous cannula insertion); (ii) the extra-corporeal liver can be continuously monitored to detect deterioration early; (iii) ELC can be stopped safely and immediately if necessitated by the clinical situation. ELC is not a new idea. Previous attempts at ELC more than two decades ago were limited by primitive perfusion technology and 90% of the donor livers being wild-type xenografts[29].

Two main developments now address these issues: (i) modern organ perfusion technology reliably supports multi-day perfusion[30,31]; (ii) improved access to donor livers (human donor livers declined for transplantation[32] and transgenic porcine livers[33]) that can provide short-term hepatic function. This thesis revisits the concept of ELC in the context of these developments.

Liver cell type	Functions of systemic relevance
Hepatocyte	Carbohydrate metabolism • glycogenesis; glycogenolysis; gluconeogenesis; fructose and galactose metabolism Lipid metabolism • β-oxidation; de-novo lipogenesis; ketogenesis; cholesterol and lipoprotein synthesis Protein/amino acid metabolism • urea cycle (ammonia detoxification); trans-/de-amination; plasma protein synthesis Bile production • bilirubin conjugation; secretion of bile acids, phospholipids, cholesterol Phase I and II bio-transformation of drugs, hormones, xenobiotics • cytochrome P450-mediated oxidation/reduction followed by conjugation Storage • glycogen; triglycerides; vitamin D, $B_{12;}$ trace metals (iron, copper) Hormone metabolism • T_4 -> T_3 conversion; inactivation of insulin, glucagon
Cholangiocyte	Bile modification • secretion of bicarbonate, water; reabsorption of bile acids, glucose
Liver sinusoidal endothelial cell	Ultrafiltration • fenestrated endothelium permits plasma into space of Disse Scavenging • high endocytic capacity to remove soluble macromolecules, nanoparticles 'Tolerogenic' antigen presentation • immune tolerance to gut-derived antigens
Kupffer cell	Phagocytosis • senescent erythrocytes, pathogens, endotoxin, damage-associated molecular patterns Cytokine production • orchestrate innate immune response of liver Immunosurveillance • antigen presentation
Hepatic stellate cell	Vitamin A storage
Pit cells/Natural killer cell	Cytotoxic defence • virus-infected, malignant cells Cytokine release • IFN-γ, TNF-α modulate Kupffer and stellate cell response
Dendritic cell, T-/B-cell	Adaptive immunity Tolerance induction

Table 1: key functions of the liver with systemic relevance according to cell type[34-41].
T_4: thyroxine. T_3: triiodothyronine. IFN-γ: interferon-γ. TNF-α: tumour necrosis factor-α.

1.2 Acute liver failure

ALF is defined by the European Association for the Study of the Liver (EASL) as: rapid development of coagulopathy (INR > 1.5) and hepatic encephalopathy secondary to severe liver injury in the absence of pre-existing liver disease[42,43]. The period between onset of jaundice and development of encephalopathy varies according to the definition chosen (e.g. 1 week for hyperacute liver failure, 1 – 4 weeks for acute liver failure, and 4 – 12 weeks for subacute liver failure[44]). The incidence is 1 – 8 cases per million person years in the United Kingdom (UK) (70 – 540 cases per year) [45-47].

The most common cause in the UK is paracetamol overdose, with other causes including viral infections, drug-induced liver injury, acute ischaemic injury, and autoimmune hepatitis[43,48]. The pathophysiology of ALF combines loss of normal hepatic function with activation of a systemic inflammatory response that results in multi-organ failure.

Loss of detoxification function results in accumulation of endogenous and exogenous toxins, including those bound to albumin[49,50]. Hepatic encephalopathy occurs due to a combination of hyperammonaemia (secondary to loss of the hepatic urea cycle), additional neurotoxins, increased cerebral endothelial permeability, and altered cerebral blood flow[45]. Loss of excretory function causes jaundice and reduced toxin clearance. Loss of synthetic function results in failure to produce a range of proteins; clinically, this is assessed by monitoring INR (a measure of coagulation factors II, VII, IX, X), although reduced production of both pro- and anti-thrombotic factors means that a bleeding tendency is not typically observed[51].

Hepatocyte necrosis releases damage-associated molecular patterns (DAMPs) (e.g. cell-free DNA, histones, high mobility group box-1 (HMGB1)), whilst pathogen-associated molecular patterns (PAMPs) may derive from gut organisms (normally extracted by the hepatic reticulo-endothelial system) and heterotopic viruses. DAMPs and PAMPs establish an innate immune response both locally and systemically that generates reactive oxygen species and pro-inflammatory cytokines (e.g. TNF-α, interleukin-1β). This recruits immune cells to the liver, furthering hepatic damage, and reduces systemic vascular resistance, causing hypotension. Microcirculatory vasodilatation impairs pulmonary oxygen exchange and oxygen delivery to tissues, contributing to lactic acidosis. The effect on renal vascular tone in combination with hypotension contributes to acute kidney injury, which occurs in 70% of patients with ALF[43,52]. Systemic inflammation may induce mitochondrial dysfunction in peripheral organs[2].

A late compensatory anti-inflammatory response combined with loss of hepatic immunologic and reticulo-endothelial function increases susceptibility to infection. Interleukin-4, interleukin-10, and TGF-β impair monocyte and neutrophil function. Reduced hepatic synthesis of complement proteins

impairs immune cell recruitment and encapsulated organism clearance, whilst reduced fibronectin production impairs Kupffer cell clearance of microbes[52].

Medical management primarily comprises intensive supportive therapy of the major organ systems[45]. The underlying aetiology is treated where possible, however liver-directed therapies, except N-acetylcysteine infusion in paracetamol overdose, are limited[42]. Liver transplantation is potentially curative. Those least likely to survive with medical management alone are identified based on scoring systems (e.g. UK revised criteria[45]; APACHE II > 19; Model for End-Stage Liver Disease (MELD) score > 32), aetiology (drug-induced liver injury; autoimmune hepatitis; hepatitis B reactivation; acute Budd-Chiari), and clinical status (hepatic encephalopathy grade III-IV; multi-organ failure)[53].

28-day survival in ALF is 70%, with deaths skewed to early timepoints[43]. In large cohort studies, about 35% of patients with ALF are listed for transplant[48,54]. Of those listed, about two-thirds receive a transplant after a median of 3 days[24] with a 30-day survival post-transplant of 86%[55]. Less severe organ failure at the time of transplant is associated with better post-transplant outcome[56,57]. 15% of those listed die without receiving a transplant and 20% recover spontaneously[58]. The median time-to-death of listed patients who do not receive a transplant is 3 days[54].

1.3 Acute-on-chronic liver failure

ACLF occurs when acute decompensation of pre-existing liver disease leads to (multi-)organ failure[1]. As with ALF, multiple definitions exist. The EASL-Chronic Liver Failure (EASL-CLIF) consortium defines ACLF as: occurrence of organ failure (per the modified Sequential Organ Failure scoring system (CLIF-SOFA)) in a patient with acutely decompensated (ascites, encephalopathy, and/or gastrointestinal haemorrhage) cirrhosis[1] (*figure 1*).

The UK incidence of ACLF is not directly reported. However the prevalence of cirrhosis in England and Wales is 60,000[59], 12% of whom decompensate annually[60]. 31% of decompensated patients develop ACLF[1], giving an estimated UK ACLF incidence of 2,500/year. The Intensive Care National Audit & Research Centre (ICNARC) reports that in the UK 3,500 – 4,500 patients are admitted to critical care with a background of severe liver disease annually[61-63]. The incidence of ACLF is

approximately 10 times that of ALF. The annual intensive care costs amount to approximately £50 million in the UK[64] and $3 billion in the USA[65].

Alcohol-related liver disease and hepatitis C infection are the most common underlying liver disease aetiologies[66], although the proportion due to hepatitis C is falling with improved anti-viral therapies[67]. In 60% of ACLF cases, acute deterioration is triggered by an identifiable precipitating event, most commonly bacterial infection, acute alcoholic hepatitis, or gastrointestinal haemorrhage[1,2,68]. These stimulate intense systemic inflammation that is the defining characteristic of ACLF and correlates with disease severity[69]. In patients in whom a trigger cannot be identified, the cause may be translocation of bacterial by-products from the intestinal lumen[70]. DAMP- and PAMP-driven inflammation result in (multi-)organ failure through direct tissue injury[71] and mitochondrial dysfunction[72]. Nitric oxide-driven splanchnic vasodilatation results in homeostatic activation of endogenous vasoconstrictors to maintain the effective arterial blood volume. In turn, this triggers renal vasoconstriction, hypoperfusion, and type 1 hepatorenal syndrome[73,74].

Treatment is primarily intensive supportive medical care combined with addressing the precipitant. In a large multi-centre cohort study, 28-day survival was 67%[1]. Mortality in ACLF is skewed towards the first week[2] and severity of organ failure at days 3 – 7 from ACLF onset predicts outcome better than severity at onset[75]. For those that survive to hospital discharge, the 12-month transplant-free survival rate is 61%[76].

Fewer ACLF patients receive urgent transplants compared to those with ALF: a multi-centre observational study found that 9% received a transplant within 28 days[1], whilst on meta-analysis only 3.8% of patients with cirrhosis admitted to critical care received a transplant within 6 months[20]. For patients with ACLF grade II – III who are not transplanted, 28-day survival is only 23%[75].

Reasons for the lower transplant rate in ACLF include increased age, co-morbidities, uncontrolled infection, and active alcohol use[20]. However, there is increasing recognition that carefully selected patients can benefit substantially from transplantation. In the UK, highly selected patients with severe ACLF (predicted 28-day survival < 50%) but favourable post-transplant prognostic factors are eligible for waitlist prioritisation. During a 30-month prospective study, 52 patients were listed (about 1% of all patients with ACLF in the UK during that time). 81% of patients received a transplant at a median

of 2 days with 28-day post-transplant survival of 93% and 12-month survival of 77%. As observed in ALF[56,57], improved physiological status at the time of transplant was associated with better post-transplant outcomes. 19% did not receive a transplant, all of whom died after a median of 7 days[20].

Organ System	1 Point	2 Points	3 Points
Liver	Bilirubin <6 mg/dl	Bilirubin 6.0–11.9 mg/dl	Bilirubin ≥12 mg/dl
Kidney	Creatinine <1.5 mg/dl Creatinine 1.5–1.9 mg/dl	Creatinine 2.0–3.4 mg/dl	Creatinine ≥3.5 mg/dl or RRT
Brain (West Haven criteria)	Grade 0	Grade 1–2	Grade 3–4
Coagulation	INR <2.0	INR 2.0–2.4	INR ≥2.5
Circulation	MAP ≥70 mm Hg	MAP <70 mm Hg	Vasopressor requirement
Respiration	Pao_2/Fio_2 >300 Spo_2/Fio_2 >357	Pao_2/Fio_2 201–300 Spo_2/Fio_2 215–357	Pao_2/Fio_2 ≤200 Spo_2/Fio_2 ≤214

Patient Group	Prevalence	28-Day Mortality	Assigned Grade
	% of patients		
Absence of OF	68.3	4.4	Absence of ACLF
Single, nonkidney OF without KD or BD	9.9	6.3	
Single KF	6.7	18.6	ACLF-1
Single, nonkidney OF with KD or BD	4.2	27.8	ACLF-1
Two OFs	7.5	32.0	ACLF-2
Three OFs	1.9	68.0	ACLF-3
Four to six OFs	1.4	88.9	ACLF-3

Figure 1: European Association for the Study of the Liver-Chronic Liver Failure (EASL-CLIF) consortium organ-failure score (left), used to define acute-on-chronic liver failure diagnosis and grade (right).
Light grey denotes organ dysfunction. Dark grey denotes organ failure. OF: organ failure. KD: kidney dysfunction; BD: brain dysfunction[1,2].

1.4 Artificial liver support systems

Artificial liver support systems usually combine renal replacement therapy (to remove small molecule toxins), albumin dialysis (to clear albumin-bound toxins) ± DAMP/PAMP adsorption (*table 2*).

In the Molecular Adsorbent Recycling System (MARS), the patient's blood passes over an albumin impermeable 50 – 60 kDa cut-off membrane, which is dialysed against a solution containing 20% exogenous albumin. Molecules < 50 – 60 kDa pass through the membrane down their concentration gradients and albumin-bound toxins are transferred to the toxin-free exogenous albumin. The latter is detoxified by passing through charcoal and anion exchange columns, and recycled. Small molecules are removed by passage through a standard dialysis circuit[77-79]. A randomised controlled trial (RCT) (*n = 102*) demonstrated that MARS therapy in ALF patients improved bilirubin, haemodynamics, and encephalopathy, but a survival benefit was not shown. The short time between treatment initiation and transplantation (median: 16.2 hours) may have limited the power of the study to demonstrate survival benefit[80]. Secondary analysis did find that patients who received ≥ 3 MARS sessions (*n = 16*) had improved survival compared to those who received < 3 sessions, suggesting that ≥ 3 MARS sessions

may be considered for ALF patients who are not candidates for LT[53]. An RCT of MARS in 189 ACLF patients found that whilst MARS improved creatinine, bilirubin, and encephalopathy, again this did not translate into a survival benefit. MARS did not lower the level of circulating cytokines[11].

The Prometheus device first fractionates blood by filtering out plasma and proteins < 250 kDa. The separated plasma is then purified by passing through natural resin and anion-exchange columns, before passing through a standard renal replacement therapy circuit and returning to the patient. In the HELIOS RCT of 145 ACLF patients, Prometheus reduced serum bilirubin but did not increase overall survival. In subgroup analysis, survival was improved for patients with type-1 hepatorenal syndrome and those with MELD score > 30[12].

Single Pass Albumin Dialysis (SPAD) uses a standard renal replacement therapy circuit with a dialysate containing 4% albumin to extract toxins from the patient's albumin. A small RCT compared SPAD to MARS. Both treatments reduced bilirubin. MARS additionally reduced bile acids, creatinine, and urea, whilst SPAD was associated with increased metabolic derangements and electrolyte disturbances[81].

In the DIALIVE device, blood first passes through a filter that performs ultrafiltration to remove albumin and cytokines. The extracted albumin is replaced with 20% exogenous albumin. A second filter adsorbs DAMPs (e.g. HMGB1) and PAMPs (e.g. endotoxins) before the blood is returned to the patient. An RCT of 30 patients with alcohol-related ACLF was performed, with patients receiving a minimum of three 8 – 12-hour sessions. Treated patients demonstrated a reduction in endotoxaemia, biomarkers of systemic inflammation, and improvement in albumin function. Degree of organ failure and time to resolution of ACLF were reduced, with no difference in overall survival[14]. A subsequent trial of DIALIVE is due to commence recruitment in 2025.

There are two active clinical trials of an artificial liver support system, Double Plasma Molecular Adsorption System (DPMAS), which adsorbs bilirubin and cytokines: NCT05030571 (Thailand); NCT05129904 (China)[82,83].

The fundamental limitation of artificial liver support systems is that, though they may remove harmful substances associated with liver failure (toxins, DAMPs, PAMPs), they cannot replace the synthetic, metabolic, immunologic, and regulatory functions of an intact liver.

Therapy	Sample size (ITT)	Pathology	Number of sessions	Session duration (hrs)	Total duration (hrs)	Blood exchange (mL/min)	Bili	INR	NH_3	Cr	HE	Vasopressor	TFS	OS
MARS[11]	95 *v.* 94	ACLF	6.5	6.8	42.3	100 – 250	↓	—	NR	↓	—	NR	—	—
Prometheus[12]	77 *v.* 68	ACLF	8.1	5.7	46.2	200	↓	—	NR	—		NR	—	—
SPAD[81]	32 (cross-over)	ALF, ACLF, PNF	2.0	7.25	14.5	100 - 150	↓	—	—	—	—	—	NR	NR
DIALIVE[14]	17 *v.* 15	ACLF	3.0	8.0	24	200	↓	↓	—	↑	↓	—	NR	—
ELAD[16]	96 *v.* 107	SAH	Cont.	Cont.	≤ 120	≤ 200	↓	—	NR	—	NR	NR	NR	—
HepatAssist[84]	85 *v.* 86	ALF, PNF	2.9	6	17.4	100[85]	↓	—	—	—	—	—	—	—
HV PLEX[86]	92 *v.* 90	ALF	2.4	6	14.4	~ 50	↓	↓	↓	↓	NR	↓	↑	↑
SV PLEX[87]	20 *v.* 20	ALF	2.15	3.9	8.4	~ 40	↓	↓	↓	—	NR	NR	↑	↑
SV PLEX[88]	120 *v.* 258	ALF	3	NR	NR	NR	—	↓	↓	↓	NR	↓	—	—

Table 2: liver support device clinical trials: clinical impact.
Intervention *versus* control where applicable. Impact assessed as significant if p-value < 0.05. Average values quoted (mean or median depending on which provided by the study). Total duration calculated by multiplying average session duration by average number of sessions. ITT: intention-to-treat. Bili: bilirubin. INR: international normalised ratio. NH_3: ammonia. Cr: creatinine. HE: hepatic encephalopathy. TFS: transplant-free survival. OS: overall survival. HV PLEX: high-volume plasma exchange. SV PLEX: standard-volume plasma exchange. ALF: acute liver failure. ACLF: acute-on-chronic liver failure. PNF: primary non-function. SAH: severe alcoholic hepatitis. Cont.: continuous. NR: not reported.

1.5 Bio-artificial liver support systems

In bio-artificial devices, plasma is separated from the patient's blood and passed through a hollow-fibre dialysis chamber containing hepatocytes: human hepatoblastoma cells lines (HepG2/C3A) in the case of Extra-corporeal Liver-Assist Device (ELAD) and porcine hepatocytes in the case of HepatAssist. Plasma flows through the hollow fibres in free exchange with the extra-capillary space that contains the hepatocytes (*table 2*).

A 2004 RCT assessed the treatment of patients with ALF and primary non-function post-transplant with HepatAssist (*n = 171*). The study found no improvement in overall survival. However, subgroup analysis of patients with ALF (*n= 147*) combined with a co-variate time-dependent proportional hazard model (to take account of the effect of transplantation) found a possible survival benefit with

HepatAssist (*RR = 0.56, p = 0.048*)[84]. A phase-3 RCT of 203 patients with severe alcoholic hepatitis failed to demonstrated an overall survival benefit with ELAD (*51.0% vs 49.5%, p = 0.90*)[16]. Bio-artificial devices are currently limited for a number of reasons[17]. Hepatocytes alone cannot reproduce the many non-hepatocyte roles of a liver: whilst specific hepatocyte function (e.g. ammonia detoxification) may be preserved, many of the roles of a liver (e.g. excretory, reticulo-endothelial, and immunologic function) are not. In a porcine ALF model, a bio-artificial liver worsened conjugated bile acid accumulation due to lack of biliary excretion[89]. Primary human hepatocytes, the cell of choice, are limited in availability and quality; hepatoblastoma lines are less functionally active than primary hepatocytes and porcine hepatocytes are associated with the risks of xenotransplantation including physiological incompatibilities and zoonotic transmission. The hepatocytes are maintained in culture, lacking the neuro-humoral and paracrine stimuli that influence function; physiological zonal differentiation of hepatocyte function within the hepatic lobule is not replicated. The challenge of generating adequate hepatocyte mass for effective treatment, as well as the cost of the equipment involved, are additional hurdles to clinical adoption[17].

1.6 Plasma exchange

Plasma exchange exchanges patient plasma with allogenic fresh frozen plasma in discrete (usually daily) sessions (*table 2*). High-volume plasma exchange exchanges a plasma volume of 15% ideal body weight (8 – 12 L) per session. An RCT of high-volume plasma exchange (up to 3 consecutive sessions) in 182 ALF patients demonstrated an improvement in overall hospital survival (*58.7% vs. 47.8%; p = 0.008; HR 0.56; 95% CI 0.36–0.86*)[86], associated with a reduction in circulating cytokines and DAMPs[86]. Standard volume plasma exchange (1.5 – 2.0 plasma volumes (about 5 L) per session) also improved 21-day transplant-free survival in ALF (*75% vs. 45%; p = 0.04, HR 0.30, 95% CI: 0.01-0.88*), associated with reduced DAMPs, endotoxin, and modulation of the innate immune response[87]. Studies of plasma exchange in ACLF have largely been limited to patients with hepatitis B in China[90,91], in which different ACLF criteria make extrapolation challenging[92].

Although plasma exchange may improve the patient's plasma composition during a session, transient improvements in the plasma proteome deteriorate between sessions[93]. Plasma exchange does not replace the role of the liver: 'off-the-shelf' plasma cannot respond dynamically to the patient's physiology or perform the regulatory functions of a liver. In a real-world cohort of 378 consecutive ALF patients admitted to the UK tertiary liver centres, plasma exchange was associated with a reduction in vasopressor requirement but did not improve overall or transplant-free survival[88].

1.7 Auxiliary liver transplantation

Auxiliary liver transplantation has been used as a bridge to native liver recovery in ALF. The aim is to allow the native liver to recover and then withdraw immunosuppression. Auxiliary transplantation can be orthotopic (in which case a partial native hepatectomy is performed) or heterotopic. Auxiliary transplant is not generally considered in ACLF, where the presence of underlying cirrhosis favours standard orthotopic liver transplant.

A single-centre retrospective cohort study of 48 ALF patients who underwent orthotopic auxiliary liver transplantation reported a 1-year survival rate of 81%. Of patients surviving greater than 12 months and avoiding re-transplantation (*n = 39*), 82% achieved complete immunosuppression withdrawal after a median of 29 months; none underwent graft explant. However, this is a complex operation to perform in severely unwell patients: 47.9% of patients experienced Clavien-Dindo ≥ IIIa complications and 25% required re-laparotomy. Moreover, only grafts that would have been suitable for standard orthotopic liver transplantation were selected (brain-dead donors of median age 44 years)[26]. Heterotopic auxiliary transplantation avoids the need to resect part of the native liver, but is complicated by the constraints of space that predispose to vascular compression, as well as suboptimal venous drainage of the graft due to anastomosis lower on the inferior vena cava (IVC). This technique is rarely performed[27,28].

The fundamental limitations of auxiliary transplantation for ALF are: (i) surgical complexity of implantation and (ii) requirement for graft quality comparable to that for standard orthotopic transplantation (to avoid the risk of subjecting the recipient to major explant surgery shortly after

implantation). The latter necessitates competition for grafts that could be used for standard orthotopic liver transplantation.

1.8 Normothermic machine perfusion of the liver

Normothermic machine perfusion of the liver was developed as a technique of organ preservation prior to transplantation. Organ preservation is traditionally achieved by static cold storage, in which the liver is preserved in an organ preservation solution at approximately 4°C. The liver is ischaemic during this period. The cold temperature reduces metabolic activity, but adenosine triphosphate (ATP) is depleted and metabolites (e.g. succinate) accumulate that generate reactive oxygen species on reperfusion and contribute to ischaemia-reperfusion injury (IRI)[94].

During normothermic perfusion, the liver is provided with blood, oxygen, and nutrients at body temperature. Cellular respiration is maintained, which replenishes ATP stores and reduces the accumulation of reactive oxygen species-generating toxins. Compared to static cold storage, normothermic liver preservation is associated with reduced early allograft injury, despite longer preservation times and increased organ utilisation[95].

Maintenance of metabolic activity enables assessment of graft viability during organ preservation. A variety of viability criteria exist, which consider aspects of liver function that include: glucose metabolism; lactate clearance; pH maintenance; bile production; and hepatocellular damage[96]. Measurement of these parameters during perfusion can predict graft function after implantation: for example, perfusate 2-hour alanine transaminase (ALT), 2-hour lactate, bicarbonate requirement during first 4 hours, and peak bile pH correlate with post-transplant Model for Early Allograft Function (MEAF) score[3]. Assessment of cholangiocyte bicarbonate secretion and glucose reabsorption by measuring bile pH and glucose concentration informs the decision to decline livers at highest risk of non-anastomotic strictures, although there remains a significant false-negative rate[97]. Prediction of primary non-function is statistically limited by its low incidence[3].

During normothermic perfusion in pre-clinical studies, livers maintained function across the range of *in vivo* hepatic physiology over multiple days:

(i) Metabolic: glucose and lactate metabolism; appropriate response to glucagon and insulin[30].

(ii) Detoxification: ammonia conversion to urea[98]; cytochrome P450 activity[99].

(iii) Excretion: bile production; bilirubin excretion[30].

(iv) Synthetic: factor V, factor VII, fibrinogen, and anti-thrombin synthesis[100].

(v) Immunologic: complement synthesis[98].

(vi) Reticulo-endothelial: clearance of free haemoglobin[30].

Several groups have now perfused human livers for ≥ 7 days[30,101], and a liver has been transplanted after 3 days of normothermic preservation[31].

1.9 Extra-corporeal liver cross-circulation

The capacity of a liver to maintain a range of hepatic function over multiple days during *ex situ* normothermic perfusion provides the rationale for ELC. Blood from the systemic circulation of a patient is exchanged with a donor liver perfused on a machine outside the body (*figure 2*). The aim is for the donor liver to temporarily replace the life-sustaining functions of the patient's failing liver until either it recovers or transplantation becomes possible.

The hypothesis is that a whole perfused liver provides more comprehensive replacement of lost hepatic function than can be achieved with artificial and bio-artificial systems, whilst avoiding the major surgery associated with auxiliary transplantation. Since only temporary function is required and the system can be immediately disconnected if required, grafts with time-limited function (e.g. sub-optimal human donor livers and xenografts) may be used.

131 ALF patients underwent ELC between 1964 and 2001 with mixed results: neurologic improvement in 67%, survival > 48 hours in 59%, and complete recovery in 40%. Long-term survival was 28%. However, the majority of these cases occurred before 1990. Results after 1990 were better: neurologic improvement 82%, survival > 48 hours 64%, complete recovery 64%, and long-term survival 56%[29].

Several aspects contributed to poor outcomes: number of cases per centre was low; median perfusion duration was 5 hours; and only 11% of cases used human donor livers, with the remainder using wild-

type xenografts. Improved outcomes were associated with use of human/baboon livers and ELC duration greater than 10 hours[29].

Table 3 describes the clinical cases that have been reported since 1990. The largest case series assessed ELC as a bridge to allotransplantation[102]. 14 ALF patients with grade 4 encephalopathy underwent ELC with a total of 16 livers (8 human; 8 wild-type porcine). The median perfusion durations were 55 and 36 hours for the human and pig livers, respectively. Plasma ammonia improved with both types of graft, but only human livers achieved sustained improvement in bilirubin. In contrast to human livers, the pig livers demonstrated centrilobular necrosis with mild deposition of IgM, IgG, and complement. Plasma-only (rather than whole-blood) ELC exchange was used for 6/8 pig liver perfusions, but was not associated with improved histological appearance or function. 9/14 patients (64%) were successfully bridged to transplant, and 7/9 (78%) of these were alive at a median of 48 months follow-up. For four of the five patients who died, it was decided during ELC that transplantation was contra-indicated and support was withdrawn[102].

A single study describes ELC using transgenic porcine livers, which expressed human complement regulatory proteins (CD55, CD59). Two patients with ALF were treated with ELC for short durations (6.5 – 10 hours), and bridged to allotransplantation. In both cases, ELC improved haemodynamic stability and bilirubin, with variable effects on INR and ammonia. Xenograft biopsies at the end of ELC demonstrated moderate-severe sinusoidal haemorrhage with minimal hepatocyte necrosis and abundant platelet aggregates. Infiltrating immune cells were mainly neutrophils with mild T-cell-predominant lymphocytic infiltration. There was IgG and C3 deposition in both cases, and IgM deposition in one case[103].

No further clinical studies have been published since 2001. These early studies suggest the potential for ELC. Several advances make ELC a potentially viable therapy in the current era.

Clinical experience with liver perfusion has expanded significantly in recent years: 32% of donor livers in the UK now undergo machine perfusion[104]. Modern normothermic perfusion technology reliably supports multi-day perfusion (although currently mainly in the pre-clinical setting)[30,31,101]; at least several-day connection is likely required for consistent clinical benefit in ALF and ACLF. Perfusion durations achievable during ELC may exceed those during *ex situ* perfusion due to

restoration of physiological support for the graft (e.g. endocrine influences of vascular and hepatocyte function; waste product management; exposure to metabolic stimuli; improved red blood cell oxygen handling[105]). This concept has been demonstrated experimentally to improve *ex situ* normothermic preservation of declined human donor livers by performing ELC with an anaesthetised wild-type pig[106].

Since earlier studies of ELC, the National Organ Retrieval Service (NORS) now co-ordinates human livers declined for transplantation in the UK, which will facilitate their use for ELC[32]. Graft function can be predicted based on behaviour during normothermic perfusion; modification of the criteria used to determine suitability for transplant[3] could select which declined livers may provide effective ELC before connecting to a patient. In parallel, advances in CRISPR-cas9 gene editing have generated porcine donors with multiple gene edits that enhance compatibility with humans[107]. These edits make short-term liver xenografting, using an immunosuppression regimen suitable for patients with ALF and ACLF, a realistic prospect.

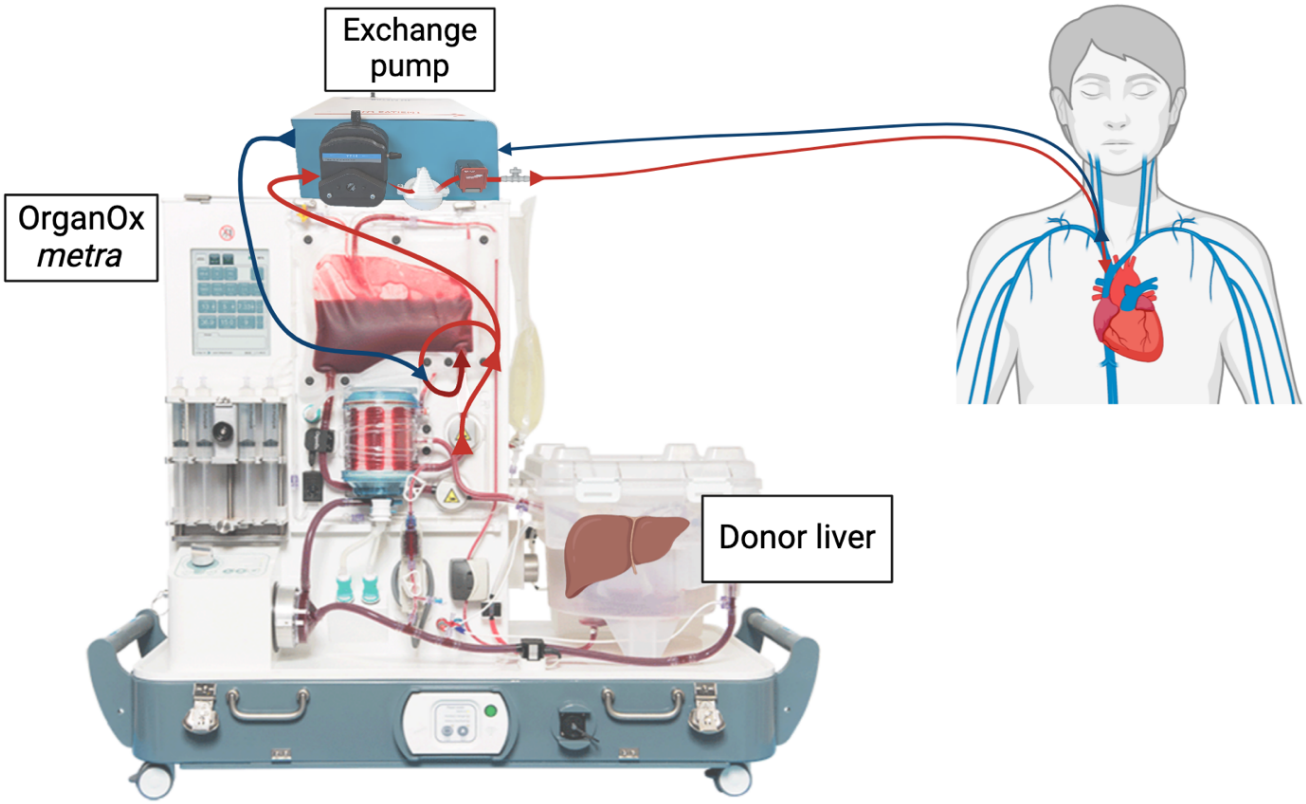


Figure 2: *metra*-ELC system.

Study	*N*	Pathology	Donor	ELC duration (hrs)	Exchange vessels	ELC circuit	Graft perfusion (mL/min)	Perfusion vessels	Anticoag.	Bili	INR	NH_3	Cr	HE	MAP	Plts ($x10^9$/L)	TEAE	Outcome
Fox[108]	*3*	ALF	Human (*3*)	47 (20-72)	V-V	NR	NR	NR	NR	↓	NR	↓	NR	↓/—	NR	NR	NR	2/3: OLT 1/3: death
Chari[109]	*4*	ALF, ACLF, PNF	Pig (*8*)	3.6 (0.5-6)	V-V	Direct (with bypass)	500-700/kg	PV	Heparin (ACT 200)	↓	NR	↓	NR	↓	NR	50 (30-81)	NR	1/4: OLT 3/4: death
Horslen[102]	*14*	ALF	Pig (*8*) Human (*8*)	36 (7-68) 55 (20-92)	V-V	Reservoir (*8*) OR plasma filter (*10*)	1000/kg	PV + HA	ACT 180 - 220	↓/— ↓	NR	↓ ↓	NR	NR	↑/— ↑/—	NR	NR	9/14: OLT 5/14: death
Levy[103]	*2*	ALF	h*CD55/59* pig (*2*)	8.3 (6.5-10)	V-V	Direct (with bypass)	800	PV (*1*) PV + HA (*1*)	NR	↓	↑/↓	↑/↓	NR	NR	↑	NR	NR	2/2: OLT
Argibay[110]	*1*	ALF	Pig (*1*)	5	NR	PAN filter	NR	NR	NR	↓	NR	↓	NR	NR	—	NR	NR	1/1: OLT
Tector[111]	*2*	ACLF, ALF	Pig (*2*)	8 (4.5-11.5)	V-V	NR	NR	PV	Heparin	NR	NR	NR	NR	NR	↑/NR	NR	NR	1/2: OLT 1/2: death

Table 3: clinical reports of extra-corporeal liver cross-circulation (ELC) since 1990 (1993 – 2001).
The blood exchange rate between patient and circuit is not reported for any study. N: number treated. Anticoag.: anticoagulation regimen. Bili: bilirubin. INR: international normalised ratio. NH_3: ammonia. Cr: creatinine. HE: hepatic encephalopathy. MAP: mean arterial pressure. Plts: platelet count during ELC. TEAE: treatment-emergent adverse events. ALF: acute liver failure. ACLF: acute-on-chronic liver failure. PNF: primary non-function. PV: portal vein. HA: hepatic artery. ACT: activated clotting time. OLT: orthotopic liver allotransplantation. NR: not reported. PAN: polyacrylonitrile.

1.10 Source of donor livers for ELC: allografts ('allo-ELC')

Each year in the UK, 570 livers offered for transplantation from deceased donors undergoing retrieval surgery are declined (2014 – 2024 average). 387 of these livers are not retrieved from the donors, whilst 183 are retrieved but subsequently not transplanted (*figure 3*)[32]. Between 1 January 2013 and 31 October 2023, 6,144 livers were not retrieved from deceased donors that donated at least one other organ (NHSBT registry data, request HD29844, *table 4*). In 85% of cases the kidneys were transplanted. The most common reasons recorded for declining the livers were past medical history, age, and steatosis. Since this registry only includes donors where at least one organ was donated, few livers were declined for a primary reason that would be an absolute contra-indication for ELC (e.g. malignancy, virology, infection). However, the NHSBT registry provides limited resolution to analyse the reasons for non-utilisation: only one reason is recorded per liver (in reality, the decision is often multi-factorial) and a specific reason is not provided in 25% of cases (either 'other' or 'used for research').

In the last three years in the USA (2022 – 2024), the annual number of deceased donor livers declined pre- and post-retrieval were 5,092 and 1,053, respectively[112]. Between 2010 and 2021, the most

common reasons recorded for non-retrieval were findings during retrieval surgery (16%), graft not being accepted (11%), and poor organ function (9%). Those for decline post-retrieval were biopsy findings (steatosis and/or fibrosis, 39%), prolonged warm ischaemia (9%), and anatomic abnormalities (8%)[113].

Many livers are declined due to concern regarding complications in the medium-to-long term: in particular, the increased risk of ischaemic cholangiopathy with extended criteria donor livers[114-117]. ELC only requires donor livers that function for a number of days. The tools used to predict short-term function post-transplant (e.g. risk of primary non-function, early allograft dysfunction[118-120]) were developed in cohorts of transplanted livers and so cannot be usefully applied to declined liver registry data. They also require either donor data that are not present in the registry[118] or recipient data[119,120]. To estimate the proportion of declined livers that may provide effective temporary hepatic support, it is preferable to extrapolate from studies that specifically assess this population of grafts.

In a UK study of livers declined post-retrieval (VITTAL), the most common reasons for non-utilisation were steatosis (42%), fibrosis/cancer (12%), prolonged warm ischaemia (9%), and logistics (8%). 32% were declined for reasons that would have likely been contra-indications for ELC. 71% of the livers accepted to the study met pre-defined transplantability criteria during *ex situ* normothermic perfusion and were transplanted, with 100% 90-day graft survival[121]. Although 18% developed non-anastomotic strictures within 6 months, this is not relevant when considering liver suitability for temporary ELC. Extrapolating from this study, 68% of declined livers may be free of contra-indications to consideration for ELC, 71% of which may have sufficient short-term hepatic function: 48% of declined livers may therefore provide effective ELC (270 per year).

This calculation is an approximation based on a small sample size that makes several assumptions. The first is that the quality of declined livers has not changed since the VITTAL study in 2016 – 2018. Advances in machine perfusion have enabled utilisation of more marginal grafts[95,122-135], which would be expected to reduce the quality of the remaining declined livers. Although the proportion of donor livers that are declined in the UK and USA has actually risen over the last decade, this is at least partly driven by more extended criteria donors[32,112]. The second assumption is that the livers declined pre-retrieval will have comparable levels of short-term function to those declined post-retrieval in the

VITTAL study. It might be expected that the former will be higher risk grafts. The NHSBT registry data suggest that more livers declined pre-retrieval are in the highest risk category of the UK donor liver index[136], although this index predicts graft survival in the long- rather than short-term and it could not be calculated for 76% of donor livers due to missing data (*table 4*). Functional data regarding livers declined pre-retrieval are not available because they are not offered for research use in the UK. The third assumption is that the seven livers in the VITTAL trial that met criteria for early allograft dysfunction[119] would have provided clinical benefit in the context of ELC. 4/7 met transaminase criteria but how many of these (if any) also met functional (i.e. bilirubin, INR) criteria is not reported; one of the three remaining livers developed hepatic artery thrombosis requiring revascularisation. All went on to recover good function. Moreover, the VITTAL trial excluded 25 livers (20% of those without absolute contra-indications) that were considered too low-risk for study inclusion, but would be good candidates for ELC.

There is no study that specifically addresses the function of declined livers over a duration relevant to ELC because it is not of major clinical relevance in allotransplantation. The predicted 270 per year that may provide effective ELC is necessarily extrapolated from registry data and studies that were not designed to answer this question. Until this has been empirically evaluated, it is acknowledged that this figure remains an estimate.

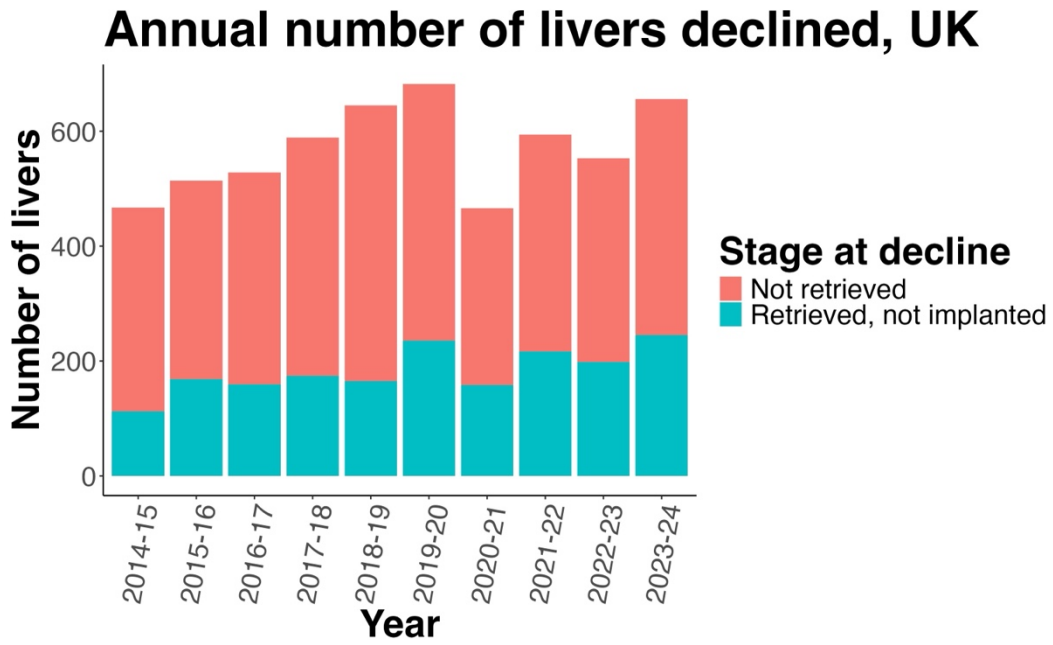


Figure 3: Number of livers from deceased donors undergoing retrieval surgery declined per year in the United Kingdom, 2014 – 2024.

Variable	Value	Number	Percentage (%)
Cause of death	Intra-cranial haemorrhage	2587	42
	Hypoxic brain damage	2049	33
	Intra-cranial thrombosis	235	4
	Intra-cranial – type unclassified	204	3
	Respiratory failure	141	2
Donor type	DBD	1824	30
	DCD	4320	70
Blood type	O	2778	45
	A	2509	41
	B	590	10
	AB	259	4
	NR	8	-
Solid organ retrieved	Yes	6144	100
	No	0	0
Solid organ transplanted	Yes	5293	86
	No	851	14
Kidney retrieved	Yes	5927	96
	No	217	4
Kidney transplanted	Yes	5201	85
	No	943	15
Liver offered	Yes	5417	88
	No	727	12
Liver accepted	Yes	2724	50
	No	2734	50
	NR	686	-
Liver retrieved	Yes	1896	31
	No	4248	69
Liver transplanted	Yes	0	0
	No	6144	100
FWIT > 30 mins	Yes	269	9
	No	2608	91
	NR (DCD)	1443	-
UK DLI quartile: all livers	1	77	5
	2	98	7
	3	125	8
	4	1196	80
	NR	4648	-
UK DLI quartile: decline pre-retrieval	1	44	4
	2	57	5
	3	72	6
	4	950	85
	NR	3125	-
UK DLI quartile: decline post-retrieval	1	33	9
	2	41	11
	3	53	14
	4	246	66
	NR	1523	-
UK DLI1 quartile: all livers	1	19	5
	2	49	13
	3	73	20
	4	225	61
	NR	5778	-
Reason for liver decline	Past medical history	1417	23
	Other	1089	18
	Age	697	11
	Steatosis	653	11
	Poor function	501	8

	Used for research	416	7
	No suitable recipients	216	4
	Size	211	3
	Anatomical	155	3
	Family permission refused	138	2
	Warm ischaemia time	110	2
	Cold ischaemia time	104	2
	Organ damage	81	1
	Coroner permission refused	78	1
	No response to fast track	39	0.6
	Poor perfusion	34	0.6
	Tumour	28	0.5
	Centre already retrieving/transplanting	27	0.4
	Virology	20	0.3
	Organ unsuitable for transplant	16	0.3
	Other disease	15	0.2
	Family permission not sought	12	0.2
	Donor unstable	11	0.2
	Infection	11	0.2
	Organ fibrotic	9	0.1

Table 4: NHS Blood and Transplant registry data for all livers declined from deceased donors in which at least one organ was retrieved 1 January 2013 – 31 October 2023 in the United Kingdom.
N = 6,144. Registry request: HD29844. Percentage values are expressed as a fraction of the cases for which data were available. NR: not reported. DBD: donation after brainstem death. DCD: donation after circulatory death. FWIT: functional warm ischaemic time (time between systolic blood pressure < 50 mmHg and start of perfusion). DLI: donor liver index. DLI1: donor liver index for 1-year graft survival.[136]

1.11 Source of donor livers for ELC: xenografts ('xeno-ELC')

The annual number of declined deceased donor human livers is about one-tenth the incidence of ACLF. If ELC proves effective, declined allograft availability will limit the number of patients that can be treated.

Advances in CRISPR-cas9 gene editing have enabled production of porcine clones with multiple gene edits that enhance compatibility with humans. The latest generation of genetic edits include triple glycan knock-out (α-Gal; Neu5Gc; SDa) to avoid hyperacute rejection due to pre-formed antibodies and insertion of human transgenes to address incompatibilities with the human complement, coagulation, and immune systems[33].

1.11.1 Kidney and heart xenotransplantation

1.11.1.1 Development of current generation genetic edits

Pigs have become the standard organ donors for xenotransplantation. Although non-human primates are more closely related to humans, several logistical factors favour pigs: straightforward breeding in captivity, rapidly reach reproductive maturity (4 – 8 months), short gestation period (4 months), and large litters (5 – 12). Rearing of pigs for human consumption is well-established, compared to the significant ethical concerns regarding the use of non-human primates. Pig organs are anatomically and physiologically similar to human organs (relative to other large animals) and there is a lower risk of zoonotic transmission compared to non-human primates[33,137].

Transplantation of wild-type pig organs into non-human primates typically results in failure within minutes to hours due to hyperacute rejection[138]. Preformed anti-pig antibodies recognise xenoantigens on the vascular endothelium, triggering antibody-mediated complement activation. Porcine complement and coagulation regulatory proteins do not appropriately modulate the human systems, contributing to their activation in the xenograft[139]. The result is interstitial haemorrhage, thrombus formation, and tissue ischaemia, with widespread immunoglobulin and terminal complement deposition[140,141].

Genetic engineering efforts over the past 35 years have addressed the incompatibilities between pig organs and humans that result in early graft loss. Non-human primate xenotransplant studies using these transgenic pig organs have focussed on the kidney and heart.

The first transgenic pig donors were made to express human complement regulatory proteins (CD55, CD46, CD59) by direct injection of DNA fragments into the embryo nucleus[142,143]. Kidneys from these donors avoided hyperacute rejection in non-human primates, but mostly failed within 90 days[144-146].

The next milestone combined gene targeting with somatic cell nuclear transfer to produce pigs with inactivated α*1,3GT* gene[147]. The enzyme α(1,3)galactosyltransferase generates the cell membrane carbohydrate α-Gal, which is a prominent target for pre-formed anti-pig antibodies in humans[148-151]. In contrast to pigs (and most other mammals), humans, apes, and Old World monkeys lack α*1,3GT* and so their cells do not express α-Gal[152,153]; α-Gal produced by the intestinal microbiome stimulates

production of the pre-formed antibodies[154]. α-Gal knock-out (Gal KO) pig hearts and kidneys transplanted into non-human primates mostly avoided hyperacute rejection, with graft survivals of weeks to months[155,156].

Pigs with α-Gal knock-out that also expressed human complement regulatory proteins were then produced. Combining these edits with immunosuppression incorporating co-stimulatory blockade and screening for pre-formed anti-pig antibodies achieved the longest graft survivals. Life-supporting transplant of Gal KO.h*CD55* kidneys into macaques with CD154 blockade achieved survival up to 10 months[157]. Heterotopic transplant of Gal KO.h*CD46* hearts into baboons resulted in a median graft survival of 100 days (maximum 8 months)[158].

Although combining α-Gal knock-out and human complement regulatory protein insertion extended heart and kidney xenograft survival, eventual graft failure occurred due to coagulation dysregulation and thrombotic microangiopathy[33]. Thrombomodulin (gene: *THBD,* protein: TM), a multi-functional glycoprotein expressed on the luminal surface of endothelial cells, binds circulating thrombin to inhibit its interaction with fibrinogen and increase its affinity to bind and activate protein C. Activation of protein C is further increased by binding extra-cellular protein C receptor (gene: *PROCR*, protein: EPCR), also expressed on the luminal endothelial membrane. Activated protein C acts as an anticoagulant through proteolytic deactivation of factors Va and VIIIa[33]. Porcine thrombomodulin binds human thrombin, but the porcine thrombomodulin : human thrombin complex fails to activate protein C[159]. This failure contributes to xenograft-associated dysregulated coagulation and thrombotic microangiopathy[160].

Gal KO.h*CD46*.h*THBD* pigs were produced, with h*THBD* expression controlled by the endogenous pig endothelium-specific *THBD* promoter[161]. This was associated with markedly improved xenograft survival: heterotopic heart xenotransplantation into non-human primates achieved graft survival > 2.5 years[162]. Orthotopic Gal KO.h*CD46*.h*THBD* pig heart-to-baboon cardiac xenotransplantation achieved survival up to 195 days[163].

Xeno- (and allo-)transplantation are associated with inflammation that contributes to graft rejection and coagulation dysregulation[33,164]. IRI, haemolysis, and haem release induce expression of haem

oxygenase-1 (gene: *HMOX1*, protein: HO1) that regulates this inflammatory response[165]. Pigs expressing h*HMOX1* driven by the SV40 promoter were produced: h*HMOX1* pig kidneys demonstrated longer organ viability during *ex situ* whole blood perfusion studies than wild-type pig kidneys[33].

In pig-to-non-human primate xenotransplantation, porcine cells are vulnerable to phagocytosis by recipient macrophages: porcine CD47 is not recognised as 'self' by SIRP-α on non-human primate macrophages[166-168]. To overcome this, pigs expressing h*CD47* were produced[169]. In non-human primate renal xenotransplant studies, podocyte h*CD47* expression reduced proteinuria (an important limiting factor in renal xenotransplantation), although high renal tubular expression of h*CD47* provoked an inflammatory response[170].

Despite Gal KO, insertion of human complement/coagulation regulatory genes, and increasingly sophisticated immunosuppression, delayed xenograft rejection was still observed in non-human primate studies with evidence of anti-pig antibody deposition [33]. Two additional major porcine carbohydrate xenoantigens were identified: n-glycolylneuraminic acid[171] (Neu5Gc, generated by cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH)) and sialyl-dimeric antigen[172] (SDa, formed by β-1,4-N-acetyl-galactosaminyltransferase 2 (β4GalNT2)). Pre-formed anti-Neu5Gc and anti-SDa antibodies have been detected in humans[172,173]. Neu5Gc is present in most mammals, including all Old World primates except humans[174]. Old World primates such as baboons and macaques cannot therefore be used as recipients to assess the impact of CMAH KO in porcine donors. Triple knock-out pigs with Gal KO.CMAH KO.β4GalNT2 KO were generated using CRISPR-cas9 gene editing. An *in vitro* study of porcine endothelial cells from single, double, and triple knock out pigs demonstrated 72%, 92%, and 97% reductions in IgG binding when exposed to human serum relative to wild-type pigs, respectively[33]. The majority of renal transplant waiting list patients had a negative cross-match with triple knock-out pigs[175]. By contrast, baboon and macaque sera often demonstrate greater antibody binding to triple-KO than double-KO (Gal KO.β4GalNT2 KO) porcine cells, and the CMAH KO increases immunogenicity in baboon xenotransplant studies[176-178].

Zoonotic transmission is a risk inherent to xenotransplantation. Pigs used for xenotransplantation must be raised in a designated pathogen free facility and screened for potential zoonoses, including herpes virus-gamma, swine influenza virus, porcine cytomegalovirus (pCMV), hepatitis E (HEV), and porcine endogenous retrovirus (PERV). PERVs are integrated into the porcine genome and all pigs express PERV-A and PERV-B; they cannot be eliminated by treatment, vaccination, epidemiological measures, or genetic selection[179]. PERVs are expressed in organs for xenotransplant and so there is theoretical potential for transmission to a human recipient[180]. A variety of human cells have been infected with PERV *in vitro*, but without evidence of viral replication[181]. A single *in vitro* case of PERV infection of human cells with viral replication has been reported: a pig cell line (containing PERV-A, -B, -C) was co-cultured for more than 6 months with human embryonic kidney cells (HEK 293) lacking the viral restriction factor APOBEC3. A PERV-A/C recombinant virus infected the HEK 293 cells[182]. PERV has never been shown to infect non-human primates or humans *in vivo*[183]. One approach to mitigate the risk has been to breed out the PERV-C virus[33]. The alternative approach has been to inactivate the reverse transcriptase *pol* gene from all 59 PERV elements in the porcine genome[107].

As longer graft survivals are anticipated, size mismatch between the porcine donor and human recipients may become relevant if the grafts continue to grow in the recipient. Rapid porcine donor growth also limits the window during which donation of a suitably sized organ can occur. This has been mitigated by either use of minipig donors or knockout of the growth hormone receptor (*GHr*) from Large White pigs: double *GHr* knock-out reduces pig size by 40%, although this is associated with 80 – 90% reduction in insulin-like growth factor 1 and transient juvenile hypoglycaemia[33,184]. CRISPR-cas9 has enabled multiple genetic edits to be combined. Kidneys from Yucutan minipig donors with triple glycan knock-out (Gal KO.CMAH KO.β4GalNT2 KO), seven human transgene insertion (*THBD.PROCR.CD46.CD55.CD47.HMOX1.TNFAIP3*) ± PERV *pol* inactivation (designated 3KO.7TG±RI) were transplanted into macaques that had undergone bilateral nephrectomy. Immunosuppression comprised induction T-cell depletion (anti-thymocyte globulin (ATG)), B-cell depletion (anti-CD20) and maintenance co-stimulatory blockade (anti-CD154), mycophenolate

mofetil, tacrolimus (2 months), and corticosteroids (1 month). Despite the limitations of testing CMAH KO organs in Old World primates, survival beyond 6 months was achieved in 7/15 recipients, with 1 case surviving more than 2 years[107].

1.11.1.2 Decedent model

The decedent model has been used to evaluate xenotransplantation in humans who have been declared dead by neurologic criteria and ruled out as potential organ donors[185,186]. This enables assessment of transgenic pig organs in the context of human physiology and immunology. The model is discussed in detail in *7.1*.

Gal KO pig thymo-kidneys (with sub-capsular autologous thymic tissue) were implanted into two decedents with immunosuppression that comprised methylprednisolone and mycophenolate mofetil. The xenografts were monitored for 54 hours, and demonstrated evidence of renal function without hyperacute or antibody-mediated rejection[187].

10GE pig kidneys with triple glycan knock-out, six human transgene insertion (*THBD.PROCR.CD46.CD55.CD47.HMOX1*), and growth hormone receptor knock-out were transplanted into decedents following bilateral native nephrectomy with 3-day ($n = 2$) and 7-day ($n = 1$) observation. Immunosuppression comprised ATG, rituximab, tacrolimus, mycophenolate, and steroids, with complement inhibition (eculizumab) in the final two. The first study demonstrated persistent renal dysfunction with thrombotic microangiopathy and C5b-9 deposition in the graft. The 7-day case demonstrated successful graft function, although with C5b-9 deposition possibly due to subtherapeutic eculizumab dosing[188-191].

A Gal KO pig thymo-kidney was transplanted into a decedent and observed for 61 days. The graft functioned well in the first month. In the second month, renal function declined and biopsy confirmed antibody-mediated rejection. Standard immunosuppression management resolved the rejection episode and the graft appeared healthy at the end of the study[33,192].

Two 10GE pig hearts were transplanted into decedents under immunosuppression including eculizumab. The duration of both studies was 66 hours. No cell- or antibody-mediated rejection was

observed. One heart exhibited graft dysfunction – possibly due to not using *ex situ* perfusion during preservation and/or size mismatch with the recipient[193].

For these decedent studies, donor pigs were raised in bio-secure facilities. Neither zoonotic transmission nor micro-chimerism were detected[33].

1.11.1.3 Clinical cases

The first clinical transgenic pig heart xenotransplantation occurred in January, 2022 under an FDA expanded-access Investigational New Drug Application. The recipient was an ECMO-dependent 57-year-old patient with non-ischaemic cardiomyopathy, who was not considered a candidate for allotransplantation or a ventricular-assist device due to non-adherence with medical treatment. The donor was a 10GE pig. Induction immunosuppression was ATG and rituximab, complement C1 esterase inhibitor, anti-CD40 co-stimulatory blockade, and methylprednisolone. Maintenance immunosuppression was anti-CD40, steroids, and mycophenolate (replaced with tacrolimus for days 21 – 52). The donor heart was smaller than the explant. The xenograft functioned well until abrupt deterioration on day 49. Life support was withdrawn on day 60. Xenograft failure has been attributed to antibody-mediated rejection, with the day 50 endomyocardial biopsy demonstrating severe endothelial injury, red cell extravasation, IgM, IgG, and C4d deposition. This may have been due to reduced immunosuppression dosing (due to pancytopenia), administration of intravenous immunoglobulin, and/or precipitated by porcine cytomegalovirus infection of the graft[194,195].

The second 10GE pig heart-to-human clinical xenotransplantation occurred in September, 2023. The recipient was a 58-year-old man with end-stage cardiac failure who was not eligible for allotransplantation. Similar to the first case, this xenograft also developed presumed antibody-mediated rejection and the patient died 6 weeks post-operatively[196]. Rejection may have been precipitated by reduced immunosuppression dosing and/or administration of blood products that were later found to contain substantial anti-pig antibodies[33].

A 3KO.7TG.RI pig kidney was implanted into the first clinical recipient of a transgenic pig kidney in March, 2024. Immunosuppression included T- and B-cell depletion, anti-CD154, and anti-C5, in

addition to maintenance with tacrolimus, mycophenolate, and prednisolone. Acute T-cell mediated rejection occurred on day 8, which was managed with methylprednisolone, anti-C3, anti-interleukin 6, and increased tacrolimus and mycophenolate. The xenograft maintained the recipient's estimated glomerular filtration rate 40 – 50 mL/minute/1.73m^2 until the recipient died on day 52 post-transplant due to sudden cardiac death secondary to dysrhythmia in the context of severe ischaemic cardiomyopathy. The xenograft was functioning at the time of death, with focal fibrosis in the absence of active T-cell, antibody-mediated rejection, or thrombotic microangiopathy[197].

There have been a further three clinical transgenic pig kidney xenotransplants: (i) Gal KO thymo-kidney – removed day 47 due to vascular kinking; (ii) 10GE kidney – removed day 130 due to acute T-cell mediated rejection; (iii) 3KO.7TG.RI kidney, ongoing 4-months post-transplant (as of May, 2025[198]).

1.11.2 Liver xenotransplantation

Unlike in renal and cardiac xenotransplant, liver xenotransplant studies in non-human primate studies have failed to achieve prolonged graft survival[199-202] (*table 5*). The longest survival in orthotopic liver xenotransplant in the non-human primate model is 29 days. This was achieved with Gal KO pig livers using a combination of T-cell and complement depletion, anti-CD154, and maintenance methylprednisolone and tacrolimus. Continuous Octaplex (human prothrombin complex concentrate, containing coagulation factors II, VII, IX, X, protein C, protein S) infusion was required to support coagulation function[203]. The challenge of liver xenotransplantation is that the graft must not only avoid the xeno-immune response, but replace the many, complex roles of a liver that are fundamental to the wider physiology of the organism. This is perhaps best exemplified by synthetic function: the xenograft must produce hundreds of proteins that function within the biochemical cascades of the recipient; many non-human primate liver xenotransplant studies were complicated by coagulopathy[204]. These studies were also consistently complicated by rapid, severe thrombocytopenia. Nonetheless, with the support of an Octaplex infusion, the liver xenografts provided life sustaining function (e.g. bilirubin clearance) in non-human primates up to 29 days[203].

Whilst long-term liver xenotransplant is not yet a feasible clinical target, short-term xenografting may provide effective temporary hepatic support. The hypothesis is that, although physiological incompatibility may limit the capacity of the liver xenograft to support human life for multiple months, over a period of days – week(s) the xenograft may provide life-sustaining function that is more comprehensive than artificial and bio-artificial liver support systems. Two spectra of hepatic functions are relevant to this: (i) how closely specific functions are conserved between pigs and humans, from the well-conserved (e.g. ammonia detoxification) to the poorly-conserved (e.g. synthesis of porcine proteins with 60 – 70% homology with the human equivalents); (ii) the time period over which specific functions are necessary to sustain life, from those required to sustain life over days (e.g. ammonia detoxification) to those required to sustain life over months (e.g. vitamin storage). Assuming that the xeno-immune response can be attenuated using the latest generation genetic edits and complications (e.g. thrombocytopenia) controlled, the duration over which xenografts provide effective temporary hepatic support will be determined by the overlap between well-conserved functions and those required to sustain life over a given time period.

A gene-edited (triple glycan knock-out; *CD46*, *CD55*, *THBD* human transgene insertion) pig liver was recently implanted into a human decedent with their native liver intact for 10 days. The heterotopic implantation included resection of a segment of the recipient IVC to anastomose the xenograft portal vein and supra-hepatic cava. The xenograft was removed after 10 days and the IVC reconstructed with an artificial graft. Immunosuppression comprised T- and B-cell depletion, complement C5, and TNF-α blockade, and maintenance methylprednisolone, mycophenolate, and tacrolimus. There was evidence of hepatic function, although at lower than physiological levels (bile production 2.5 mL/hour on day 10; porcine albumin ~ 2 g/L on day 8). As with the non-human primate studies, rapid, severe thrombocytopenia developed. Day-10 xenograft biopsies demonstrated preserved parenchyma and moderate antibody deposition without tissue complement activation[205]. This study showed that the xeno-immune response to the porcine liver could be controlled over 10 days but, by retaining the native liver *in situ*, the study was not designed to assess the extent to which the xenograft could support hepatic function. The immunosuppression regimen and implantation procedure would be substantial undertakings in an unwell patient with ALF or ACLF.

A clinical transgenic pig liver auxiliary xenotransplant occurred in 2024. The recipient was a 71-year-old male undergoing right hepatectomy. Little is known regarding this case as it has not yet been formally published; the purpose of the auxiliary xenotransplant was most likely to bridge the patient to regeneration of the native remnant liver. The xenograft was 514 g; the genetic edits are not known. 2 weeks after the operation, the recipient was reported as 'doing well' and the xenograft was producing 200 – 300 mL/day of bile[206].

Reference	Gene edits	Immunosuppression	Adjuncts	Survival (d)
Ramirez 2000[207]	*CD55*	CyP Csp St Spl		4 - 8
Ramirez 2005[208]	*CD55/CD59/HT*	CyP Csp St MMF anti-CD25 anti-CD20 Spl		0.5 - 1
Ekser 2010[202]	Gal KO + *CD46*	ATG CVF CyP St MMF Tac Spl	Heparin, clodronate	0 - 7
Kim 2012[200]	Gal-KO	ATG CVF St AZA Tac anti-CD154 Spl	ε-aminocaproic acid	6 - 9
Navarro-Alvarez 2016[199]	Gal-KO	ATG CVF St Tac anti-CD154 Spl	hPCC/rFVIIa	1 - 7
Shah 2017[203]	Gal-KO	ATG CVF St Tac CTLA4-Ig/anti-CD40 Spl	hPCC	5 - 29
Lee 2023[209]	TKO ± *CD46.THBD*	ATG CVF St mTORi anti-CD20 anti-CD154 anti-TNF	LMWH, aspirin	0 - 7

Table 5: summary of transgenic pig liver-to-non-human primate orthotopic liver xenotransplantation studies. HT: H-transferase. CyP: cyclophosphamide. Csp: ciclosprin: St: steroids. Spl: splenectomy. MMF: mycophenolate mofetil. ATG: ant-thymocyte globulin. CVF: cobra venom factor. Tac: tacrolimus. AZA: azathioprine. mTORi: mTOR inhibitor. hPCC: human prothrombin complex. rFVIIa: recombinant activated factor VII. LMWH: low molecular weight heparin.

1.12 Thesis overview

The aim of this thesis is the pre-clinical development and evaluation of a novel ELC protocol and system (*metra*-ELC) using allografts and transgenic xenografts.

The *metra*-ELC system adapts the OrganOx *metra* (a CE-marked class IIa, FDA-approved liver normothermic preservation device) to provide ELC. The disposable perfusion set is modified to allow blood to be exchanged between the patient and the extra-corporeal liver circuit by a bespoke exchange system (*figure 2*).

To enable the donor liver to recover from the initial IRI and assessment of graft function prior to commencing ELC, the donor liver is initially reperfused *ex situ* (*figure 4*). During normothermic liver perfusion, the perfusate electrolyte concentrations are non-physiological: in particular, severe perfusate hyperkalaemia may occur due to the use of stored packed red cells and washout of organ preservation solution. Rapid infusion into a critically unwell patient would risk cardiac arrythmia.

Modification of standard liver normothermic perfusion by incorporation of renal replacement therapy (RRT) could enhance safety by correcting perfusate electrolyte abnormalities during the isolated normothermic perfusion phase prior to commencing ELC (*figure 4*). In *chapter 3*, three techniques of RRT during *ex situ* liver normothermic perfusion are assessed to determine the optimal approach to correct perfusate composition for this purpose.

In *chapters 4* and *5*, the *metra*-ELC system is evaluated in a large animal (porcine) model using allografts. It is first assessed in healthy pigs over 3 hours to ensure that the system can be used safely *in vivo* (*chapter 4*). Allo-ELC is then assessed in pigs with ALF over 24 hours to test the extent to which it can replace lost native hepatic function (*chapter 5*).

Chapters 6 and *7* explore the potential of using transgenic porcine livers (3KO.7TG.RI) to provide ELC. The feasibility of *ex situ* isolated normothermic xenoperfusion of transgenic porcine livers with human whole blood for 48 hours is determined. This *ex situ* xenoperfusion model is also used for a pilot investigation of the mechanism of liver xenograft-associated thrombocytopenia (*chapter 6*). The feasibility of 72-hour xeno-ELC using transgenic porcine livers is assessed in human decedents with intact native livers. Its capacity to replace native hepatic function for 48 hours is then tested in an anhepatic decedent (*chapter 7*).

These experiments provide the basis for two planned phase-1 clinical trials that will determine the safety and feasibility of ELC in the treatment of ACLF. One phase-1 trial will use human donor livers declined for transplantation. The protocol for this trial is described in *chapter 8*. The other phase-1 trial will use transgenic porcine livers (*chapter 9*).

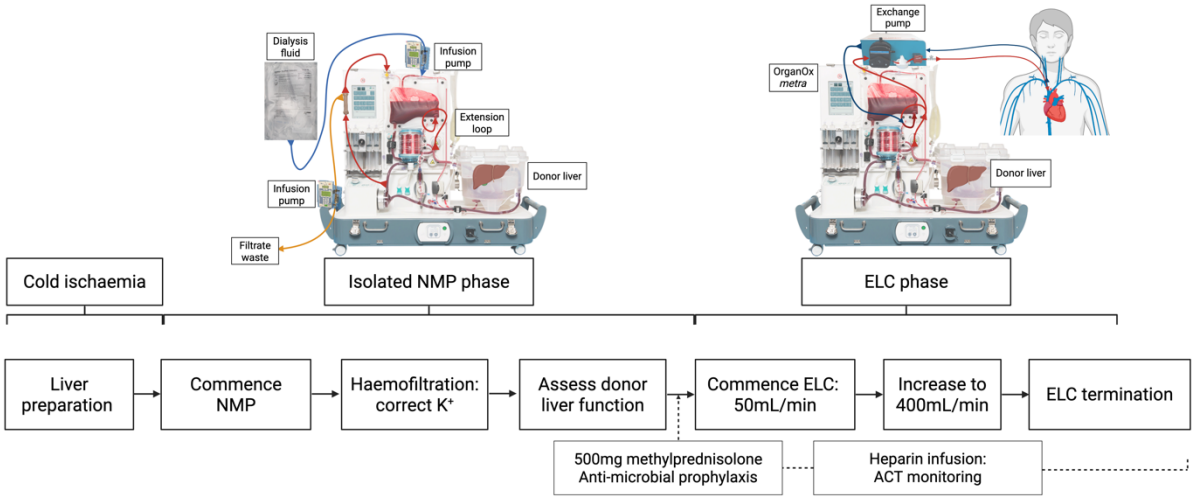


Figure 4: *metra*-ELC protocol for donor liver at low risk of ischaemia-reperfusion injury.
NMP: normothermic machine perfusion. ELC: extra-corporeal liver cross-circulation. K^+: potassium. ACT: activated clotting time.

2 Methods and techniques

This chapter describes common methods and techniques used in the experiments of this thesis.

2.1 Porcine liver retrieval technique

2.1.1 Slaughterhouse pig liver retrieval (*chapters 3, 4*)

The slaughterhouse pig liver retrieval technique has been described previously[210]. Following electrical stunning, pigs (50 – 70 kg) were exsanguinated via stab incision of the heart and great vessels in compliance with the Welfare of Animals at the Time of Killing (England) Regulations 2015 (WATOK). Whole blood was collected into a container with 20,000 IU unfractionated heparin (Wockhardt UK, Wrexham, UK). The pigs were cleaned in a hot water bath (60°C) for 2 – 6 minutes. The thoraco-abdominal viscera were removed en bloc via a thoraco-laparotomy by an incision along the anterior vertebral plane.

On the back-table, slushed ice was placed around the liver. The infra-hepatic IVC was divided. The portal vein was identified deep to the pancreatic parenchyma at the junction of the splenic vein and superior mesenteric vein. The portal vein was incised, canulated, and flushed with 2 L heparinised, cooled University of Wisconsin (UW) solution (Bridge to Life, Wandsworth, UK). The hepato-duodenal ligament was dissected off the duodenum and lesser curve of the stomach to the oesophagus, taking care to maintain an adequate length of common bile duct (CBD) to allow subsequent cannulation. The remaining diaphragmatic attachments were divided. The posterior wall of the aorta was opened to visualise the origin of the coeliac axis, which was detached as a patch from the remainder of the aorta. The liver was inspected for anatomical variations or injury. The liver was then stored in UW solution, surrounded by ice, in an ice-box (static cold storage). Autologous whole blood was leuco-depleted using the Macopharma blood collection system (FQE614B, Macopharma, Tourcoing, France).

2.1.2 Anaesthetised pig liver retrieval (*chapters 5 – 7*)

Induction and maintenance of general anaesthesia is described in *5.3.2*. After induction of general anaesthesia, the donor pig was placed in dorsal recumbency. 500 mg meropenem (Pfizer, Kent, UK) was administered intravenously.

The neck was prepared with chlorhexidine and draped. Unilateral neck dissection was performed to insert a 7 Fr triple-lumen central venous catheter into the right internal jugular vein (for fluid/medication administration and CVP monitoring) and a 3 Fr arterial line (Vygon, Swindon, UK) into the right external carotid artery (for invasive arterial blood pressure monitoring).

The abdomen was prepared with chlorhexidine and draped. A midline laparotomy was performed from xiphisternum to pubic symphysis. For *chapters 6* (xenoperfusions 4 – 9) and *7* (decedents 2 – 4), this was extended to include midline sternotomy to improve access to the abdominal cavity. The intestine was packed into an intestinal bag (E6533S, Exact medical, Nieuwkuijk, Netherlands), and retracted caudally and to the left. The hepatoduodenal ligament was dissected: the CBD was divided close to the duodenum and mobilised to the liver hilum. The common hepatic/coeliac artery was identified and dissected to reveal several hepatic branches, all of which were preserved in continuity with the liver. The gastro-duodenal artery, left gastric, and other arterial branches were ligated and divided. The portal vein was dissected from pancreas to liver hilum. All other structures, including extensive lymphoid tissue, were meticulously ligated and divided to ensure complete haemostasis. The infra- and retro-hepatic IVC was mobilised, and the right and left lobes of the liver mobilised by division of peritoneal attachments. The right-sided colon was mobilised, and the intestine reflected medially to enable dissection of the distal abdominal aorta. Both renal arteries and the superior mesenteric artery were identified at their points of origin from the aorta.

25,000 IU heparin was administered intravenously and allowed to circulate for 3 minutes. The aorta was cannulated (KP-16-22-26, Bridge to Life, Wandsworth, UK) and connected sequentially to 1000 mL bags of cold UW solution. The diaphragm and pericardium were opened and left lung mobilised to enable identification of the thoracic aorta and IVC. The donor was exsanguinated into the thoracic cavity via the IVC, at which point the thoracic aorta was cross-clamped. Clamps were placed on the

origins of the superior mesenteric and renal arteries to maximise perfusion of the liver with UW solution.

After commencing aortic cold perfusion, the portal vein was clamped at the level of the duodenum and divided, to enable simultaneous cold perfusion of the liver via the portal vein. Once at least 2 litres of UW solution had been perfused via each of the aorta and portal vein, final mobilisation of the liver was carried out by dividing: (i) the diaphragm/crura, (ii) infra-hepatic IVC, and (iii) coeliac artery.

2.2 Porcine liver back-table preparation

The liver was transferred into a sterile basin with UW solution surrounded by crushed ice for back-table cannulation. The supra-hepatic IVC was inspected, and phrenic veins identified and suture-ligated, before closing the supra-hepatic IVC with running 4/0 prolene (Ethicon, Johnson and Johnson MedTech, Raritan, NJ, USA). The coeliac artery, portal vein, and infra-hepatic IVC were cannulated, after excluding all air from the cannulas. A further 2 L UW solution was perfused through each of the hepatic artery and portal vein. The CBD was cannulated. Cholecystectomy was not performed in order to minimise risk of later bleeding, given the partial intra-hepatic location of the porcine gallbladder and anticoagulation used during normothermic perfusion. In *chapters 3 – 5*, the cystic duct was suture-ligated, taking care to preserve the cystic artery. In *chapters 6* (xenoperfusions 4 – 9), and *7*, the cystic duct was not suture ligated; instead, an additional cannula was secured in the gallbladder and connected to the cannula draining the CBD to reduce the risk of gallbladder ischaemia. A peripheral wedge liver biopsy was taken and the site over-sewn with 4/0 prolene. The liver was perfused with 1 L gelofusine (B. Braun Medical, Sheffield, UK, *chapters 3 – 5*) or 1 L 5% human albumin solution (*chapters 6* and *7*) via each of the coeliac artery and portal vein at the end of back-table preparation to flush out remaining UW solution prior to reperfusion on the *metra*.

2.3 Normothermic machine perfusion of the porcine liver

Normothermic machine perfusion of the porcine livers was performed using the *metra*[4] (OrganOx, Oxford, UK, *figure 5*). The *metra* is class IIa CE-marked (up to 24 hours) and FDA-approved (up to 12 hours) for normothermic liver preservation prior to transplantation[211].

The liver is perfused with oxygenated blood-based perfusate at body-temperature. Perfusate drains via the IVC of the liver to a centrifugal pump that passes the perfusate via a heat exchanger/oxygenator to the soft-shell portal reservoir or into the hepatic artery. The portal reservoir drains under gravity into the portal vein (low-pressure, high flow). The portal vein pressure is determined by the height of perfusate in the portal reservoir. A bubble sensor on the line draining the portal reservoir will close a pinch valve if air is detected to prevent air from an empty portal reservoir entering the portal vein. Pressure in the hepatic artery is monitored by a pressure sensor and regulated by a pinch valve on the line between oxygenator and portal reservoir (target hepatic artery pressure: 65 – 70 mmHg). The hepatic artery supply is high-pressure, low flow. IVC pressure is monitored by a pressure sensor and is regulated between -2 and +2 mmHg by modulating the centrifugal pump speed. Continuous blood gas monitoring is performed by a shunt sensor (CDI510H, Terumo, Camberley, UK) on a bypass line between the hepatic artery inflow and IVC outflow, enabling automated control of gas exchange (target perfusate pO_2: 12 – 18 kPa; pCO_2: 4 – 6 kPa) and perfusate temperature (target: 37°C). In-line flow sensors monitor perfusate flow in the portal vein and IVC; hepatic artery flow is automatically calculated as the difference between these. The graphical user interface provides real-time read-out of perfusion parameters, including vessel flows, pressures, temperature, perfusate pO_2, pCO_2, pH, and centrifugal pump revolutions per minute (rpm). A recirculation roller pump collects fluid leaking from the surface of the perfused liver and returns it to the portal reservoir at a rate of 50 mL/minute. In the standard *metra* set-up, bile produced by the liver passes through a flow sensor and is collected in a separate chamber within the liver bowl. In the experiments described in this thesis, bile is collected outside the liver bowl to enable manual measurement of rate of production and avoid contamination of stagnant bile in the interior of the bowl over prolonged perfusions.

When the *metra* is used in the context of clinical liver transplantation, the perfusate consists of 3 units of packed red cells and 500 mL gelofusine or 5% human albumin solution. 10,000 IU heparin, 10 mL 10% calcium gluconate (B. Braun Medical, Sheffield, UK) and anti-microbials (e.g. 500 mg

meropenem and 100 mg fluconazole) are administered at the start of perfusion. 8.4% sodium bicarbonate (B. Braun Medical, Sheffield UK) is added to titrate the perfusate pH to 7.25 – 7.35. Infusions of heparin (833 IU/hour), insulin (6.67 IU/hour actrapid (Novo Nordisk, Bagsvaerd, Denmark)), bile salts (150 mg/hour sodium taurocholate (OrganOx, Oxford, UK)), and epoprostenol (8.33 µg/hour Flolan (GlaxoSmithKline, London, UK)) continue during the perfusion. Parenteral nutrition (Nutriflex Special (B. Braun Medical, Sheffield, UK)) is infused at a rate of 4 mL/hour when the perfusate glucose concentration is less than 10 mmol/L.

In the experiments in this thesis, the perfusate composition, bolus, and infusion medications varied depending upon the study.

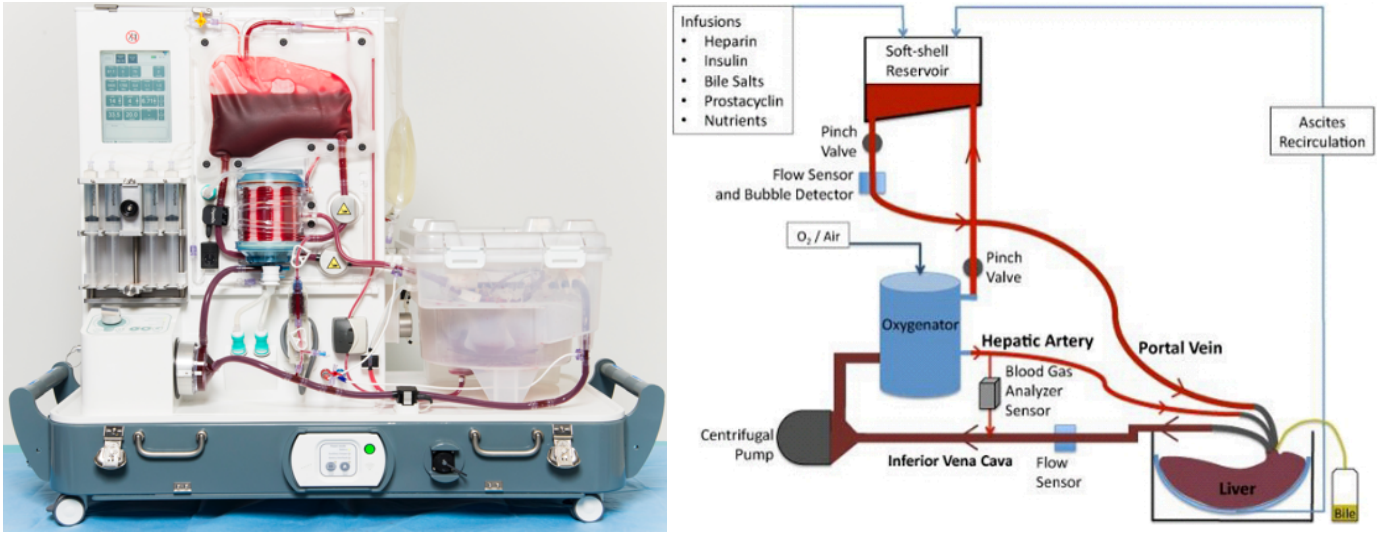


Figure 5: OrganOx *metra* liver normothermic machine perfusion device and circuit diagram.

2.4 *metra*-ELC system

The *metra* is adapted for ELC through modification of the disposable perfusion set to allow blood to be exchanged between the patient and the extra-corporeal liver circuit by a bespoke exchange pump ('*metra*-ELC system', *figures 2, 6*). The underlying *metra* device and software are unchanged.

An extension tubing loop is inserted at the inflow of the portal reservoir to connect to a bypass circuit. This tubing loop ensures adequate separation of the from- and to-patient lines. Blood is exchanged

between the central venous system of the patient and the portal reservoir of the *metra* via the bypass circuit using the exchange pump. The exchange pump comprises: roller pumps (GFS265, Oriental Motor, Tokyo, Japan) (2); pressure (Pendotech, Plainsboro, NJ, USA) (2); flow (2); bubble (2) sensors (Sonoflow CO.56/080, Sonotec, Halle (Saale), Germany); arterial filter (AF02, Terumo, Tokyo, Japan) (1).

The sensors ensure safety cut-off in the event of excess pressure, flow imbalance, or air entrained in the line. The occlusive nature of roller pumps means that a safety stop prevents flow of blood between patient and device. An arterial filter on the to-patient bypass line prevents particulate matter reaching the patient and enables clearance of any entrained air. The bypass lines are connected to central venous catheters in the patient (e.g. femoral and internal jugular vein catheters, or a single dual-lumen catheter).

Software control of the exchange pump balances flows to and from the patient according to real-time monitoring by the flow sensors. The user continuously supervises the net volume exchange to ensure stability: the portal reservoir filling height (discounting IVC collapse episodes, during which volume is transiently taken up by the liver (see *chapter 9*)) reflects the volume status of the *metra* perfusion circuit. The pre-connection reservoir height is marked, with a margin of ± approximately 100mL. If a net volume exchange in excess of this margin is detected, the user can fine-tune the flow balance by manual offset of the 'to-patient' pump rate relative to the 'to-*metra*' pump.

Exchange of the patient's blood with the portal reservoir decouples the exchange rate from the vascular flows to the donor liver. This means that the exchange rate can be started low (device minimum: 50 mL/minute) and incrementally increased to the maximum tolerated by the recipient (device maximum: 500 mL/minute), and allows the exchange to be paused if required, without affecting perfusion of the donor liver.

Exchange of the patient's central venous blood with the portal reservoir means that the portal supply of the donor liver is partially deoxygenated. The pO_2 of the donor liver portal supply is determined by the ratio of the exchange rate to the portal flow, combined with the ratio of the recipient central venous blood pO_2 to the post-oxygenator perfusate pO_2. This is a more physiological arrangement than the standard *metra* set-up, in which the portal vein receives fully oxygenated blood. Partial

deoxygenation of the portal vein has been associated with improved haemodynamics during *ex situ* perfusion[212] and is a stimulus for physiological metabolic zonation within the hepatic lobule[213] (see *9.3.6*).

The occlusion of the exchange roller pumps is set to the minimum occlusion needed to generate a proximal vacuum in a clamped tube (i.e. 'just occlusive'). Whilst roller pumps are associated with greater haemolysis than centrifugal pumps during adult cardiopulmonary bypass[214], evidence from neonatal, paediatric ECMO, and *in vitro/in silico* analysis suggests that this relationship is reversed at the lower flows used in the *metra*-ELC system[215-217]. Neonatal ECMO flows (300 – 400 mL/minute), in particular, closely reflect the *metra*-ELC system[215].

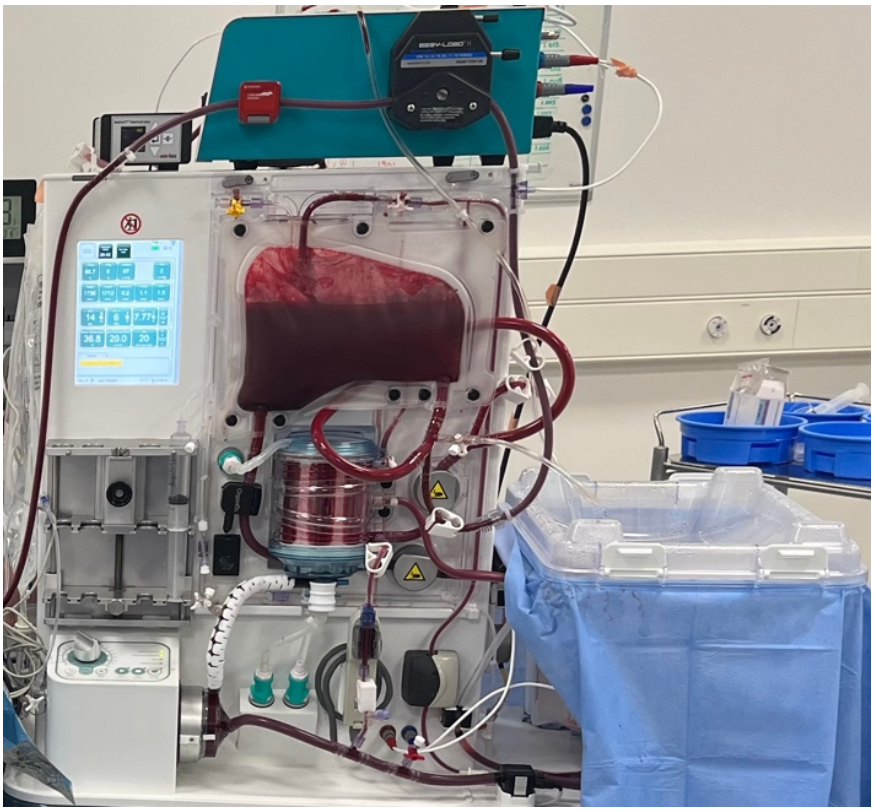


Figure 6: *metra*-ELC exchange system.

2.5 *metra*-ELC protocol

The *metra*-ELC protocol has been developed over the course of this thesis and the details therefore evolved from *chapters 4* to *8*. However, the general approach was consistent in the main experimental chapters (*chapters 5* and *7*) where ELC was evaluated (*figure 4*).

The standard *metra* perfusion circuit was modified to allow ELC. Prior to priming, the following two modifications were performed: (i) extension tubing loop inserted at portal reservoir inflow (see *2.4*); (ii) bypass line containing a haemoconcentrator (DP03, Medica, Modena, Italy) inserted between centrifugal pump and portal reservoir in parallel to the main perfusion circuit to perform *ex situ* perfusate haemofiltration prior to commencing ELC (*figure 7*).

The *metra* was primed with 1.5 L autologous leuco-depleted porcine blood (*chapter 5*) or 3 units packed human red cells and 500 mL 5% human albumin solution (*chapter 7*). Bolus medications were administered to the perfusate: 500mg meropenem; 50mg caspofungin (Wockhardt UK Ltd, Wrexham, UK); 10mL 10% calcium gluconate; heparin (*chapter 5*: nil; *chapter 7*: decedents 1 – 3: 10,000 IU; decedent 4: 5,000 IU). The *metra* was prepared for liver reperfusion by warming to 37°C per the Instructions for Use[4].

Ex situ haemofiltration of the perfusate (at a rate of 999 mL/hour) was commenced 30 (*chapter 5*) or 60 (*chapter 7*) minutes prior to liver reperfusion, and continued until ready for patient connection. Filtrate was drawn from the haemoconcentrator using an Alaris SE 7130 infusion pump (BD, Wokingham, UK), and discarded. Dialysate (*chapter 5*: multiBic K4; *chapter 7*: NxStage PureFlow RFP-404 (Fresenius Medical Care, Bad Homburg v. d. Höhe, Germany)) was infused into the portal reservoir using a second Alaris SE 7130 pump at a rate equal to the filtrate removal rate.

The donor liver was retrieved from the anaesthetised porcine donor and prepared for perfusion under static cold storage (see *2.1.2, 2.2*). The graft underwent initial isolated normothermic perfusion on the *metra*, during which perfusate potassium concentration was corrected by *ex situ* haemofiltration; the haemofiltration rate was titrated during this phase based on perfusate biochemistry, haematocrit, and activated clotting time (ACT). The following perfusate medications were infused during this phase: epoprostenol (8.33 μg/hour); insulin (*chapter 5*: nil; *chapter 7*: decedents 1 – 3: 3.33 IU/hour; decedent 4: 1.33 IU/hour); heparin (*chapter 5*: nil; *chapter 7*: decedents 1 – 3: 833 IU/hour; decedent 4: nil). The bile salt infusion was omitted because there are no clinically approved formulations for intravenous delivery. Metabolic function, perfusate chemistry, haematocrit, ACT, and haemodynamic parameters were monitored during the isolated normothermic perfusion phase. If the perfusate ACT

fell below 200 seconds, 1,000 IU heparin was administered to prevent device clotting. Once the perfusate potassium was within the physiological range, the *ex situ* haemofiltration system was disconnected from the perfusion circuit and all *metra* infusions other than epoprostenol were stopped. Graft function was confirmed by measuring perfusate lactate, glucose, pH, ALT (*chapter 5*), and portal vein flow. In both *chapters 5* and *7*, graft function was assessed at the end of the isolated normothermic perfusion phase (during which continuous *ex situ* haemofiltration had been performed throughout), due to the low index of suspicion regarding IRI of these grafts. This differs from the situation for declined human donor livers, in which the index of suspicion for IRI is higher and graft assessment must be performed before commencing *ex situ* haemofiltration to facilitate interpretation of perfusate viability markers (see *4.5.3* for further discussion and *figure 68*).

The ELC bypass tubing was connected to the portal inflow extension tubing loop and placed in the exchange pump. The bypass tubing was primed with perfusate using the exchange pump, and air expelled from the portal reservoir. Additional leuco-depleted blood from the blood-only donor pig (*chapter 5*) or packed red cells/5% human albumin solution (*chapter 7*, see *7.5*) were administered to the *metra* circuit during priming to ensure the portal reservoir remained 50 – 90% full.

2,500 IU heparin, 500 mg methylprednisolone (Pfizer, Kent, UK), and anti-microbial prophylaxis were administered intravenously to the recipient. The recipient's ACT was confirmed to be ≥ 200 seconds. The ELC bypass sash was divided to the correct length and connected under sterile conditions to the recipient's central venous cannulas (recipient to *metra* flow: right internal jugular cannula (16 Fr); *metra* to recipient flow: left internal jugular cannula (16Fr) (*chapter 5*) or right common femoral vein cannula (15 Fr) (*chapter 7*)), after excluding all air using heparinised saline. The exchange rate commenced at 50mL/minute and was increased in 50mL/minute increments every 10 minutes to 400mL/minute. Haemodynamic stability of the recipient was confirmed at each exchange rate increment before increasing further.

During ELC, an intravenous heparin infusion was administered to the recipient to target an ACT 160 – 200 seconds. ACT was measured at least hourly (more frequently when indicated). The only *metra* infusion that continued during ELC was epoprostenol. For ELC durations longer than 24 hours (i.e. *chapter 7*), 500 mg intravenous methylprednisolone was administered daily and bile salts excreted by

the extra-corporeal liver were replaced with enteral bile salts (4 mg/kg ursodeoxycholic acid 8-hourly) administered to the recipient.

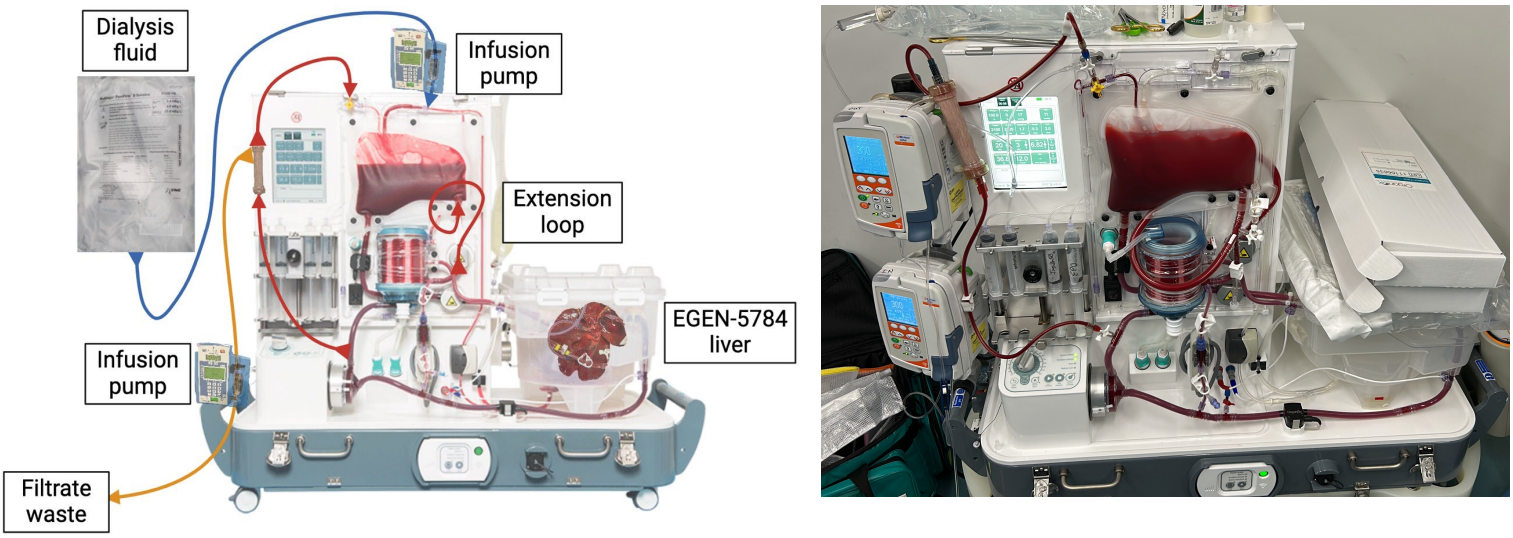


Figure 7: *metra* perfusion circuit modified to enable *ex situ* haemofiltration and connection to the extra-corporeal cross-circulation bypass circuit.
Ex situ haemofiltration was enabled by insertion of a tubing loop containing a DP03 paediatric haemoconcentrator (Medica, Modena, Italy) between centrifugal pump and portal reservoir, in parallel to the main perfusion circuit. Filtrate was drawn from the haemoconcentrator using an Alaris SE 7130 infusion pump (BD, Wokingham, UK), and discarded. Dialysate was infused into the portal reservoir using a second Alaris SE 7130 pump at a rate equal to the rate of filtrate removal. The *ex situ* haemofiltration circuit was disconnected from the *metra* circuit at the end of the isolated normothermic perfusion phase, prior to connection to the recipient.
The extension tubing loop at the inflow of the portal reservoir enabled connection of the *metra* circuit to the extra-corporeal cross-circulation bypass circuit.
EGEN-5784: donor transgenic pig liver used in *chapter 7*.

2.6 Sample collection and processing

Perfusate was collected from the sampling port on the hepatic artery-to-IVC bypass line of the *metra* perfusion circuit. Recipient blood was collected from an internal carotid arterial line. Perfusate and blood samples were collected in SST (serum separator), EDTA (ethylenediaminetetraacetic acid), citrate tubes (BD, Wokingham, UK), and 1 mL syringes (point-of-care testing). Perfusate and blood SST, EDTA, and citrate tubes were processed to serum (SST) and plasma (EDTA, citrate) by centrifugation (plasma: immediate; serum: post-clotting) at 4°C for 10 minutes, followed by freezing (temporarily -20°C, then -80°C). EDTA samples for full blood count analysis were not centrifuged; these samples were stored at 4°C until analysis.

Bile produced by the extra-corporeal liver was collected from the biliary drainage tube, and either analysed immediately (point-of-care testing) or frozen (temporarily -20°C, then -80°C).
Donor liver biopsies were either peripheral (pre- and during perfusion) or central wedge biopsies (end of perfusion). Liver tissue samples were divided into three sections for: (i) fixation in 10% formalin; (ii) preservation in RNAlater; (iii) snap-freezing in liquid nitrogen. Formalin-fixed liver tissue samples were paraffin-embedded and sectioned in preparation for staining.

2.7 Data analysis

Statistical analysis and graphical presentation were performed using R (v. 4.4.2 (2024-10-31)).

3 *Ex situ* renal replacement therapy during normothermic porcine liver perfusion

3.1 Background

The blood-based perfusate used for normothermic liver perfusion consists of packed red cells and colloid, with additional bolus and infusion medications. This results in a perfusate composition that differs from *in vivo* whole blood. In particular, the use of stored packed red cells combined with washout of organ preservation solution often results in severe hyperkalaemia: in the COPE liver normothermic preservation RCT[95], the mean 1-hour perfusate potassium concentration with a recordable result was 13.5 (3.5 – 20.9) mmol/L; in 19 of 91 cases, the perfusate potassium was unrecordably high. Whilst perfusate hyperkalaemia may not significantly impair perfusion of transplantable livers, rapid infusion of perfusate with an unrecordably high potassium level into a critically unwell patient with liver failure upon commencing ELC would risk cardiac dysrhythmia and haemodynamic instability. Although measures such as washing red cells pre-priming can reduce the perfusate potassium level[97], this does not guarantee that it falls within a safe range at the time of patient connection. In addition to perfusate hyperkalaemia, there is time-related deterioration in perfusate quality during perfusion: metabolic waste (urea) accumulates and osmolality increases[98,218]. Renal replacement therapy (RRT) techniques were developed to correct electrolyte abnormalities, fluid imbalance, and remove urea in patients with renal failure[7]. Applications of the concepts that underpin RRT to normothermic liver perfusion may correct perfusate hyperkalaemia and attenuate the time-related deterioration in perfusate quality. Different techniques of continuous RRT exist: (i) haemofiltration: solute clearance by convection as ultrafiltrate from the patient's blood is driven across the filter membrane by a hydrostatic pressure gradient; replacement fluid is infused to replace lost volume. (ii) haemodialysis: solute clearance by diffusion as dialysate flows counter-current to the patient's blood, separated by a semi-permeable membrane. (iii) haemodiafiltration: solute clearance by both diffusion and convection as dialysate counter-current flow is combined with ultrafiltration[219]. Standard dialysis filters contain semi-permeable membranes with an approximate molecular weight cut-off of 5 kDa. This allows removal of ions and small molecules (e.g. urea), but not cytokines or

albumin (for example). Convective techniques are associated with greater clearance of higher-molecular weight substances (i.e. 1.5 – 50 kDa)[219]. High cut-off filters have been developed, with increased pore size, to increase removal of larger molecules (up to 60 kDa) without albumin (66.5 kDa) loss in conditions such as multiple myeloma with light chain disease[220,221].

Incorporation of haemodialysis in a 'donation after circulatory death' rat liver normothermic perfusion model was associated with increased graft bile production, reduced perfusate transaminase levels, and less apoptosis[222]. Since then, there have been reports of multi-day perfusion of declined human livers supported by incorporation of haemodialysis[30] or haemofiltration[218,223]. During these prolonged perfusion studies, incorporation of RRT was associated with maintenance of stable perfusate biochemistry, however the impact of RRT was not directly assessed and different techniques were not compared.

There is increasing recognition of the phenomenon of *ex situ* reperfusion injury[224-226]: upon normothermic reperfusion of donor livers *ex situ*, there is release of cytokines and DAMPs into the perfusate that may potentiate IRI. Infusion of perfusate inflammatory mediators into a recipient upon commencing ELC could worsen the pre-existing systemic inflammatory state associated with ALF and ACLF.

Addition of cytokine and DAMP adsorption columns to normothermic perfusion circuits has been trialled to attenuate the impact of this inflammatory process. Incorporation of the Cytosorb filter to adsorb cytokines during normothermic perfusion of declined human kidneys resulted in a less pro-inflammatory graft transcriptomic signature[227]. Studies of slaughterhouse pig liver normothermic perfusion that incorporated either the Cytosorb filter[228] or NucleoCapture filter[224] (to adsorb nuclear DAMPs such as cell-free DNA, extra-cellular histones, neutrophil extra-cellular traps) improved perfusion parameters. A single study of haemofiltration during declined human liver normothermic perfusion demonstrated that this technique could remove interleukin-6, but did not assess other cytokines or DAMPs[223].

Haemolysis is common with extra-corporeal blood perfusion circuits[229], and is well-described in the context of *ex situ* normothermic liver perfusion[230]. The result is a gradual decline in perfusate quality due to a decrease in oxygen-carrying capacity and (once the liver's reticulo-endothelial system

clearance capacity is exceeded) accumulation of cell-free haemoglobin, which acts as a DAMP[231]. The latter may be particularly problematic during kidney perfusion due to the absence of an intrinsic reticulo-endothelial system and nephrotoxicity of cell-free haemoglobin[232]. The mechanism that causes haemolysis is incompletely understood, but is likely a combination of high shear stress, negative pressures, heat generation by the centrifugal pump, exposure to foreign surfaces, and turbulent flow[233]. Rising perfusate osmolality with perfusion duration may increase the sensitivity of red cells to haemolysis. Maintaining physiological perfusate osmolality during perfusion by incorporation of RRT may reduce the rate of haemolysis. Further, it may be possible to remove cell-free haemoglobin using a combination of a high cut-off filter and applying a transmembrane hydrostatic pressure gradient during RRT (i.e. haemofiltration or haemodiafiltration).

3.2 Aims

The primary aim was to determine the optimal RRT technique to correct perfusate potassium concentration during isolated normothermic liver perfusion using the OrganOx *metra* prior to ELC. The secondary aims were to assess the impact of different RRT techniques on: (i) perfusate electrolyte concentrations, osmolality, and urea concentration; (ii) haemolysis; (iii) removal of perfusate cytokines. (i) and (iii) are relevant to optimising perfusate composition prior to ELC. (i) – (iii) may support prolonged (multi-day) *ex situ* normothermic liver preservation.

3.3 Methods

3.3.1 Study design

Three *ex situ* RRT techniques were evaluated during 28-hour normothermic perfusion of slaughterhouse pig livers: (i) haemofiltration using haemoconcentrator ($n = 3$); (ii) haemodiafiltration using standard dialysis filter ($n = 3$); (iii) haemodialysis using high cut-off filter ($n = 3$). These were compared to control perfusions that underwent standard normothermic perfusion without RRT ($n = 3$) (*figure 8*).

Slaughterhouse pig livers and autologous blood were retrieved per *2.1.1*. The livers were stored under static cold storage during transport to the study site. Back-table preparation was performed per *2.2*, followed by normothermic perfusion using autologous leuco-depleted blood per *2.3* (modified to enable *ex situ* RRT according to treatment group). *Ex situ* RRT commenced 30 minutes prior to liver reperfusion and continued until 12 hours of perfusion. It was then paused for 8 hours and resumed between hours 20 and 28 of perfusion.

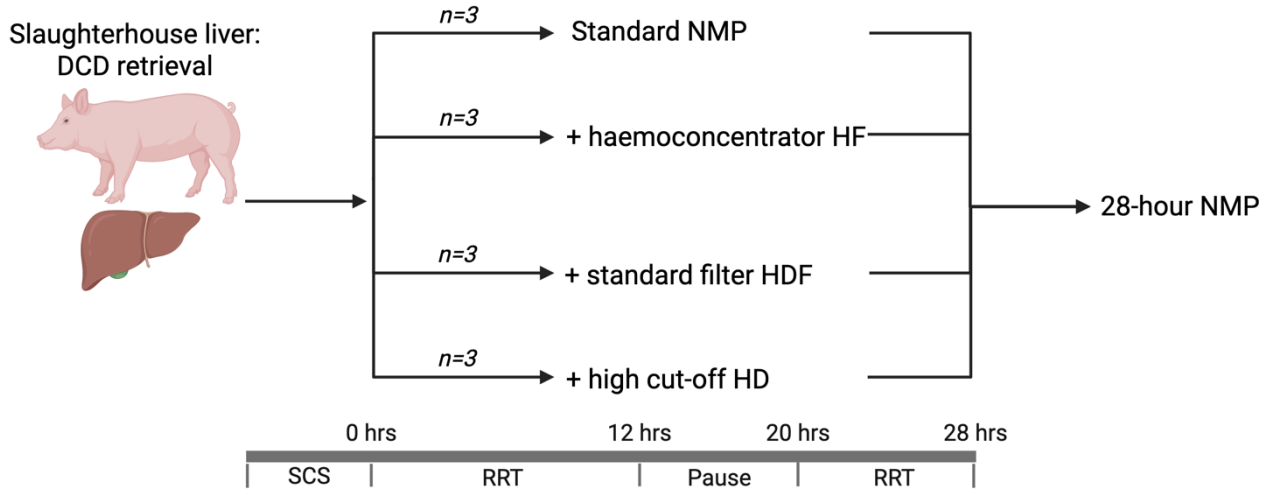


Figure 8: study design: *ex situ* renal replacement therapy during normothermic liver perfusion.
DCD: donation after circulatory death. SCS: static cold storage. RRT: renal replacement therapy. NMP: normothermic machine perfusion. HF: haemofiltration. HDF: haemodiafiltration. HD: haemodialysis. hrs: hours.

3.3.2 *Ex situ* haemofiltration

To enable *ex situ* haemofiltration, the standard *metra* perfusion circuit (*figure 5*) was modified by inserting a bypass line containing a haemoconcentrator (DP03) between the centrifugal pump and portal reservoir in parallel to the main perfusion circuit (*figures 9, 11*). Filtrate was drawn from the haemoconcentrator using an Alaris SE 7130 infusion pump, and discarded. Dialysate (multiBic K4) was infused into the portal reservoir using a second Alaris SE 7130 pump at a rate equal to the filtrate removal rate. The haemofiltration rate was maintained at 999 mL/hour in this experiment.

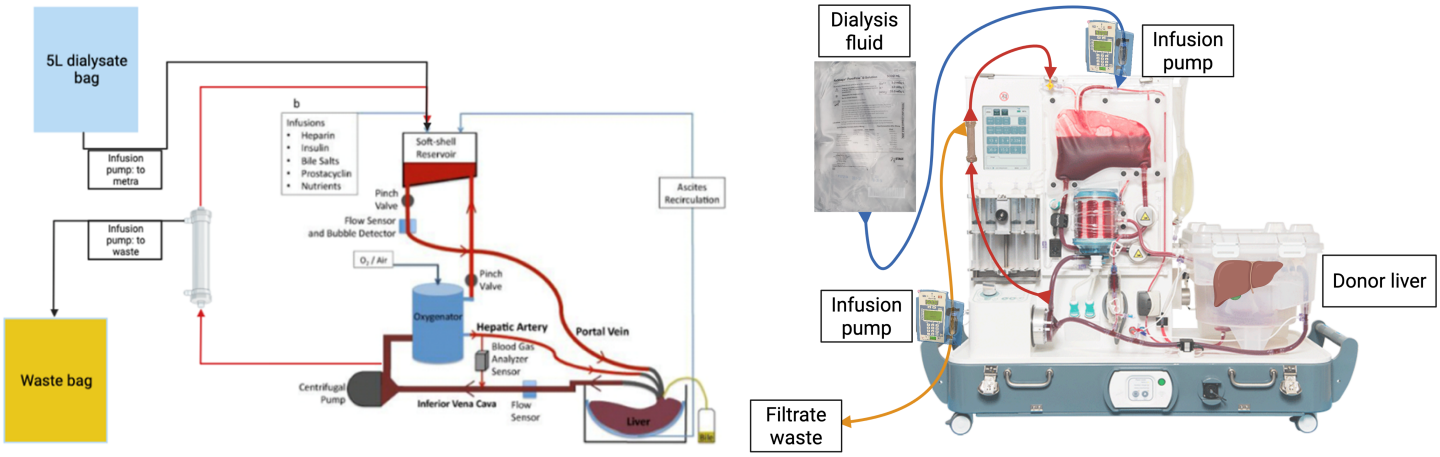


Figure 9: *ex situ* haemofiltration during normothermic liver perfusion circuit diagram.

3.3.3 *Ex situ* haemodialysis and haemodiafiltration with dialysate recirculation

Ex situ haemodialysis and haemodiafiltration were performed using a novel *ex situ* dialyser system consisting of three independently programmable roller pumps. Two of the pumps recirculate dialysate counter-current to perfusate flow through the bypass line. Transmembrane pressure is generated by offsetting the rate of these two pumps, thereby enabling haemodiafiltration: negative transmembrane pressure is generated if the efflux roller pump rate exceeds the influx pump rate. The third pump pre-dilutes the perfusate and enables neutral fluid balance in the setting of negative transmembrane pressure (*figures 10, 11*).

In this experiment, this system was used in two modes: (i) haemodiafiltration (dialysate efflux pump rate > dialysate influx pump rate; pre-dilution pump rate = efflux pump rate – influx pump rate) using a standard dialysis filter (FX100 classix, Fresenius Medical Care, Bad Homburg v. d. Höhe, Germany); (ii) haemodialysis (dialysate efflux pump rate = dialysate influx pump rate; pre-dilution pump rate = 0) using a high cut-off filter (Theranova, Baxter, Deerfield, IL, USA). In both cases, a single 5 L bag of dialysate (multiBic K4) was recirculated throughout the experiment.

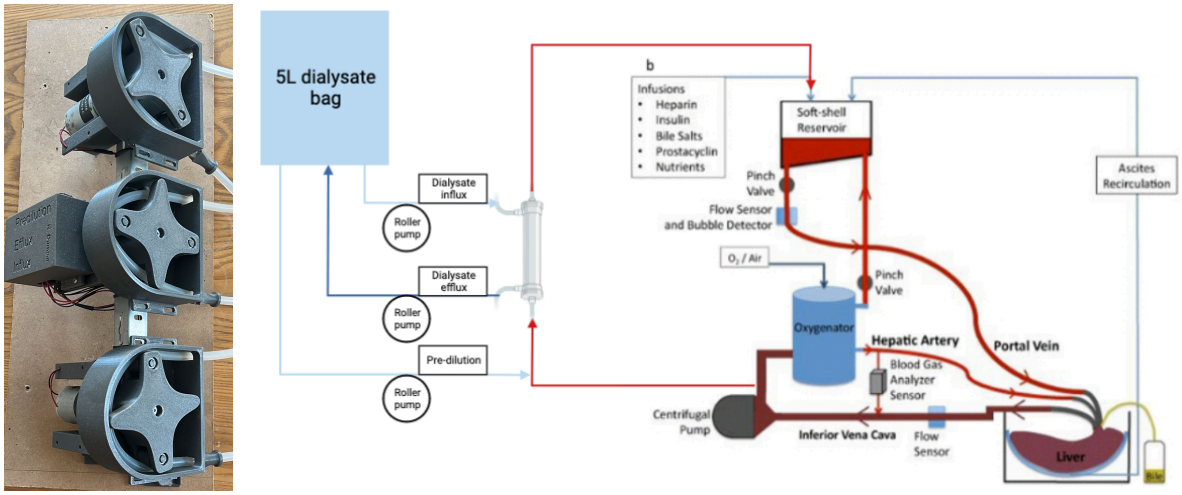


Figure 10: *ex situ* haemodialysis and haemodiafiltration with dialysate recirculation during normothermic liver perfusion is enabled by a bespoke system consisting of three independently programmable pumps.

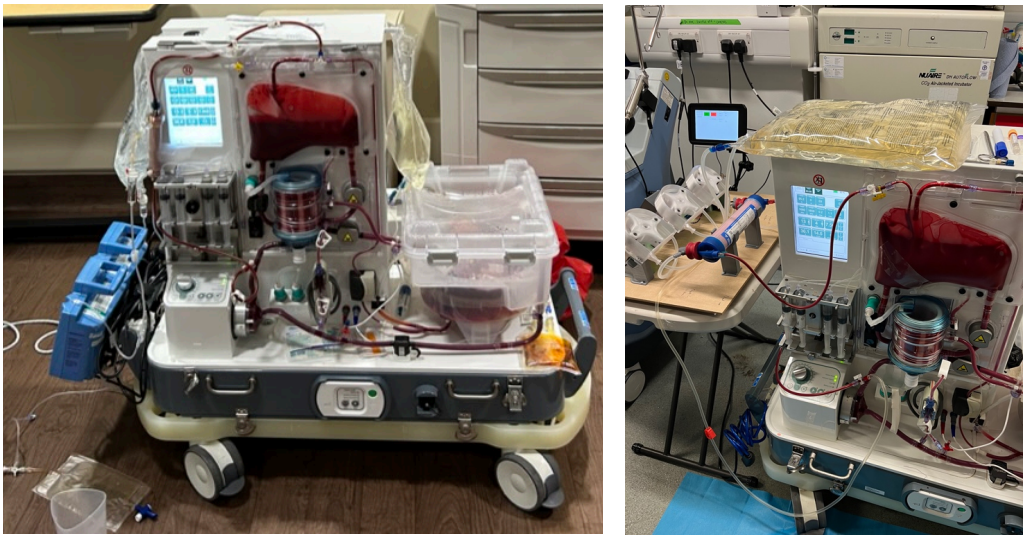


Figure 11: *ex situ* haemofiltration (*left*) and *ex situ* haemodialysis with dialysate recirculation using standard dialysis filter (*right*).

3.3.4 Sampling schedule and analysis

During normothermic liver perfusion, perfusate and filtrate/dialysate samples were taken 4-hourly. For *ex situ* haemofiltration, filtrate samples were collected in 10 mL syringes from the filtrate line leaving the haemoconcentrator. For *ex situ* haemodialysis and haemodiafiltration, recirculating dialysate was sampled from the line leaving the dialysate bag. Perfusate samples were collected into SST (1), EDTA (2) tubes, and for point-of-care analysis, processed per *2.6*. Filtrate/dialysate samples

were collected for point-of-care analysis and into Eppendorf tubes for storage (frozen temporarily at -20°C, followed by -80°C).

Point-of-care perfusate and filtrate/dialysate samples were analysed using an ABL90 Flex arterial blood gas analyser (Radiometer, Crawley, UK) for: pH, PO_2, pCO_2, haemoglobin, haematocrit, lactate, glucose, potassium, sodium, ionised calcium, and osmolality. Perfusate serum and filtrate/dialysate samples were analysed in the Clinical Biochemistry Laboratory, Oxford University Hospitals for urea, ALT, albumin, bilirubin, and insulin. Cytokine and DAMP concentrations in perfusate plasma and filtrate/dialysate samples were analysed using ELISAs: ESIL-6 (interleukin-6); KSC3011 (TNF-α); ESIL1B (interleukin-1β); KSC0101 (interleukin-10); ES12RB (interleukin-18) (Thermo Fisher Scientific, Waltham, MA, USA); abx154938 (HMGB1) (Abbexa, Cambridge, UK). Since dialysate was recirculated in the *ex situ* haemodialysis and haemodiafiltration techniques, cytokine/DAMP analysis was limited up to 8 hours; beyond 8 hours protein degradation in the dialysate maintained at close-body temperature would underestimate dialysate cytokine/DAMP concentrations. Perfusate serum samples were analysed using the ABL90 Flex arterial blood gas analyser to provide serum cell-free haemoglobin.

The *metra* recorded (every second): hepatic artery flow; portal vein flow; inferior vena cava flow; hepatic artery pressure; inferior vena cava pressure; and perfusate pO_2; pCO_2; pH using a shunt sensor (Terumo). Perfusate flow on the RRT bypass line was measured using a BioProt TT flow sensor (em-tec, Finning, Germany). Bile production was measured manually.

3.3.5 Statistical analysis

This experiment was a pragmatic assessment of *ex situ* RRT techniques during normothermic liver perfusion, with the primary aim of determining the most feasible technique to correct perfusate potassium concentration prior to ELC; the experiment was not designed with sufficient numbers to rigorously compare outcomes between techniques. Outcomes are described as mean (range) and presented graphically as mean values with individual study datapoints; statistical comparison between treatment groups is not performed.

3.4 Results

3.4.1 Normothermic liver perfusion parameters

The warm ischaemic times were 14 – 40 minutes. Cold ischaemic times were 4 – 8 hours. The portal vein and hepatic artery flows are demonstrated in *figures 12* (mean values for each treatment group) and *13* (individual studies presented). The livers maintained physiological hepatic artery and portal vein flows in all groups. Fluctuation in portal vein flow was greater during high cut-off haemodialysis and, especially, standard filter haemodiafiltration (*figure 13*). The flows in the bypass lines were: haemoconcentrator-haemofiltration: 100 – 130 mL/minute; standard filter haemodiafiltration: 150 – 250 mL/minute; high cut-off filter haemodialysis: 50 – 120 mL/minute. The total dialysate volume used per perfusion was: haemoconcentrator-haemofiltration: 20 L; standard filter haemodiafiltration: 5 L; high cut-off filter haemodialysis: 5 L.

Perfusate ALT levels remained below the threshold to consider a human liver suitable for transplantation[3]. All livers cleared lactate, and demonstrated evidence of glucose metabolism (although this was reduced in the final 8 hours of perfusion in the control group). All livers produced bile except one in the haemoconcentrator-haemofiltration treatment group (*figure 14*).

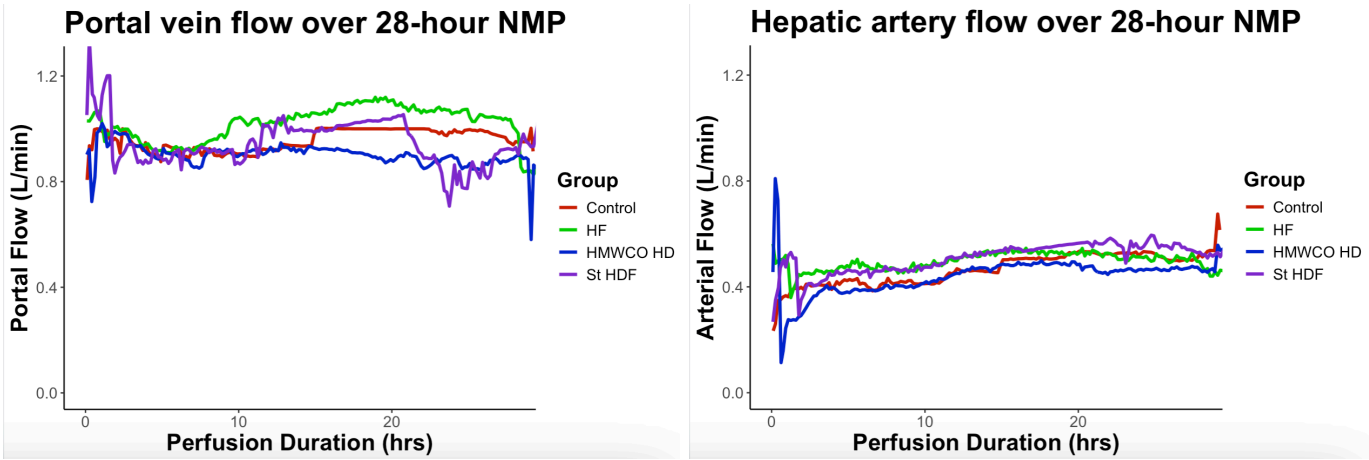


Figure 12: perfusion haemodynamics (mean portal vein, hepatic artery flow) during liver normothermic perfusion with *ex situ* renal replacement therapy.
HF: haemoconcentrator-haemofiltration; HMWCO HD: high cut-off filter haemodialysis; St HDF: standard filter haemodiafiltration. NMP: normothermic machine perfusion.

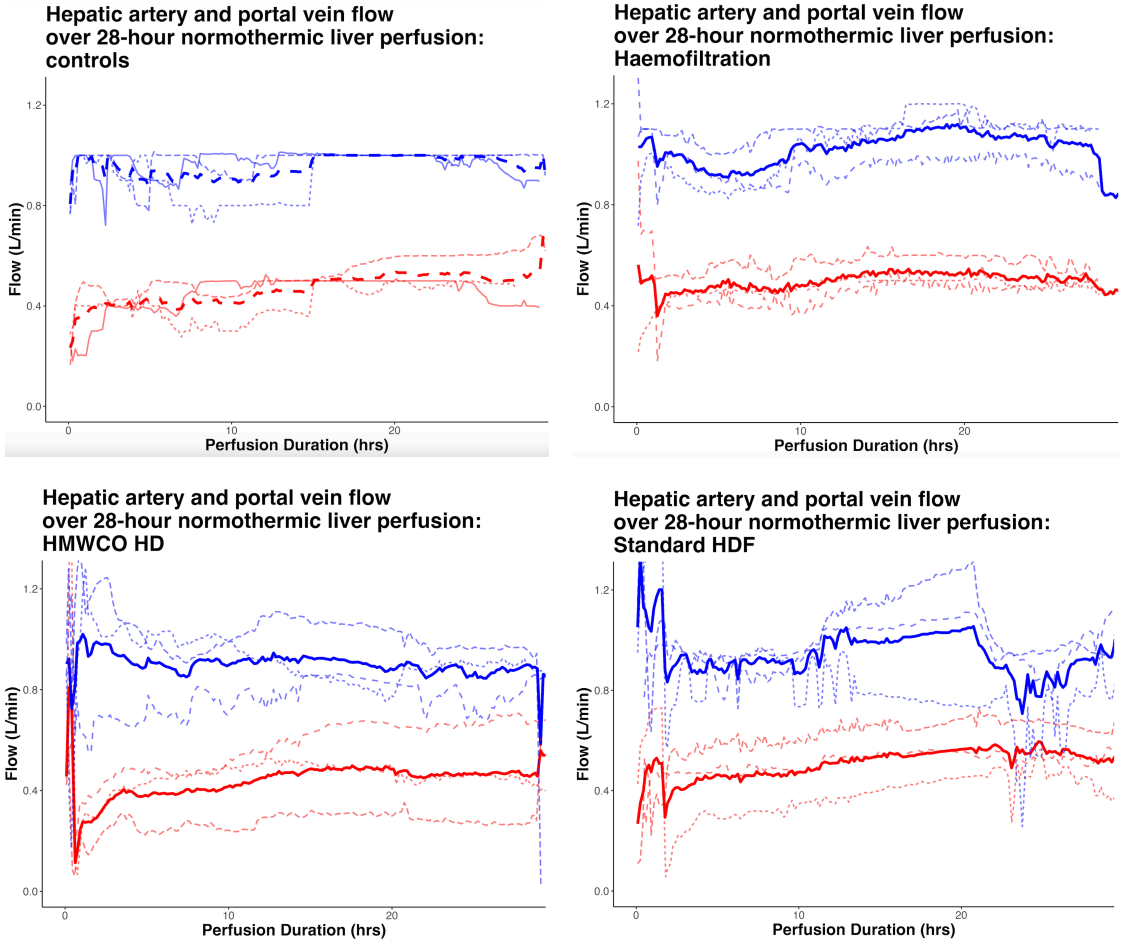


Figure 13: perfusion haemodynamics (portal vein (blue), hepatic artery (red) flow) during liver normothermic perfusion with *ex situ* renal replacement therapy.
Ex situ renal replacement therapy was paused between hours 12 and 20. Dashed lines: individual studies; solid lines: mean of each treatment group. Haemofiltration: haemoconcentrator-haemofiltration; HMWCO HD: high cut-off filter haemodialysis; Standard HDF: standard filter haemodiafiltration.

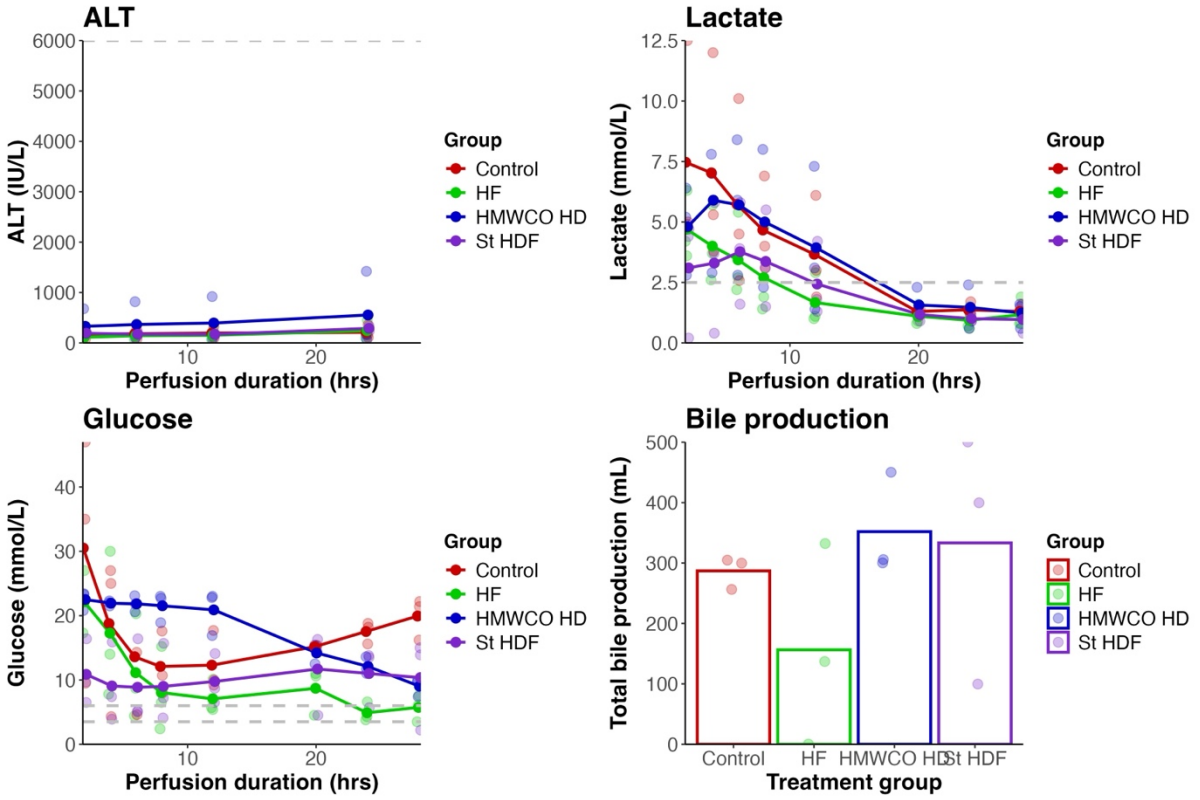


Figure 14: Perfusate alanine transaminase (ALT), lactate, glucose concentration, and total bile production during liver normothermic perfusion with *ex situ* renal replacement therapy (mean, individual studies).
Horizontal dashed line: perfusate ALT threshold considered to indicate transplantability of a human liver. HF: haemoconcentrator-haemofiltration; HMWCO HD: high cut-off filter haemodialysis; St HDF: standard filter haemodiafiltration.

3.4.2 Effect of *ex situ* RRT on perfusate composition

The *ex situ* RRT techniques all maintained physiological perfusate concentrations of potassium, sodium, and ionised calcium, in contrast to the control perfusions (*figure 15*). *Ex situ* RRT slowed accumulation of urea (*figure 15*), which was associated with maintenance of physiological perfusate osmolality and a reduction in serum cell-free haemoglobin as a fraction of total perfusate haemoglobin (a marker of haemolysis, *figure 16*). The cell-free haemoglobin concentration in the filtrate/dialysate of the RRT perfusions remained low (28-hour filtrate/dialysate cell-free haemoglobin concentration: haemoconcentrator-haemofiltration: 0.1 (0 – 0.1) g/L; standard filter haemodiafiltration: 0.1 (0.1 – 0.2) g/L; high cut-off haemodialysis: 0.2 (0 – 0.3 g/L)).

At 8 hours of perfusion, the dialysate of the high cut-off haemodialysis group removed a substantial proportion of the perfusate interleukin-6, TNF-α, and interleukin-1β, but low-to-undetectable amounts

of interleukin-10, HMGB1, and interleukin-18. The filtrate of the haemoconcentrator-haemofiltration group contained a similar proportion of the perfusate interleukin-6 as high cut-off haemodialysis, but low-to-undetectable amounts of the other cytokines/DAMPs. Standard filter haemodiafiltration did not remove significant amounts of any cytokine/DAMP (*figure 17*). Accounting for the amount of filtrate/dialysate used by each technique, high cut-off haemodiafiltration was the most efficient technique at removing interleukin-6, TNF-α, interleukin-1β, and interleukin-10 (*figure 18*). The perfusate concentration of interleukin-6 was lower in the haemoconcentrator-haemofiltration group than the other treatment groups; there was substantial intra-group variability in the perfusate concentration of the other cytokines/DAMPs, limiting inter-group comparison (*figure 19*).

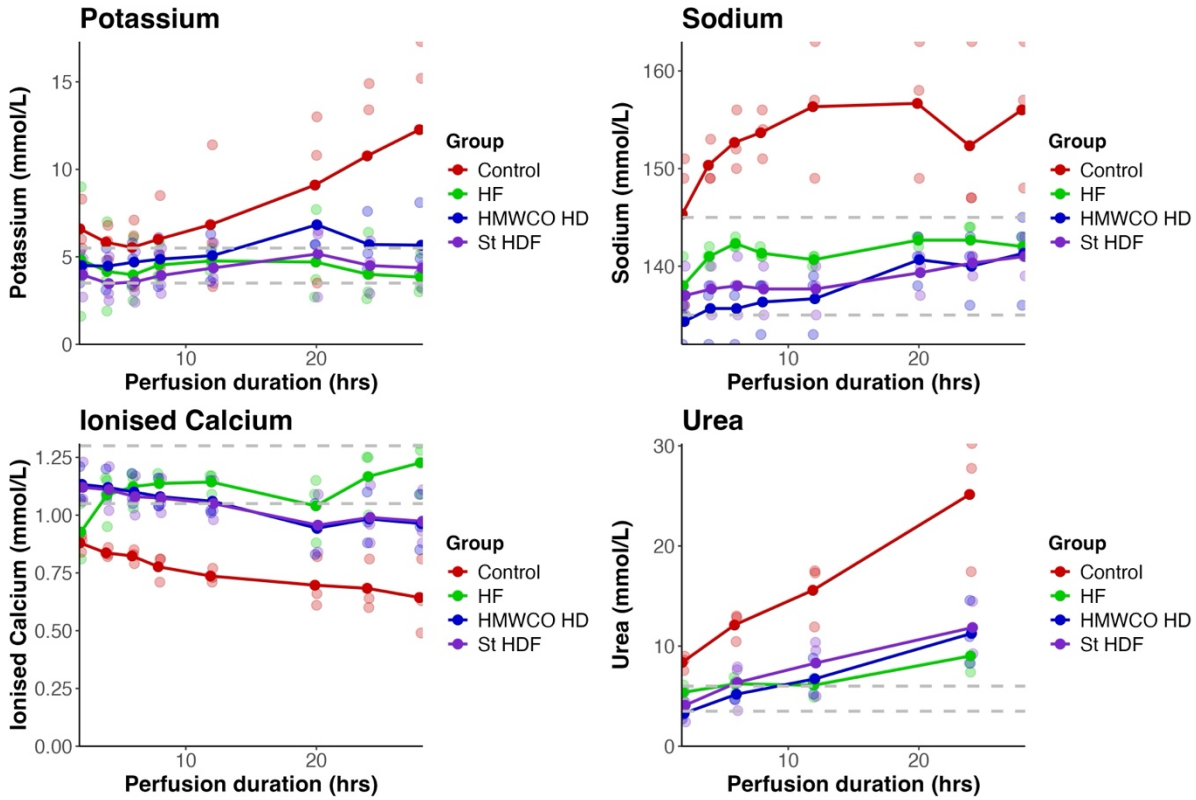


Figure 15: Perfusate potassium, sodium, ionised calcium, and urea concentration during liver normothermic perfusion with *ex situ* renal replacement therapy (mean, individual studies).
HF: haemoconcentrator-haemofiltration; HMWCO HD: high cut-off filter haemodialysis; St HDF: standard filter haemodiafiltration.

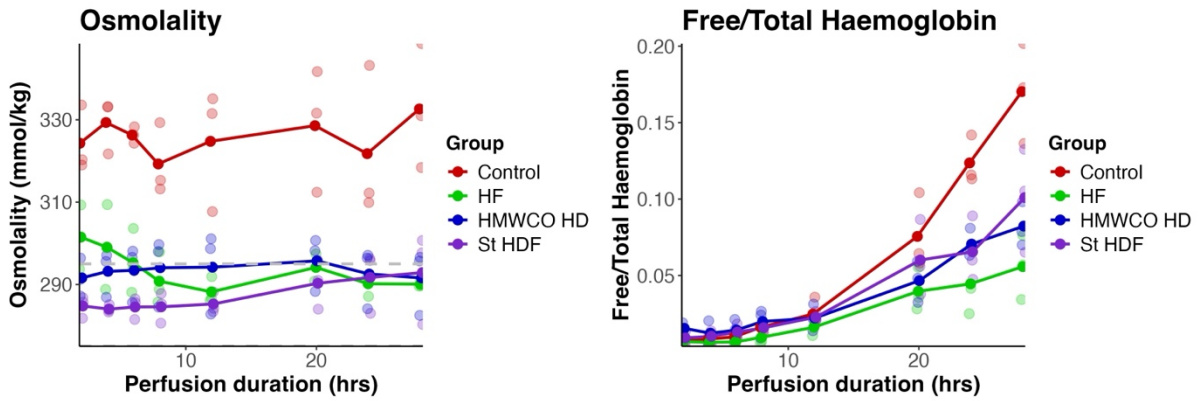


Figure 16: Perfusate osmolality and serum cell-free haemoglobin as a fraction of total perfusate haemoglobin (marker of haemolysis) during liver normothermic perfusion with *ex situ* renal replacement therapy (mean, individual studies).
HF: haemoconcentrator-haemofiltration; HMWCO HD: high cut-off filter haemodialysis; St HDF: standard filter haemodiafiltration.

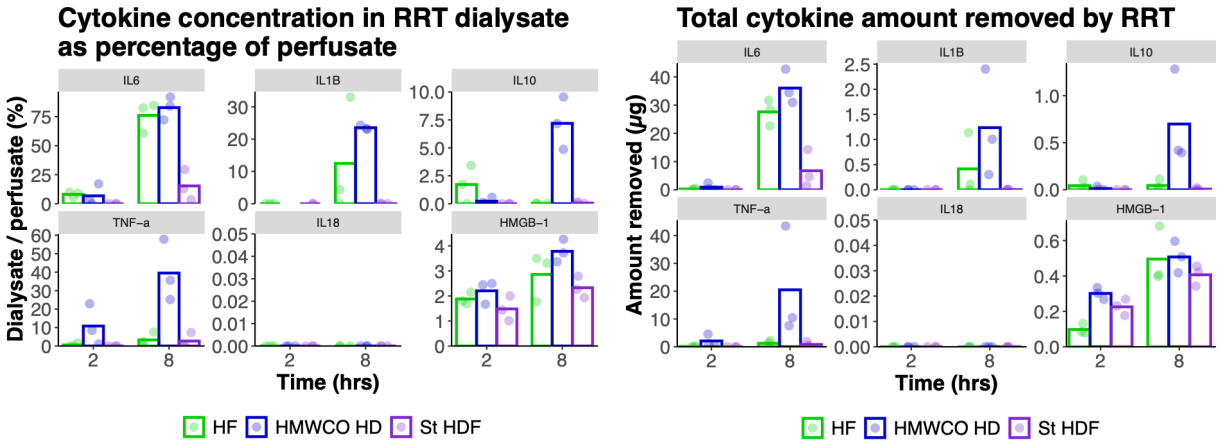


Figure 17: Cytokine/DAMP concentration in the filtrate/dialysate as a fraction of perfusate concentration (*left*) and total amount of each cytokine/DAMP removed (*right*) after 2 and 8 hours of liver normothermic perfusion with *ex situ* renal replacement therapy (mean, individual studies).
RRT: renal replacement therapy. DAMP: damage-associated molecular pattern. HF: haemoconcentrator-haemofiltration; HMWCO HD: high cut-off filter haemodialysis; St HDF: standard filter haemodiafiltration. ILx: interleukin-x. TNF-a: tumour necrosis factor-α. HMGB-1: high mobility group box-1.

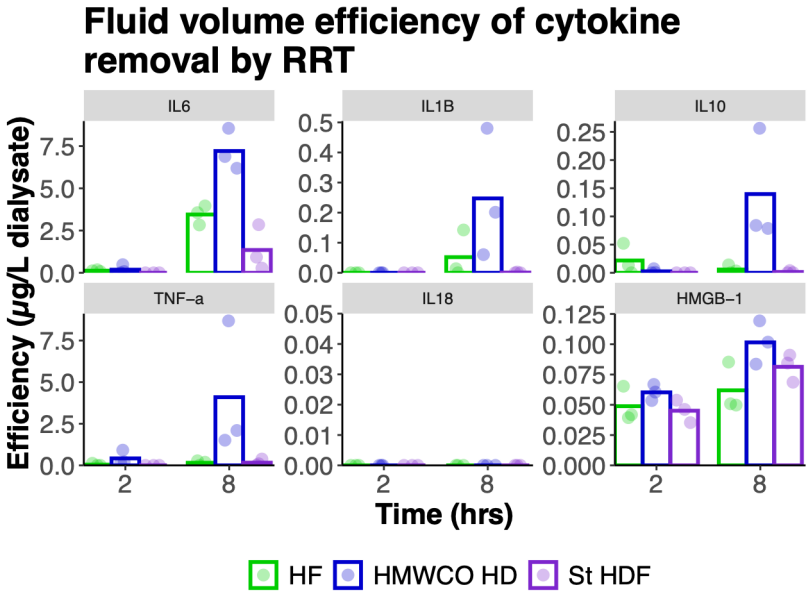


Figure 18: Amount of each cytokine/DAMP removed per litre of filtrate/dialysate used after 2 and 8 hours of liver normothermic perfusion with *ex situ* renal replacement therapy (mean, individual studies).
RRT: renal replacement therapy. DAMP: damage-associated molecular pattern. HF: haemoconcentrator-haemofiltration; HMWCO HD: high cut-off filter haemodialysis; St HDF: standard filter haemodiafiltration. ILx: interleukin-x. TNF-a: tumour necrosis factor-α. HMGB-1: high mobility group box-1.

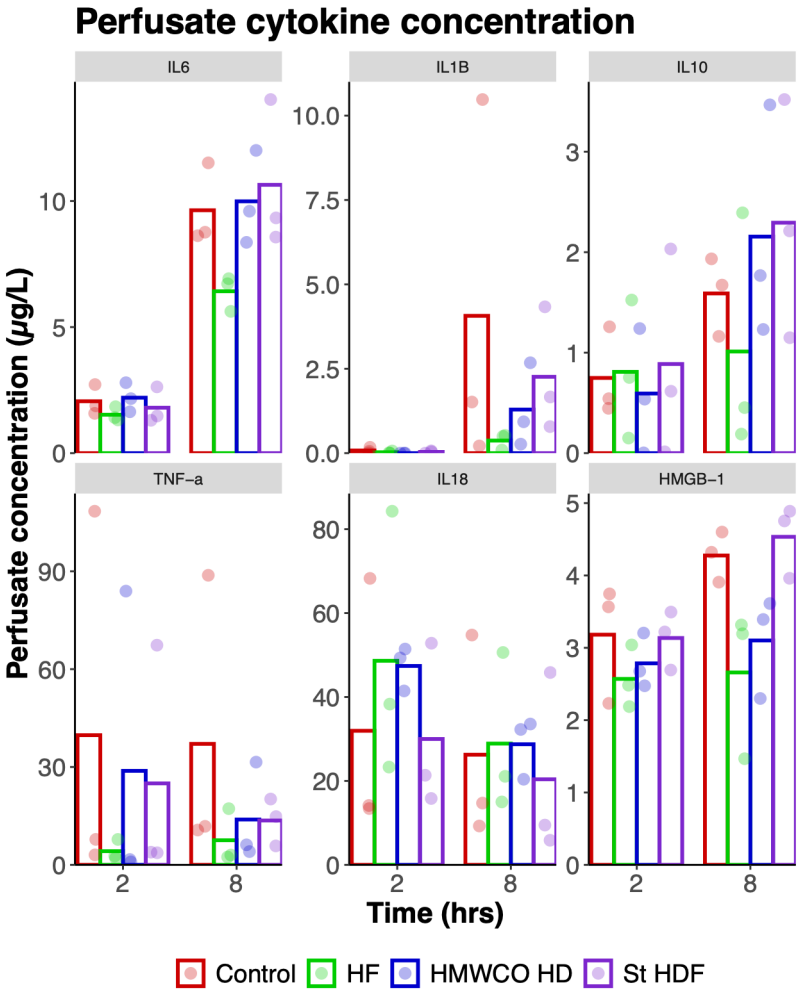


Figure 19: Perfusate concentration of each cytokine/DAMP after 2 and 8 hours of liver normothermic perfusion with *ex situ* renal replacement therapy (mean, individual studies).
DAMP: damage-associated molecular pattern. HF: haemoconcentrator-haemofiltration; HMWCO HD: high cut-off filter haemodialysis; St HDF: standard filter haemodiafiltration. ILx: interleukin-x. TNF-a: tumour necrosis factor-α. HMGB-1: high mobility group box-1.

3.4.3 Effect of *ex situ* RRT on additional molecules

The *ex situ* RRT techniques did not remove substantial amounts of perfusate albumin, ALT, or bilirubin: all three molecules remained below the limit of assay detection in the filtrate/dialysate (albumin < 3.0 g/L; ALT < 4 IU/L; bilirubin < 1.8 µmol/L). Haemoconcentrator-haemofiltration removed perfusate insulin: the filtrate level at 8 hours of liver NMP was approximately 100 – 400% of the perfusate level ($n = 3$) (*figure 20*).

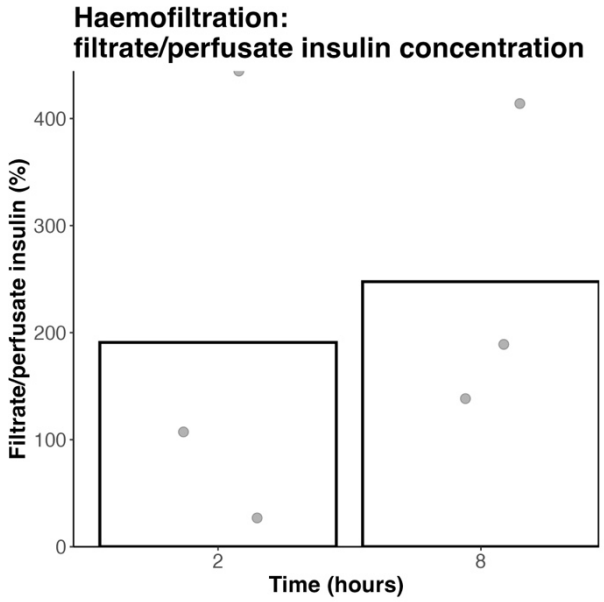


Figure 20: Insulin concentration in the filtrate as a percentage of perfusate concentration after 2 and 8 hours of liver normothermic perfusion with haemoconcentrator-haemofiltration (mean, individual studies).

3.5 Discussion

Haemoconcentrator-haemofiltration, standard filter haemodiafiltration, and high cut-off haemodialysis were feasible during normothermic porcine liver perfusion using the OrganOx *metra*; all three *ex situ* RRT techniques maintained physiological perfusate electrolyte (most importantly, potassium) concentrations and osmolality (by slowing accumulation of urea).

Liver portal vein flows were stable during haemoconcentrator-haemofiltration, whereas notable fluctuation was observed during standard filter haemodiafiltration and high cut-off haemodialysis. Portal vein flow fluctuation occurred due to variation in the portal reservoir height secondary to fluid shift between the perfusate and dialysate compartments. The novel *ex situ* dialyser system used for haemodialysis and haemodiafiltration was a prototype, 3-D printed and engineered in-house: the roller pumps were not completely occlusive and generated mildly inconsistent flows. Given the dialysate and perfusate flow rates, relatively small inconsistencies result in volume shifts that cause appreciable changes in the portal reservoir height and therefore portal vein flow. The use of the *ex situ* dialyser

system therefore requires constant supervision to maintain perfusion stability. This contrasts with the haemoconcentrator-haemofiltration system, which uses commercially-available infusion pumps designed to deliver intravenous medications to patients: the haemofiltration system reliably ensures volume neutrality between filtrate removal and dialysate fluid replacement.

Ex situ haemodialysis and haemodiafiltration clear small molecules from the perfusate flowing through the RRT bypass line by diffusion across the filter membrane down their concentration gradients to the dialysate (with additional convective clearance for haemodiafiltration)[219]. The sieving coefficient for potassium is 1.00, allowing close-to complete clearance of perfusate over the length of the filter. The perfusate potassium clearance rate therefore approximates to the bypass flow rate (~100 mL/minute). Since the perfusate total volume is about 1.5 L, initial equalisation of the perfusate potassium concentration with the dialysate (4 mmol/L) occurs within ~15 minutes. This contrasts with the haemoconcentrator-haemofiltration technique, which replaces the perfusate fluid/small molecule compartment at a rate of 999 mL/hour (16.65 mL/minute): it takes about 90 minutes to replace the initial perfusate fluid/small molecule compartment with dialysate (during which time additional potassium may be released). The initial clearance of perfusate potassium by haemofiltration is therefore slower than haemodialysis and haemodiafiltration.

Ex situ haemodialysis and haemodiafiltration recirculate dialysate, whereas haemofiltration generates waste filtrate that must be discarded. The volume of recirculating dialysate is 5 L. For molecules that cross the filter membrane, haemodialysis and haemodiafiltration with dialysate recirculation effectively increase the total volume of distribution by a factor of 4.33. In these perfusions, this was sufficient to maintain physiological perfusate electrolyte concentrations and osmolality over 28 hours. The advantage of dialysate recirculation is that it avoids the need to dispose of waste filtrate, which can be challenging if large volumes are generated whilst a liver is perfused in transit. However, the clearance gradient decreases over time, which reduces efficiency. If the dialysate becomes saturated, the dialysate bag must be exchanged for a new one, which is, in turn, less convenient than disposal of waste filtrate. Although substantially slowed relative to controls, the perfusate urea concentration increased gradually during perfusion with all three *ex situ* RRT techniques. This may have been

prevented by dialysate exchange during perfusion with *ex situ* haemodialysis/haemodiafiltration *versus* increasing the filtration rate with *ex situ* haemofiltration.

There are several potential advantages of the *ex situ* high cut-off haemodialysis technique: (i) greater clearance of perfusate cytokines; (ii) quicker initial correction of electrolyte concentrations; (iii) no need to dispose waste filtrate. However, the greater volume stability of haemoconcentrator-haemofiltration, and concomitant lower level of supervision required, is highly valuable in the context of perfusate preparation prior to ELC due to the multiplicity of demands on the perfusionist during the isolated normothermic perfusion phase. An additional practical advantage of the haemoconcentrator-haemofiltration system is that there is no requirement to monitor clearance efficiency and change the dialysate bag once saturated. Since this experiment demonstrated that all three *ex situ* RRT techniques corrected perfusate potassium effectively and within an appropriate timeframe, the decision to use haemoconcentrator-haemofiltration for the *metra*-ELC protocol was based on these pragmatic considerations.

High cut-off haemodialysis removed a substantial fraction of perfusate interleukin-6, TNF-α, and interleukin-1β, limited amounts of interleukin-10 and HMGB-1, and no detectable interleukin-18. Clearance of cytokines/DAMPs did not correlate directly with molecule size (*table 6*). This may be due to formation of dimers/trimers or the effect of molecular characteristics such as charge on interaction with the filter membrane. Combination of haemodiafiltration with a high cut-off filter could increase clearance of larger molecules by combining diffusive and convective clearance. However, when this technique was trialled during a pilot blood-only perfusion using slaughterhouse pig blood, clearance efficiency of mid-sized molecules was less than with high cut-off haemodialysis. This may be due to haemolysis (exaggerated in this model as a consequence of the slaughterhouse blood collection process) generating red cell fragments that block the filter pores. The filter surface then becomes the red cell membrane, preventing clearance of mid-sized hydrophilic molecules.

Molecule	Molecular weight (kDa)
Meropenem	0.38
Insulin	5.8
IL-1β	17
TNF-a	17 (monomer)
IL-10	18
IL-18	18
IL-6	21-28
HMGB1	25
Gelofusine	30
ALT	57
Haemoglobin	64.5
Albumin	66.5

Table 6: molecular weight (kDa) of select perfusate molecules.
ALT: alanine transaminase. IL-x: interleukin-x. TNF-α: Tumour necrosis factor-α. HMGB1: High mobility group box-1.

Whilst studies in the literature suggest that there may be benefit to removal of cytokines/DAMPs during *ex situ* organ perfusion[227,228], the evidence remains preliminary, uses surrogate markers (e.g. graft transcriptome), and has not been validated *in vivo*. In the context of ELC, it is theoretically beneficial to minimise the inflammatory load delivered to recipients at the start of ELC: increased cytokine/DAMP levels are associated with worse outcomes in patients with ALF and ACLF[234-237]. The perfusate inflammatory load could be especially prominent when declined livers are used for ELC due to their potential for an exaggerated *ex situ* reperfusion injury[224]. The relative importance of the different cytokines and DAMPs in this process is not yet known. This makes high cut-off haemodialysis during the pre-ELC isolated normothermic perfusion phase potentially attractive, as it selects based on molecular size rather than specific molecules: many cytokines and DAMPs are smaller than the high cut-off size threshold. Conversely, if specific central mediators become identified in the future, targeted adsorption may be a preferred approach for cytokine/DAMP clearance pre-ELC (combined with *ex situ* RRT to correct perfusate electrolyte composition). Future work should include clinical-grade manufacture of the *ex situ* dialyser prototype used in this study, to produce a volume-stable platform with a straightforward dialysate bag exchange system. This would make *ex situ* haemodialysis a feasible approach during the isolated normothermic phase prior to ELC. In addition, this would enable exploration of the technique of push-pull haemodiafiltration[238] to

maximise cytokine/DAMP removal from the perfusate: transmembrane pressure oscillates between positive and negative to clear mid-sized molecules by convection, whilst dislodging red cells/red cell fragments blocking filter pores.

The *ex situ* RRT techniques evaluated in this study did not remove albumin or ALT. The former comprises the colloid component of perfusate. Loss of colloid oncotic pressure during perfusion would alter transcapillary hydrostatic pressure dynamics causing tissue oedema. If this were the case (for example, as may occur with high cut-off push-pull haemodiafiltration), perfusate albumin levels would need to be monitored and replenished during perfusion. Importantly, gelofusine is frequently used during normothermic liver perfusion as an alternative source of colloid to albumin. The molecular weight of gelofusine is about 30 kDa, meaning that it could be removed by haemoconcentrator-haemofiltration and high cut-off haemodialysis. Unlike albumin, there is no straightforward assay for gelofusine to measure the amount removed and determine replacement requirement. Therefore, perfusate should be composed using 5% human albumin solution rather than gelofusine if these *ex situ* RRT techniques are planned.

Perfusate ALT levels are often measured during normothermic liver perfusion as a measure of hepatocyte damage: a perfusate level < 6,000 IU/L is used as a threshold to guide decision-making regarding the transplantability of a perfused liver[3]. The fact that ALT is not removed by the described RRT techniques means that this criterion can still be used. The other perfusate criteria used to determine liver transplantability (lactate, glucose, pH) are however affected by *ex situ* RRT and so must be interpreted accordingly. *Ex situ* haemofiltration also removes insulin (its molecular weight is 5.8 kDa). Insulin is a standard infusion used in the *metra* liver perfusion protocol. The relevance of this to ELC is discussed in *chapter 6*.

Ex situ RRT was associated with reduced accumulation of cell-free haemoglobin in perfusate. This was expressed as a fraction of total perfusate haemoglobin to facilitate comparison between the groups. Minimal cell-free haemoglobin was detected in the filtrate/dialysate, suggesting that the lower perfusate levels were not due to increased removal in the RRT-treated perfusions. *Ex situ* RRT maintained physiological perfusate osmolality, in contrast to the hyperosmolar perfusate of the control perfusions. Increased osmolality could deform perfusate red cells, increasing susceptibility to shear

stress and haemolysis. However, this study did not account for potential differences in clearance by the reticulo-endothelial system of the liver between treatment groups.

This study was a pragmatic assessment of *ex situ* RRT techniques to determine the most feasible approach to use in the *metra*-ELC protocol. Slaughterhouse pig livers sustain severe (and often inconsistent) ischaemic injury during the slaughter/retrieval process, which occurs in a non-sterile environment. The model is best suited to preliminary evaluation of the feasibility of interventions during perfusion, rather than sophisticated analysis of subtle findings. A number of additional limitations mean that rigorous comparison between groups is not possible: (i) the small number of cases per treatment group ($n = 3$) prevents statistical comparison; (ii) warm and cold ischaemic times were minimised to increase the probability of viable perfusion, rather than strictly controlled; (iii) the amount of dialysate used for *ex situ* haemodialysis and haemodiafiltration (5 L) was less than for *ex situ* haemofiltration (20 L). These limitations may account for the perfusate interleukin-6 concentration being lower in the haemoconcentrator-haemofiltration than high cut-off haemodialysis group, despite greater total removal in the latter. Finally, the perfusate was leuco-depleted autologous blood; clinical liver perfusions usually use packed red cells with 5% human albumin solution or gelofusine, rather than plasma. Extrapolation of the conclusions to clinical perfusions must be done cautiously.

The findings of improved perfusate electrolyte composition and osmolality, cytokine removal, and a potential reduction in haemolysis warrant further investigation in the context of liver preservation prior to transplant. Incorporation of *ex situ* RRT has anecdotally demonstrated benefit in supporting prolonged perfusion[30,223]. Its potential to improve perfusion of extended criteria livers over clinical perfusion durations has not been explored. A controlled study using livers from anaesthetised porcine donors with substantial (but consistent) ischaemic damage (e.g. 1-hour warm ischaemic time, 6-hour cold ischaemic time) that undergo 24 hours normothermic perfusion (perfusate: allogenic packed red cells with 5% human albumin solution) with the same treatment groups as this study ($n = 6$ per treatment group), followed by transplant and recovery (primary outcomes: early allograft dysfunction; primary non-function) could determine whether these techniques may be beneficial in the setting of organ preservation.

This study demonstrates that all three examined *ex situ* RRT techniques correct perfusate electrolyte composition, maintain perfusate osmolality, and remove metabolic waste. This is combined with cytokine removal for high cut-off haemodialysis and, to a lesser extent, haemoconcentrator-haemofiltration. For correction of the perfusate potassium level during the isolated normothermic perfusion phase prior to ELC, haemoconcentrator-haemofiltration is the superior technique due to practical considerations related to the use of the system.

3.6 Declaration

I designed and ran the study; performed the liver perfusions and sample collection (with support from the Transplant Research Fellows and OrganOx clinical specialists included under *Acknowledgements*); laboratory analysis (except measurements of urea, albumin, ALT, bilirubin, and insulin, which were completed in the Clinical Biochemistry Laboratory, Oxford University Hospitals); data analysis; and interpretation.

4 *In vivo* assessment of the feasibility of extra-corporeal liver cross-circulation by adaptation of a commercially-available normothermic liver preservation device

4.1 Background

The OrganOx *metra* is a commercially-available, CE-marked, FDA-approved normothermic liver perfusion device[211,239]. In an RCT, normothermic preservation using the *metra* was associated with reduced early allograft dysfunction, despite longer preservation durations and increased organ utilisation[95]. Its use in liver preservation has since become well-established in both Europe and the USA. In addition to efficacy demonstrated in clinical trials[95], robust real-world safety data now exist[239,240]. The CE-mark covers liver perfusion up to 24 hours, whilst the FDA-approval permits liver perfusion to 12 hours[211,239]. However, this duration has been extended in clinical perfusions[241] and multi-day pre-clinical perfusions[242]. Software control of perfusion parameters enables semi-autonomous liver perfusion. Cannulation of the venous drainage of the liver makes the *metra* a closed circuit (except for the small amount of fluid leaking from the liver surface)[4] unlike, for example, the Liver Assist device (XVIVO, Gothenburg, Sweden)[243] (see *2.3*).

Attempts at ELC before 2000 were limited by primitive perfusion technology[29]. The OrganOx *metra* represents the current state-of-the-art of clinical liver perfusion. This, in addition to the advantages of semi-autonomous perfusion control (making perfusion management more straightforward) and a closed circuit (reducing risk of infection), makes the *metra* an attractive platform to adapt for ELC. The adaptations to enable this are described in *2.4* and *2.5*.

In order to assess the feasibility of this approach, the *metra*-ELC system was evaluated *in vivo* in a large animal model. The pig model was chosen because: (i) both recipient animal and donor liver have comparable size and haemodynamics to humans[244]; (ii) pigs are readily available with established protocols for use in scientific research and veterinary anaesthetic management[245,246]; (iii) translation of preclinical porcine organ perfusion research to clinical use is well-reported[247,248]; (iv) pigs have become the standard donors for xenografts[33]; with a view towards eventual clinical xeno-ELC, the system should perfuse porcine livers effectively.

4.2 Aims

The aim was to assess a novel ELC system (*metra*-ELC) using donor allografts with healthy anaesthetised pigs, according to: (i) achievement of the target exchange rate (500 mL/minute) and maintenance of stable exchange between recipient and perfusion device; (ii) impact on recipient haemodynamic and clinical status; (iii) impact on donor liver perfusion; (iv) avoidance of device clotting using anticoagulation.

4.3 Methods

4.3.1 Study design

Three healthy, anaesthetised Large White x Landrace pigs (55 – 60 kg) underwent ELC with slaughterhouse pig livers for a pre-defined duration of 3 hours.

4.3.2 Recipient pig preparation

This experiment was performed under UK Home Office license PBB30C3AB in an animal surgical suite at the Biomedical Sciences Building, University of Oxford under the anaesthetic care of Veterinarians. The protocol had been approved by the University of Oxford AWERB.

Recipients were brought to the study site on the morning of surgery. Initial sedation was with ketamine 10 mg/kg, medetomidine 0.02 mg/kg, and midazolam 0.1 mg/kg, followed by total intravenous anaesthesia using propofol (4 mg/kg/hr) and fentanyl (0.02 mg/kg/hr). Maintenance fluids comprised intravenous Hartmann's solution (5 mL/kg/hr). The recipient pig was A-O blood-typed (Eldon blood agglutination kit, Eldon Biological A/S, Gentofte, Denmark). 500 mg intravenous meropenem was administered prior to incision.

Bilateral neck dissection was performed to canulate the internal jugular veins with 16 Fr venous catheters (RV-40016, LivaNova, London, UK) for connection to the ELC bypass vascular lines (*figure 21*). The external carotid artery and external jugular vein were canulated with a 3 Fr arterial line and a

7 Fr central venous line to monitor arterial pressure and administer intravenous medications, respectively (*figure 22*). A mini-laparotomy was performed to insert a urinary catheter to monitor urine output.



Figure 21: bilateral cannulation of internal jugular veins with 16 Fr venous cannulas to connect to the extra-corporeal cross-circulation bypass system.

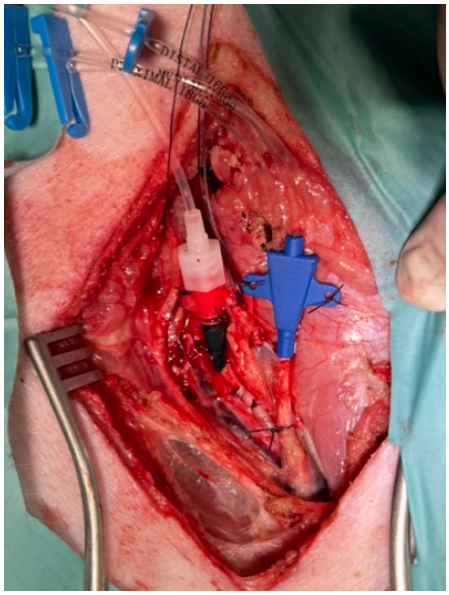


Figure 22: cannulation of external carotid artery and external jugular vein for invasive arterial pressure monitoring and intravenous medication administration, respectively.

4.3.3 *metra*-ELC protocol 1.0 (*figure 23*)

The donor livers and autologous blood were procured from 50 – 70 kg slaughterhouse pigs per *2.1.1*. The donor pigs were A-O blood-typed (Eldon blood agglutination kit) to ensure O-type pigs were selected. The liver (under static cold storage) and heparinised blood (chilled, surrounded by crushed ice) were transported back to the study site. On arrival at the study site, the liver underwent back-table preparation (see *2.2*), followed by isolated normothermic perfusion on the *metra*. The perfusate comprised autologous leuco-depleted blood. The standard *metra* bolus medications were administered (anti-microbial: 500 mg meropenem) and epoprostenol, insulin, and heparin infusions were commenced; bile salt infusion was omitted[4]. *Ex situ* haemofiltration was not performed.

Once ready for connection, the recipient pig received 10,000 IU intravenous heparin, the *metra* insulin and heparin infusions were discontinued, and the bypass lines were primed with perfusate. An

additional unit of leuco-depleted autologous blood was added during bypass line priming to maintain the portal reservoir 50 – 90% full. The recipient pig ACT was confirmed > 200 seconds.

ELC was commenced at 200 mL/minute and increased in 50 mL/minute increments as tolerated, to 500 mL/minute. Additional intravenous heparin boluses were administered to the recipient pig to maintain ACT > 200 seconds. After completion of the pre-defined 3-hour ELC duration, ELC was disconnected from the recipient. An exploratory laparotomy was performed to assess for evidence of intra-abdominal bleeding. The pig was euthanised with intravenous pentobarbitone.

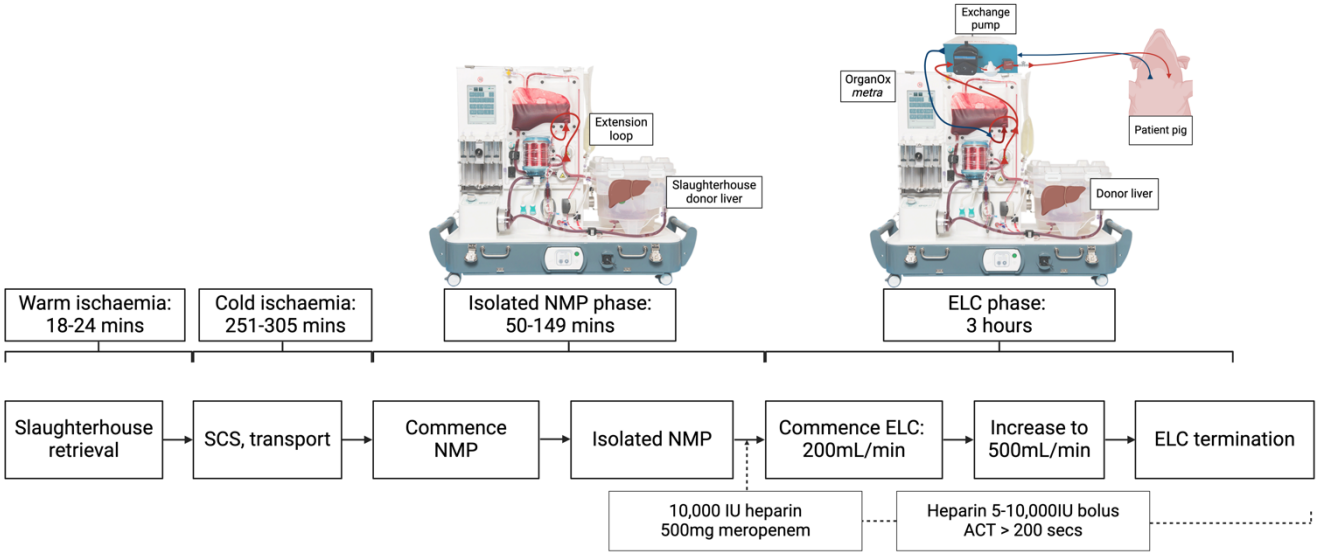


Figure 23: *metra*-ELC protocol 1.0, used for initial test of feasibility with healthy, anaesthetised pigs.
NMP: normothermic machine perfusion. ELC: extra-corporeal liver cross-circulation. SCS: static cold storage. ACT: activated clotting time.

4.3.4 Sampling schedule

Perfusate and recipient pig arterial blood samples were collected prior to commencing ELC and at 1, 2, and 3 hours during ELC into SST (1), EDTA (2), citrate (1) tubes, and for point-of-care analysis, and processed per *2.6*. Perfusate samples were also taken prior to initial liver reperfusion. Point-of-care samples were analysed with: (i) CG4+ and Chem8+ cartridges using an iSTAT device (Abbott, Maidenhead, UK) for: pH, pO_2, pCO_2, haemoglobin, haematocrit, potassium, sodium, ionised calcium, chloride, glucose, lactate, base excess, urea, creatinine, and osmolality; (ii) ACT+ cartridges on a Hemochron Signature Elite device (Werfen, Warrington, UK); (iii) Liver Panel Plus cartridges

(Abaxis, Union City, CA, USA) using serum samples on a Piccolo Xpress (Abbott, Maidenhead, UK). Prothrombin time was measured on citrated plasma samples using a Sysmex CS5100 (Sysmex, Milton Keynes, UK). Full blood counts were analysed on EDTA samples using a Sysmex-XN (Sysmex, Milton Keynes, UK). Recipient serum samples were analysed using the ABL90 Flex arterial blood gas analyser to provide serum cell-free haemoglobin. Donor liver bile produced during ELC was collected; bile chemistry was measured using the ABL90 Flex arterial blood gas analyser.

The *metra* recorded (every second): hepatic artery flow; portal vein flow; inferior vena cava flow; hepatic artery pressure; inferior vena cava pressure; and perfusate pO_2; pCO_2; pH using a shunt sensor (Terumo).

4.3.5 Statistical analysis

Since the aim of this study was determine the feasibility of ELC using the novel *metra*-ELC system, outcomes are reported individually for each study or as a range where appropriate; no comparator group is included.

4.4 Results

4.4.1 Perfusion parameters

The slaughterhouse donor livers were retrieved with warm ischaemic times 18 – 24 minute and cold ischaemic times 251 – 305 minutes. The isolated normothermic perfusion phase duration was 50 – 149 minutes. The pre-connection perfusate biochemical parameters are presented in *table 7*.

The recipient pigs underwent ELC for 3 hours. The target exchange rate of 500 mL/minute was reached in all 3 cases (*figure 24*). The donor livers maintained physiological hepatic artery and portal vein flows, except for a transient drop in portal vein flow that occurred after 2.5 hours of ELC during study 3 due to temporary emptying of the portal reservoir (*figure 25*). The end-ELC donor liver bile chemistry is presented in *table 8*: all three livers met bile pH transplantability criteria; livers 2 and 3 met bile glucose transplantability criteria[3].

Parameter	Study 1	Study 2	Study 3
pH	7.08	7.22	7.23
K^+ (mmol/L)	2.7	2.7	Low
iCa^{2+} (mmol/L)	Low	0.59	1.24
Na^+ (mmol/L)	156	149	142
Glucose (mmol/L)	High	8.4	42
Lactate (mmol/L)	6.25	3.9	N/A
Haematocrit (%)	28	13.5	N/A
Haemoglobin (g/L)	N/A	48	119

Table 7: pre-connection perfusate biochemistry at the end of isolated normothermic perfusion phase.
K^+: potassium. iCa^{2+}: ionised calcium. Na^+: sodium.

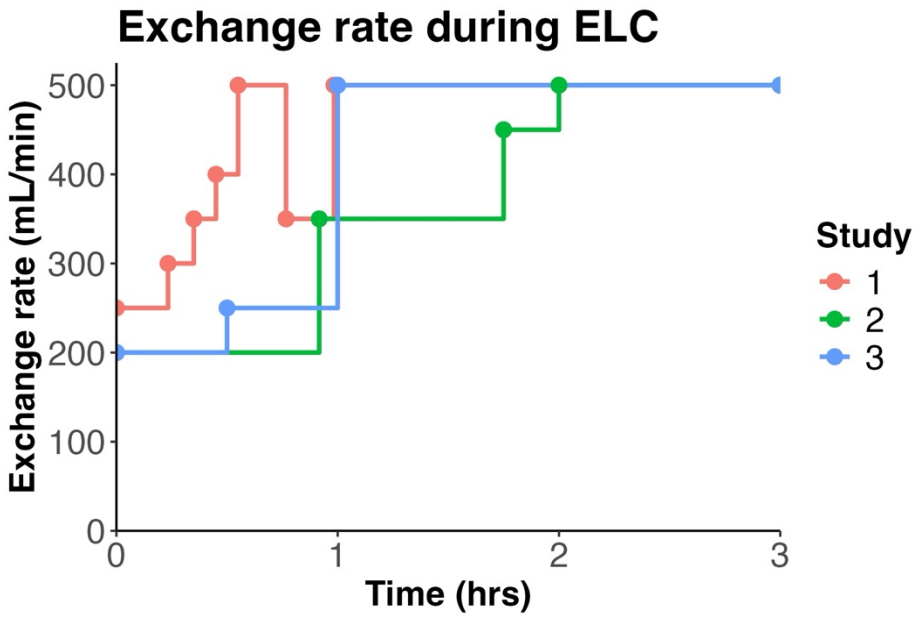


Figure 24: Extra-corporeal liver cross-circulation (ELC) exchange rate during study (between recipient pig central venous system and *metra* portal reservoir) (individual studies).

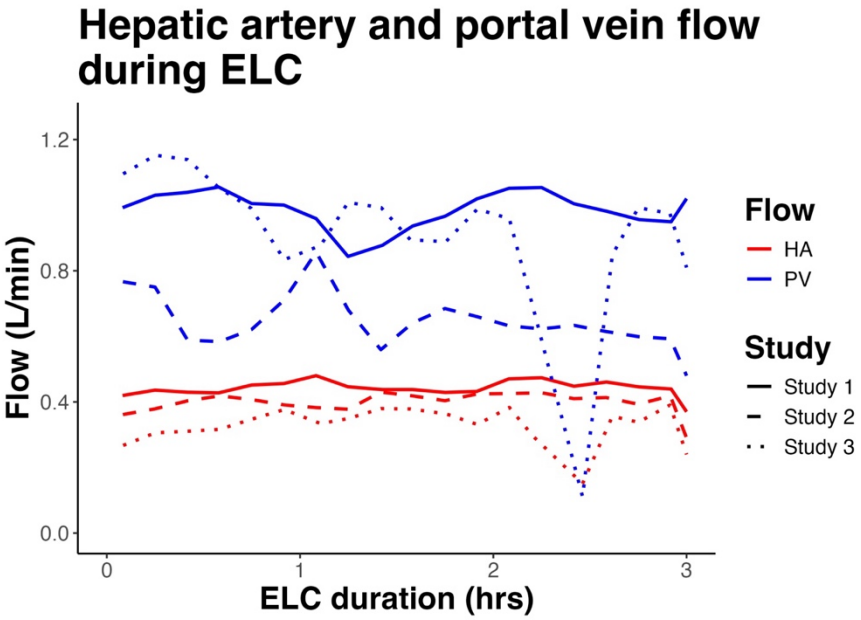


Figure 25: donor liver hepatic artery (HA) and portal vein (PV) flows during extra-corporeal liver cross-circulation (ELC) (individual studies).

3-hour ELC	Study 1	Study 2	Study 3
Bile pH	7.99	7.75	7.75
Bile glucose (mmol/L)	8.5	1.6	1.6
Blood glucose (mmol/L)	5.94	3.7	3.0

Table 8: donor liver bile chemistry and recipient pig blood glucose after 3 hours of extra-corporeal liver cross-circulation (ELC).

4.4.2 Recipient parameters

All recipient pigs were blood-type O. Recipients 1 and 3 remained haemodynamically stable throughout ELC; recipient 2's heart rate increased during ELC and the mean arterial pressure reduced at 2 hours. The hourly urine output remained above 0.5 mL/kg/hour in all three cases (*figure 26*). Recipient renal function, liver function, platelet counts, and prothrombin time measurements remained stable (the pre-connection platelet count for recipient 2 is presumed erroneous). The recipient pig haemoglobin decreased post-connection in studies 1 and 2 but not 3 (*figure 27*). The anticoagulation regime prevented device clotting or clinically-evident thrombo-embolic complication during ELC. There was no clinical evidence of bleeding, including at exploratory

laparotomy after disconnection of ELC. The recipient's serum cell-free haemoglobin level remained stable (*figure 28*).

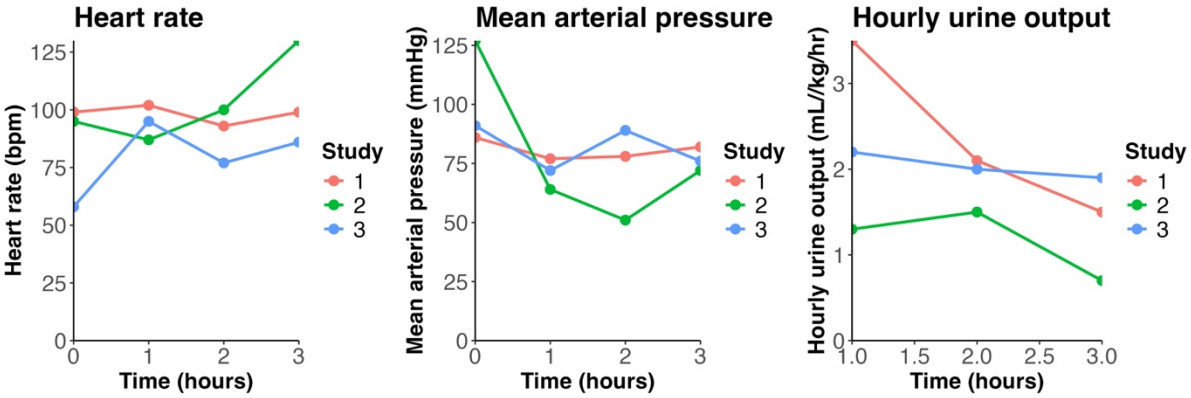


Figure 26: recipient haemodynamic parameters and hourly urine output during ELC (individual studies). bpm: beats per minute.

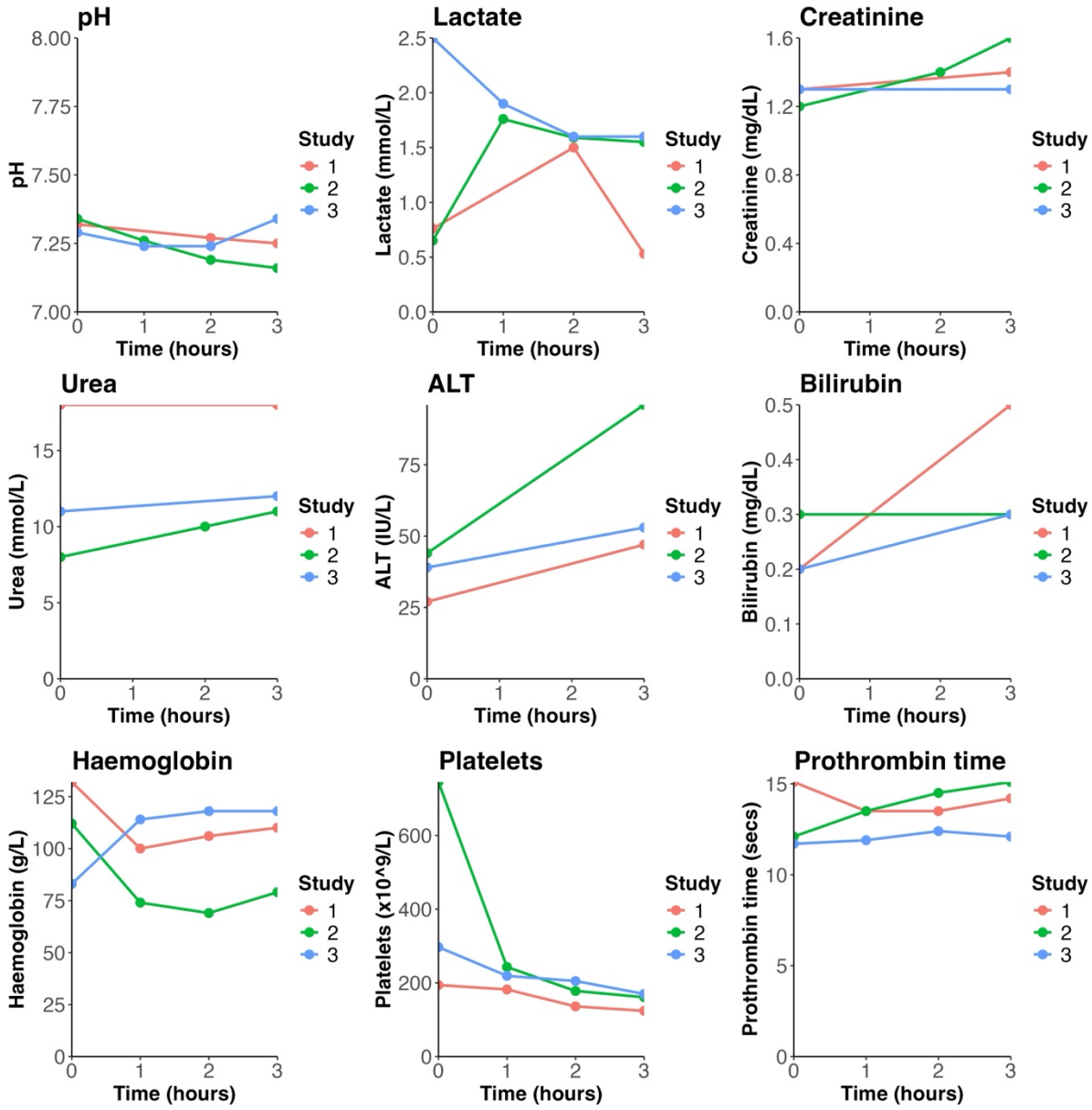


Figure 27: recipient renal, liver, haematological, and coagulation parameters during extra-corporeal liver cross-circulation (individual studies).
ALT: alanine transaminase.

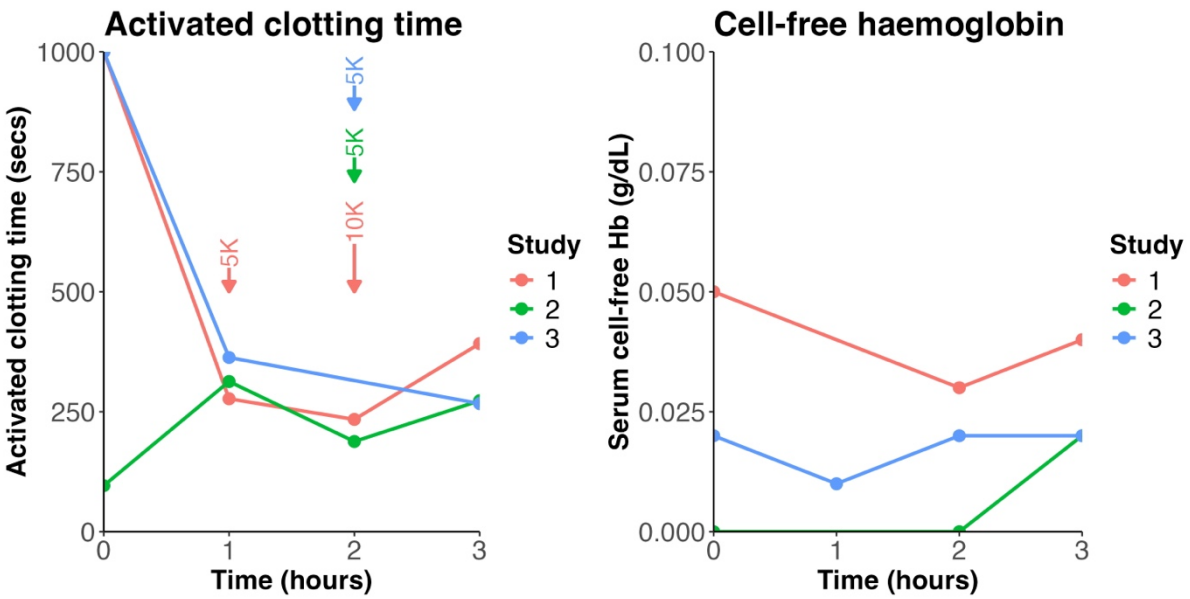


Figure 28: recipient activated clotting time and serum cell-free haemoglobin during extra-corporeal liver cross-circulation (individual studies).

4.5 Discussion

This study assessed a novel adaptation of the *metra* normothermic perfusion device to provide ELC in a large animal model. ELC at a maximum rate of 500 mL/minute was feasible over 3 hours with healthy anaesthetised pigs. There were no major complications; the anticoagulation regime avoided device-clotting without causing recipient bleeding; and the porcine neck vessel cannulation technique to enable ELC and anaesthetic management was effective. A number of necessary refinements of the *metra*-ELC system and protocol were identified over the course of this study.

4.5.1 Exchange flow balance

Automated control of donor liver perfusion by the *metra* algorithm was not affected by adaptation of the system for ELC. However, perfusion management must take consideration of the potential for the total perfusate volume to change as a result of flow imbalance between the recipient and perfusion circuit. Although the exchange pump software aims to balance the 'to-patient' and 'to-*metra*' flows, small proportional differences between the flows at exchange rates of 500 mL/minute result in

meaningful net volume shifts between the recipient and perfusion circuit: e.g. a 2% error at 500 mL/minute causes a 200 mL blood volume shift over 20 minutes.

In study 1, the exchange pump software control of the exchange rate effectively maintained net volume balance. During study 2, the 'to-*metra*' flow slightly exceeded the 'to-patient' flow, resulting in gradual accumulation of blood volume in the *metra* circuit. This manifested as a decline in recipient arterial pressure and rise in heart rate (exacerbated by the low haematocrit of the pre-ELC perfusate (see *below*)). This was detectable by the user as an increase in portal reservoir height. During study 3, the 'to-patient' flow exceeded the 'to-*metra*' flow in the final hour of ELC, resulting in emptying of the portal reservoir (exacerbated by the donor liver taking on blood volume due to ischaemic injury during retrieval). The donor liver portal flow therefore transiently declined, as demonstrated in *figure 25*. In both cases, the net volume shift was identified based on the change in portal reservoir height and corrected by manual offset of the 'to-patient' pump rate relative to the 'to-*metra*' pump: in study 2, the 'to-patient' pump rate was manually increased, returning more blood to the patient, whilst in study 3, the 'to-patient' pump rate was manually reduced to transfer blood to the perfusion circuit. This approach corrected the issue in both cases, but the delay in recognition resulted in temporary impact on the recipient's haemodynamic status and donor liver perfusion, respectively; this would not be acceptable in a clinical case.

These incidents highlighted the requirement for continuous monitoring of the portal reservoir height by the user, with early manual adjustment of the relative pump rates if required. For future uses of the *metra*-ELC system, the pre-connection portal reservoir height was marked, along with a margin of ± approximately 100 mL: the user continuously monitored the portal reservoir height (discounting IVC collapse episodes, during which volume is transiently taken on by the liver (see *chapter 9*)) and manually offset the 'to-patient' pump rate relative to the 'to-*metra*' pump if a net volume imbalance was detected.

In this study, the initial exchange rate was 200 mL/minute, which was incrementally increased to a maximum of 500 mL/minute. Although this starting exchange rate was tolerated by the healthy anaesthetised pigs in this study, unwell recipients with ALF or ACLF may be less tolerant. Achieving volume neutrality is also more challenging at faster exchange rates: a given proportional flow error

generates a greater absolute volume imbalance. For this reason, the exchange pump software was modified to start at an exchange rate of 50 mL/minute. The primary target exchange rate was reduced from 500 to 400 mL/minute for future studies.

4.5.2 *Ex situ* renal replacement therapy during isolated normothermic perfusion phase

In this study, the isolated normothermic perfusion phase of the donor liver prior to ELC did not incorporate *ex situ* renal replacement therapy (RRT). The perfusate biochemistry at the time of connection (*table 7*) was grossly abnormal: in this study (because the perfusate was composed of fresh blood rather than stored packed red cells), the perfusate was hypo- (rather than hyper-)kalaemic and the study 2 perfusate was severely anaemic (due to dilution by washout of gelofusine from the flushed liver). The latter contributed to the haemodynamic instability experienced by pig 2: the recipient's blood was diluted to a haemoglobin level ~ 70 g/L, likely explaining the persistent tachycardia despite achieving net volume neutrality by 3 hours of ELC. Since the eventual recipients of ELC will be critically unwell patients, minimising the change in their perfusate biochemistry upon commencing ELC will reduce complications associated with the therapy. *Ex situ* RRT during the isolated normothermic perfusion phase enables correction of perfusate electrolyte concentrations, pH, and (by modulating the filtrate removal *versus* fluid replacement rates) haematocrit/haemoglobin concentration. Based on the results described in *chapter 3*, the decision was made to incorporate *ex situ* haemofiltration using a haemoconcentrator during the isolated normothermic phase of the *metra*-ELC protocol for future uses.

4.5.3 Donor liver functional assessment during isolated normothermic perfusion phase

A substantial literature describes assessment of graft function during normothermic liver preservation prior to transplant (see *chapter 1*)[3,96]. The isolated normothermic perfusion phase of the *metra*-ELC protocol could be leveraged to evaluate the donor liver. In the setting of allotransplantation, the goal is to predict the risk of a range specific complications: primary non-function, early allograft dysfunction, biliary complications, and graft survival. By contrast, for ELC only short-term function (i.e. over a

number of days) is relevant. This has not yet been specifically investigated in the clinical setting, but an initial set of ELC criteria may be extrapolated from studies in the context of allotransplantation. Prediction of primary non-function could potentially be used to definitively exclude specific grafts for ELC. However, this is not possible based on normothermic perfusion criteria due to the statistical limitations imposed by its very low incidence[3]. Early allograft function post-transplantation does correlate with lactate clearance, pH maintenance, transaminase release, and peak bile pH during normothermic preservation[3]. Peak bile pH is determined by preservation of cholangiocyte bicarbonate secretion[97], which is less relevant to the immediate life-sustaining benefit of ELC. Metabolic regulatory function is, however, central: evidence of glucose metabolism during normothermic perfusion may act as a surrogate for this and is used clinically to determine suitability for transplant[3,96,121]. Vascular flows of the graft during normothermic perfusion are also variably used to determine suitability for transplant[96,121]. In the setting of ELC, the vascular flows during perfusion are relevant as they determine the rate of exposure of the patient's blood to the donor liver and affect the pO_2 of the portal supply (see *2.4*). Determination of donor liver suitability to proceed to ELC based on function during the isolated normothermic phase may therefore be guided by the following parameters: lactate clearance; glucose metabolism; maintenance of perfusate pH; ALT < 6,000 IU/L; portal vein flow > 500 mL/minute.

Perfusate pH, lactate, and glucose concentrations are affected by *ex situ* haemofiltration: the values will tend towards those in the dialysate replacement fluid. Failure of the donor liver to control these parameters does nonetheless result in measurable deterioration in the perfusate values despite haemofiltration, but the effect is delayed and milder than in standard normothermic perfusion. If there is a high index of suspicion regarding graft function (e.g. a declined human liver, see *chapter 8*), graft function must therefore be assessed (e.g. after 2 hours of normothermic perfusion) prior to commencing *ex situ* haemofiltration. If the index of suspicion is low (e.g. a porcine liver procured from an anaesthetised donor with minimal ischaemic injury, see *chapters 5, 7*), *ex situ* haemofiltration may be performed throughout the isolated normothermic perfusion phase, with careful consideration of the trends in these values over this period. If a human liver is perfused in the absence of *ex situ* haemofiltration, it is reasonable, until experience with ELC in this context has been established, to

extrapolate direct values for perfusate lactate clearance (i.e. 2-hour lactate < 2.8 mmol/L), glucose metabolism (rate of fall ≥ 1 mmol/L/hr by hour 4), and pH maintenance (> 7.2 beyond 2 hours without ongoing bicarbonate supplementation) from human transplant studies[3]. If a pig liver is perfused or *ex situ* haemofiltration used during the graft assessment period, judgement must instead be based on trends within the individual perfusion. Importantly, until these criteria have been validated in the clinical ELC setting, they remain a guide rather than a strict prescription for predicting suitability for ELC.

4.5.4 Anticoagulation regimen

The anticoagulation regimen used in this study successfully avoided device-clotting or clinically-detectable thrombo-embolic complication, without causing bleeding. However, the pigs received a significant cumulative heparin dose over 3 hours (study 1: 25,000 IU; studies 2 and 3: 15,000 IU). Since the goal of the study was to test the use of the ELC system over 3 hours, the primary aim of the anticoagulation regimen was to avoid device clotting (which would end the experiment). The target ACT (> 200 seconds, without an upper limit) was chosen accordingly. The clinical recipients of ELC (patients with ALF or ACLF) will be at higher risk of bleeding due to the potential co-existence of coagulopathy, thrombocytopenia, and gastro-oesophageal varices. The optimal ACT target for clinical ELC would be the lowest ACT that consistently avoids device clotting and thrombo-embolism. A heparin infusion is preferable to bolus administration as it minimises ACT peaks and troughs. For the subsequent experiment (*chapter 5*), an ACT target of 160 – 200 seconds with hourly ACT monitoring (more frequent where necessary) was chosen, guided by practice within the fields of ECMO and cardio-pulmonary bypass. Due to uncertainty in the expected hourly heparin dose required to achieve this in a pig with liver failure, the anticoagulation regimen for the first two pigs treated in *chapter 5* used a bolus heparin approach, which was converted to a heparin infusion for pig 3 onwards.

4.5.5 Recipient pre-medication

Pre-medication of the recipient in this experiment comprised anti-microbial prophylaxis and heparin anti-coagulation. This was a 3-hour experiment using donor livers that had sustained significant ischaemic damage (see *2.1.1*). Beyond ensuring that the perfusion dynamics of the donor liver were unchanged by the modifications of the *metra*, the function of the donor liver was not the primary focus of this study. However, this will be central to the success of ELC. In order to minimise IRI (to protect both graft and recipient), intravenous 500 mg methylprednisolone will be administered to the recipient prior to commencing ELC (*chapters 5, 7, 8).* This reflects management during liver allotransplantation[249]. To attenuate the immune response to the graft during ELC durations beyond 24 hours, further doses of intravenous 500 mg methylprednisolone will be administered daily to the recipient (*chapters 7, 8*). No additional immunosuppression is planned since function beyond a number of days is not relevant.

4.5.6 Summary of *metra*-ELC refinements

The *metra*-ELC system successfully adapts the *metra* normothermic preservation device to provide ELC in a large animal model up to 3 hours. A number of refinements have been identified: (i) improvement in management of the exchange balance by continuous monitoring of portal reservoir filling height with manual exchange pump rate offset if required; (ii) reduction in starting exchange rate from 200 to 50 mL/minute to increase initial tolerance in critically unwell recipients; (iii) target maximum exchange rate reduced from 500 to 400 mL/minute to ensure more stable exchange; (iv) incorporation of *ex situ* RRT during isolated normothermic perfusion phase to correct perfusate biochemistry ± haematocrit prior to commencing ELC; (v) assessment of donor liver function before connection to patient; (vi) rationalisation of anticoagulation regime to target ACT 160 – 200 seconds rather than > 200 seconds; (vii) pre-medicate recipient with intravenous 500 mg methylprednisolone pre-ELC, and 24-hourly thereafter. These refinements were incorporated into future uses of the *metra*-ELC system in *chapters 5, 7,* and *8*.

4.6 Declaration

I designed and ran the study; performed the liver perfusions, ELC, and sample collection (with support from the Transplant Research Fellows and OrganOx clinical specialists included under *Acknowledgements*); laboratory analysis; data analysis; and interpretation.

Recipient pig surgery was performed by Professor Peter Friend and the Transplant Research Fellows.

Recipient pig anaesthesia was performed by the veterinary staff at the Biomedical Sciences Building, University of Oxford.

5 Extra-corporeal liver cross-circulation provides comprehensive hepatic support in a porcine model of acute liver failure

5.1 Background

The *metra*-ELC system and protocol were refined based on the experience of *in vivo* use described in *chapter 4*. This included incorporation of *ex situ* RRT during the isolated normothermic perfusion phase prior to commencing ELC: the experiment reported in *chapter 3* identified *ex situ* haemofiltration using a haemoconcentrator as the optimal technique for this. The purpose of the experiment described in this chapter was to evaluate this refined *metra*-ELC system and protocol in a large animal (porcine) model of ALF.

Existing artificial and bio-artificial liver support systems have shown benefit in pig ALF models[250], but failed to translate this to survival benefit in clinical trials[14]. Such systems may demonstrate improvement in relevant biochemical parameters (ammonia, bilirubin, INR), and even survival, in the pig model. The improvement in biochemical parameters may extend to patients, but this fails to improve survival. By contrast, patients with ALF or ACLF who undergo emergency liver transplantation often show rapid, marked improvement in a range of clinical parameters[251,252], which does translate to considerably improved survival[20]. No artificial or bio-artificial liver support system has approached this level of impact. The role of the liver extends beyond the clinically-measured biomarkers (see *1.1*) and comprehensive replacement of hepatic function is needed to replicate the profound benefit of emergency transplant.

An example is the central regulatory role that the liver plays at the intersection of the body's biochemical pathways. Failure of this function has far-reaching implications on metabolic status. Proteomics and metabolomics combine advanced analytical chemistry and statistical analysis to characterise the proteins and metabolites in a sample[253]. Many of the biochemical pathways regulated by the liver involve proteins and metabolites circulating in the plasma. The plasma proteome and metabolome of patients with ALF and ACLF have been studied, but the field is relatively new. The changes that have been observed in these conditions (including in pig ALF models) are summarised in *tables 9* and *10*. In this experiment, the plasma proteome and metabolome were analysed as ALF

developed in pigs under controlled conditions to further explore these and to understand the impact of ELC beyond standard liver function tests.

Domain	Protein
Injury markers/hepatocyte metabolic enzymes	↑ LDH[1] ↑ ALDOB[1] ↑ ASL[1] ↑ PGK1[1] ↑ GPLD1[2] ↑ CNDP2[2]
Inflammatory proteins/cytokines	↑ IL6[1] ↑ IL10[1] ↑ TNF[1] ↑ MIF[1]
Oxidative stress	↑ SOD1[1] ↓ PON1[2]
Immune regulation	↑ SLPI[1,2] ↓ FETUB[1,2]
Complement	↓ C9[2] ↓ C4BPB[2] ↓ C3[2] ↓ C8G[2] ↓ C4A[2] ↓ C1QC[2] ↓ C6[2]
Coagulation	↓ F7[1] ↓ F5[2] ↓ F2[2] ↓ VTN[1,2]
Vitamin/nutrient regulation	↓ RBP4[1]
Acute phase/haematological regulation	↑ CRP[2] ↓ SAA4[1] ↓ HP[2] ↑ HBB[2]
Apolipoproteins	↓ APOC2[1,2] ↓ APOC3[2] ↓ APOC4[1]
Chaperone proteins	↓ CLU[2]
Protease inhibition	↓ ITIH4[2] ↓ SERPINA5[2] ↓ SERPINC1[2] ↓ SERPIND1[2]
ECM/cytoskeleton/cell adhesion	↓ CDH2[2] ↓ VTN[1,2]

Table 9: plasma proteomic changes reported in acute (designated: [1]) and acute-on-chronic liver failure (designated: [2]) patients and animal models[93,254-261].
Human genome organisation Gene Nomenclature Committee protein name presented.

Domain	Metabolite
↓ Bile acid excretion	↑ Conjugated bile acids[2]
↓ β-oxidation	↑ Free fatty acids[2] ↑ Acylcarnitines[1,2]
↑ ω-oxidation	↑ Dicarboxylic acids[2]
↑ Ketogenesis	↑ Ketones[2]
↑ Glycolysis	↑ Lactate[1]
↑ Pentose phosphate pathway	↑ Ribose-5-phosphate[2]
Sphingolipid metabolism	↑ O-phosphoethanolamine[1] ↓ Sphingomyelins[1,2] ↓ Sphingosine-1-phosphate[2]
Choline metabolism	↓ Phosphoryl choline[2] ↓ Phosphatidylcholines[1,2] ↓ Lysophosphatidylcholines[1,2]
↑ Aromatic amino acids	↑ Tyrosine[1,2] ↑ Phenylalanine[1,2] ↑ Tryptophan[1]
↓ Branched chain amino acids	↓ Isoleucine[1] ↓ Valine[1] ↓ Leucine[1]
↑ Proteolysis	↑ N6,N6,N6-trimethyl-L-lysine[2]
Lysine metabolism	↑ Pipecolic acid[2]

Table 10: plasma metabolomic changes reported in acute (designated: [1]) and acute-on-chronic liver failure (designated: [2]) patients and animal models[72,89,262-269].

In addition to loss of native hepatic function, ALF is characterised by a systemic inflammatory response driven by the release of DAMPs by necrotic hepatocytes, as well as PAMPs due to super-added infection or translocation of gut bacterial products[52,270]. Circulating cell-free DNA, histones, endotoxin, and cytokine levels are elevated in ALF[86,87,237,271,272]. Though this systemic inflammatory response may be reflected in routinely-measured clinical parameters (e.g. haemodynamic status, pyrexia, white cell count, c-reactive protein), these measures are influenced by multiple factors. To gain an in-depth understanding of the impact of ELC, the molecular drivers (i.e. cytokines, DAMPs, PAMPs) of this inflammatory state were measured in this study.

Based on these observations, the central goals of this study included determination of: (i) the extent to which ELC can broadly replace lost native hepatic function; (ii) the impact of ELC on the systemic inflammatory component of ALF. Several pig ALF models have been described previously (*table 11*). To evaluate these goals, hepatic inflow devascularisation was selected as the model. This combines liver-specific injury with DAMP release from necrotic hepatocytes, reproducing the biochemical[273] and systemic[274] disturbances of ALF over a predictable time course. The intensive care management

of the pigs in the study followed the EASL ALF guidelines[42] to reflect the clinical condition and support translation. There are several limitations to this model that are considered in the discussion (see *5.5*).

Model	Pig weight (kg)	Time to death (hrs)*	Biochemical changes	SIRS	Issues
Intravenous D-galactosamine[275]	40-50	77	ALT/AST; bili; NH_3; INR.	Y	• Prolonged prodrome: - prolonged GA or animal suffering - unpredictable timings
Enteral paracetamol[276]	30-40	32	Bili; INR; (NH_3 pre-terminal)	Y	• Alternative paracetamol metabolism: - methaemoglobinaemia - nephrotoxicity
Enteral carbon tetrachloride + hepatic artery ligation[277]	60-100	18	ALT/AST; bili, NH_3	Y	• Hazardous • Limited recent literature • Potential hepatotoxic plasma concentration at ELC
Hepatectomy[273]	30-40	17	NH_3, INR	N	• Lacks necrotic liver • Lacks clinical correlate • Technically challenging
Hepatic inflow devascularisation[273,274,278]	30-40	10-11	AST; bili; NH_3; INR	Y	• Lacks clinical correlate • Technically challenging

Table 11: porcine models of acute liver failure.
*Time to death presented as mean value for control pigs in the referenced studies. ALF: acute liver failure. ALT/AST: alanine/aspartate transaminase. bili: bilirubin. NH_3: ammonia. INR: international normalised ratio. SIRS: systemic inflammatory response. GA: general anaesthetic. Y: yes. N: no.

ELC is a complex, multi-parametric treatment, fundamentally distinct from artificial systems: each extra-corporeal liver will behave differently based on donor history, the retrieval process, and perfusion parameters. This study provided an opportunity to deeply analyse the behaviour of the perfused livers: in addition to perfusion dynamics and histological appearance, the tissue proteome and bile metabolome of the livers were assessed to better understand their biology and characterise the excretory function provided to the recipient.

Previous attempts at ELC[29,279], as well as wider clinical experience with extra-corporeal circuits[229,280], have identified haemolysis and thrombocytopenia as potential complications. This study enabled assessment of these in the presence of biological controls.

5.2 Aims

The primary aim was to determine the impact of ELC on the biochemical and clinical hallmarks of ALF. The secondary aims were to explore: (i) perfusate potassium correction by *ex situ* haemofiltration prior to ELC; (ii) behaviour of the extra-corporeal liver during ELC; (iii) effect of ELC on the pathophysiology of ALF and functional hepatic replacement; (iv) potential complications of ELC.

5.3 Methods

5.3.1 Study design overview

ALF was induced in 12 patient pigs by hepatic inflow devascularisation (hepatic artery and portal vein ligation; porto-caval anastomosis). Patient pigs in the control (*n=6*) and intervention (*n=6*) groups received standard intensive care medical management. For the intervention group, donor livers for ELC were retrieved from terminally anaesthetised donor pigs (*n=6*). Patient pigs in the intervention group commenced ELC 1-hour post-hepatic devascularisation and remained connected until the experiment end (*figure 29*). Patient pigs were observed for up to 25 hours after hepatic devascularisation. Pigs were maintained under general anaesthesia throughout the study.

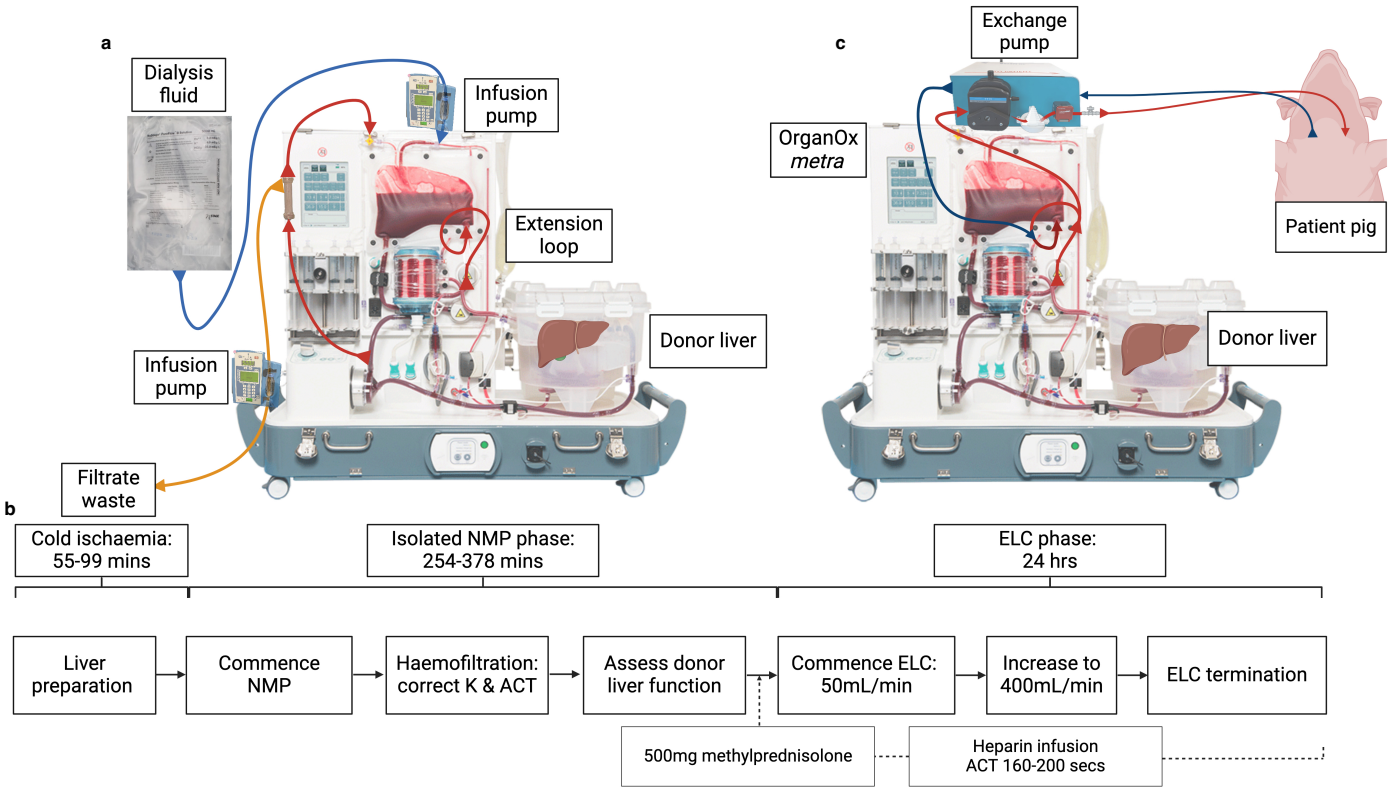


Figure 29: *metra*-ELC system and protocol.
a: *metra* circuit set-up during isolated normothermic perfusion phase. b: *metra*-ELC protocol. c: *metra*-ELC circuit set-up. NMP: normothermic machine perfusion. K: potassium. ACT: activated clotting time. ELC: extra-corporeal liver cross-circulation.

5.3.2 Animal care and anaesthesia

Animal experiments were performed under UK Home Office license PBB30C3AB in a dedicated intensive care unit at the Large Animal Research and Imaging Facility, University of Edinburgh under the medical care of specialist Veterinary anaesthetists. PBB30C3AB was approved by the AWERBs of the Universities of Edinburgh and Oxford.

Pigs were young adult 50 – 70 kg female Large White x Landrace pigs. Pigs were brought in at least 3 days prior to the procedure for acclimatisation and housed with con-specifics. They were bedded on straw, with a 12-hour light-dark cycle and received commercial grower feed *ad libitum*. The first three patient pigs received 10 mg/kg oral phenobarbitone once daily for 3 days prior to undergoing general anaesthesia to enable switching to the enteral carbon tetrachloride model[277] in case the hepatic inflow

devascularisation model had been sub-optimal (this proved not to be the case). Food was withheld for 12 – 16 hours pre-operatively. A-O blood typing was undertaken via ear- or tail-prick samples using Eldon blood agglutination tests to ensure that donor and patient pigs were A-O blood type matched. Pigs received pre-anaesthetic intramuscular sedation (ketamine 7 mg/kg; morphine 0.3 mg/kg; medetomidine 7 μg/kg; midazolam 0.3 mg/kg) followed by mask induction (isoflurane 1 – 3% in 100% oxygen). Intravenous access was gained using a 20 G cannula in an auricular vein. Endotracheal intubation was achieved using a cuffed endotracheal tube (8 – 9 mm internal diameter) after topical application of 1 – 2 mL 2% lidocaine onto the larynx. Ocular lubricant was applied. The forehead was clipped, shaved, cleaned with chlorhexidine in ethanol, and dried for bi-spectral index (BIS) sensor (BIS Vista, Medtronic, Dublin, Ireland) application (BIS is a measure of electro-encephalogram activity under general anaesthesia). The surgical site was prepared by clipping and cleaning using chlorhexidine. General anaesthesia was maintained using isoflurane in oxygen : air mixture (starting FiO_2 0.5 [0.48-0.52]) with partial intravenous anaesthesia (morphine 0.2 mg/kg/hr; ketamine 10 μg/kg/min; midazolam 0.2 mg/kg/hr; medetomidine 2.5 μg/kg/hr). In the control group, intravenous and inhalant anaesthetic doses were titrated down as liver failure developed, guided by BIS monitoring and clinical assessment of anaesthetic depth. Ventilation was volume-controlled with tidal volume 8 mL/kg (based on V_{Texp} on spirometry), inspiratory : expiratory ratio 1:2, positive expiratory end pressure 5 cmH_2O, and respiratory rate 20 – 35 breaths/minute (to maintain normocapnia (P_ECO2 5.5 [4.7-6] kPa)). Monitoring during anaesthesia included: electrocardiogram (ECG), pulse oximetry, inspired/expired gases, spirometry, invasive arterial blood pressure, central venous pressure (CVP), temperature, cardiac output, cardiac index, stroke volume, stroke volume variation, systemic vascular resistance, BIS, and hourly urine output (latter two not in donor pigs). Pig allocation was neither randomised nor blinded due to the nature and logistical requirements of ELC.

5.3.3 Induction of ALF in patient pigs

After induction of general anaesthesia, the patient pig was placed in dorsal recumbency. 500 mg meropenem was administered intravenously.

The neck was prepared with chlorhexidine and draped. Control and intervention pigs underwent neck dissection to insert a 7 Fr triple-lumen central venous cannula into the right external jugular vein (for fluid/medication administration and CVP monitoring) and a 3 Fr arterial line into the right external carotid artery (for invasive arterial blood pressure monitoring). Intervention pigs underwent bilateral neck dissection to insert 16 Fr venous cannulas (RV-40016, LivaNova) into the right and left internal jugular veins (to connect to the ELC system).

The abdomen was prepared with chlorhexidine and draped. A midline laparotomy was performed from xiphisternum to pubic symphysis. A urinary catheter was inserted and secured in the bladder. The hepato-duodenal ligament was dissected as per the liver donor operation (*see 2.1.2)* (except the CBD and non-hepatic vascular branches were preserved) to skeletonise the hepatic artery, portal vein, and CBD. The hepatic hilum was meticulously dissected with division/ligation of lymphatic and connective tissue, and all peritoneal attachments of the liver were divided to ensure no accessory vessels remained to supply the liver. A peripheral wedge biopsy of the liver was taken prior to devascularisation and haemostasis secured using diathermy. The portal vein was clamped proximally and ligated close to the hepatic hilum. An end-to-side porto-caval anastomosis was formed using 6/0 prolene (Ethicon, Johnson and Johnson MedTech, Raritan, NJ, USA). The hepatic artery was ligated proximal to all branches to the liver. Devascularisation of all hepatic lobes was confirmed by visual inspection. The laparotomy was closed.

5.3.4 Liver donor operation and back-table preparation

The liver donor operation and back-table preparation are described in *2.1.2* and *2.2*.

The *in situ* cold perfusion step was modified to allow collection of autologous blood prior to cold perfusion. The aorta was cannulated as per *2.1.2*, but connected via a three-way tap to blood-collection bags (FQE614B, Macopharma) and bags of cold heparinised UW solution. The pig was exsanguinated via the aortic cannula into sequential blood collection bags (FQE614B, Macopharma). The blood was leuco-depleted using the Macopharma blood collection system. Once exsanguination

was complete, the three-way tap was turned to the UW bags for *in situ* cold perfusion. The donor operation then proceeded as per *2.1.2*.

5.3.5 Blood-only donor operation

Blood-only donor pigs provided additional blood for priming the *metra*-ELC system and maintaining the portal reservoir 50 – 90% full during the isolated normothermic perfusion phase and ELC. One blood-only donor pig was used per intervention case.

The blood-only donor pig was prepared as per the liver donor operation. The neck was prepared with chlorhexidine and draped. Unilateral neck dissection was performed to insert a 16 Fr venous cannula (RV-40016, LivaNova) into the right internal jugular vein. 25,000 IU heparin was administered intravenously and allowed to circulate for 3 minutes. The pig was exsanguinated via the venous cannula into sequential citrated blood collection bags (FQE614B, Macopharma). The pig was placed in an extreme Trendelenburg position to maximise blood yield. The blood was leuco-depleted using the Macopharma blood collection system and stored in SAG-mannitol at 4°C until required.

5.3.6 *metra*-ELC protocol (*figure 29*)

The donor livers underwent initial isolated normothermic perfusion, during which *ex situ* haemofiltration was performed, as described in *2.3*, *2.4,* and *2.5*.

In this phase, the donor liver was perfused with perfusate that comprised autologous leuco-depleted blood. Due to administration of 25,000 IU heparin during the retrieval process and citrate present in the blood bags, this perfusate was sufficiently anticoagulated to not require administration of the standard *metra* heparin bolus or infusion during the isolated normothermic perfusion phase. *Ex situ* haemofiltration removes excess heparin and citrate. This, combined with biological clearance of heparin by binding to the liver endothelium and hepatic metabolism of circulating citrate, caused the perfusate ACT to decline during the isolated normothermic perfusion phase. If perfusate ACT reached 200 seconds, 1,000 IU heparin was administered to prevent device clotting. To reduce the exposure of

the recipient to anticoagulant (and therefore minimise bleeding risk), the target perfusate ACT at time of patient connection was 180 – 350 seconds. Once this target was reached and the perfusate potassium concentration was within the physiological range (target: 3.5 – 5.2 mmol/L), the *ex situ* haemofiltration system was disconnected. The function of the donor liver was confirmed according to the following perfusate criteria: lactate < 2.8 mmol/L; pH > 7.2; alanine transaminase < 6000 IU/L; evidence of glucose consumption; portal vein flow > 500 mL/minute.

Pigs in the intervention group commenced ELC 1-hour after hepatic inflow devascularisation. ELC was performed as per *2.5* and maintained for 24 hours.

Regarding the heparin anticoagulation regimen during ELC, for the first two pigs, bolus dosing was used. This was modified to a continuous heparin infusion for the final four pigs. The target ELC exchange rate was 400 mL/minute. In one intervention case, the exchange rate was increased to 500 mL/minute for the final hour of ELC.

5.3.7 Medical management of patient pigs

The patient pigs received 500 mg meropenem and 1mg/kg omeprazole intravenously at induction. Thereafter, 500 mg meropenem was administered 8-hourly and 1 mg/kg omeprazole 12-hourly. Maintenance fluid was Hartmann's solution, titrated to volume status.

In addition to the continuous monitoring described in *5.3.2*, arterial gases, electrolytes, acid-base status, renal function, and liver function were measured at least 4-hourly (more frequently when indicated). Plasma biochemistry was managed as follows: (i) hypoglycaemia: intravenous 50% dextrose; (ii) hypocalcaemia: intravenous 10% calcium gluconate; (iii) hyperkalaemia (potassium concentration > 6.5 mmol/L): intravenous insulin 0.5 IU/kg + 50% dextrose; (iv) hypokalaemia: intravenous 0.9% saline + 40 mmol potassium chloride. The target mean arterial pressure (MAP) was > 60 mmHg. Hypotension was managed by: (i) reducing inhalant concentration; (ii) fluid (Hartmann's and/or gelofusine) bolus; (iii) intravenous noradrenaline infusion commencing at 0.05 µg/kg/min, to a maximum 2 µg/kg/min.

Patient pigs were observed until 25 hours after hepatic inflow devascularisation, death, or an alternative end-point was reached. The alternative end-points were: (i) failure to maintain MAP > 40 mmHg with noradrenaline 2 μg/kg/min; (ii) arrythmia inconsistent with survival; (iii) failure of extra-corporeal liver perfusion. At the study end or if an alternative end-point was reached, pigs were euthanised using intravenous 40 mg/kg pentobarbital. Post-mortem examination was performed in all cases.

5.3.8 Sampling schedule

During isolated normothermic perfusion, perfusate was collected into SST (1), EDTA (2) tubes, and for point-of-care testing prior to liver reperfusion and at the end of isolated normothermic perfusion. Perfusate was additionally sampled for point-of-care testing at least 2-hourly (more frequently when indicated) during isolated normothermic perfusion.

Blood was sampled via the arterial line from patient pigs into SST (1), EDTA (2), citrate (1) tubes, and for point-of-care testing prior to hepatic devascularisation, 1-, 4-hours post-devascularisation, and thereafter 4-hourly until the experiment end. In one intervention case, final blood samples were taken 25 (rather than 24) hours post-devascularisation, after increasing the exchange rate to 500mL/minute.

The bile volume produced by the extra-corporeal liver was measured 4-hourly during isolated normothermic perfusion and ELC. Bile was sampled at the end of isolated normothermic perfusion and 4-hourly during ELC.

Peripheral wedge biopsies of the patient pig liver were taken prior to hepatic devascularisation and at the experiment end. Peripheral wedge biopsies of the donor liver were taken at the end of back-table preparation (i.e. pre-isolated normothermic perfusion) and at the end of ELC.

5.3.9 Sample processing

Perfusate and blood SST, EDTA, and citrate tubes were processed to serum (SST) and plasma (EDTA, citrate) by centrifugation (plasma: immediate; serum: post-clotting) at 4°C for 10 minutes, followed

by freezing (temporarily -20°C, then -80°C). An additional EDTA tube at each timepoint was not centrifuged (for full blood count analysis). Bile was frozen (temporarily -20°C, then -80°C). Liver tissue samples were divided into three sections: (i) fixation in 10% formalin, (ii) preservation in RNAlater, (iii) snap-freezing in liquid nitrogen. Formalin-fixed liver tissue samples were paraffin-embedded, sectioned, and stained with haematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and Perl's stains (Oxford Centre for Histopathology Research, University of Oxford).

5.3.10 Sample measurements

The *metra* device recorded (every second): hepatic artery flow; portal vein flow; inferior vena cava flow; hepatic artery pressure; inferior vena cava pressure; and perfusate pO_2; pCO_2; pH using a shunt sensor (Terumo). Proprietary software recorded the output from the Datex-Ohmeda anaesthetic monitor (GE HealthCare, Chicago, IL, USA) and BIS Vista (every second): arterial blood pressure; CVP; sO_2; FiO_2; P_ECO_2; respiratory rate; BIS, peak inspiratory pressure; positive end expiratory pressure; tidal volume; compliance; and electrocardiogram. The Most-Care Up monitor (Vygon, Swindon, UK) recorded (every 30 seconds): systemic vascular resistance; cardiac output; cardiac index; stroke volume; and stroke volume variation.

Perfusate and arterial blood samples were analysed using CG4+ and Chem8+ cartridges on an iSTAT device (Abbott). ACT was measured using ACT+ cartridges on a Hemochron Signature Elite device (Werfen). Arterial ammonia was measured using a PocketChem BA analyser (Woodley Equipment Company, Bolton, UK) (serum samples were diluted with 0.9% saline when required).

Liver function tests were measured on serum samples using Liver Panel Plus cartridges (Abaxis, Union City, CA, USA) on a Piccolo Xpress (Abbott). Prothrombin time, international normalised ratio, factor V, and VII were measured on citrated plasma samples using a Sysmex CS5100. Full blood counts were analysed on EDTA samples using a ADVIA 2120 (Siemens, Munich, Germany).

Cell-free DNA was measured using SYTOX Green Dye fluorometric assay (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA (at University of Liverpool)), extra-cellular histones with Western blotting (H3 antibody with purified histones as a standard (at University of Liverpool)),

and uric acid using a uricase-based assay in the Clinical Biochemistry Laboratory, Oxford University Hospitals. Endotoxin was assayed using a Pierce Chromogenic Endotoxin Quant Kit (Thermo-Scientific, Waltham, MA, USA). Cytokines were measured using ELISAs (Thermo Fisher Scientific, Waltham, MA, USA): ESIL-6 (interleukin-6); KSC3011 (TNFα); ESIL1B (interleukin-1β); KSC0101 (interleukin-10). Serum cell-free haemoglobin and bile glucose were measured using an ABL90 Flex arterial blood gas analyser. Haptoglobin was measured using a Tridelta phase haptoglobin assay (BioGnosis, Eastbourne, UK). Histological analysis was performed by a consultant liver histopathologist.

5.3.11 Metabolomic and proteomic analyses

Plasma metabolomic and proteomic analyses were performed on patient pig plasma samples (pre-devascularisation of patient's liver; 8-, 16-, 20-hours post-devascularisation (*n=6* per treatment group per timepoint)). Perfusate metabolomic analysis was performed on perfusate samples taken during isolated normothermic perfusion (pre-liver reperfusion; end-isolated normothermic perfusion (*n=6* per timepoint)). Bile metabolomic analysis was performed on samples of bile produced by the extra-corporeal liver at the end of isolated normothermic perfusion and 16-hours after devascularisation of the patient pig's liver (*n=6* per timepoint). Tissue proteomic analysis was performed on snap-frozen tissue biopsies of the extra-corporeal liver taken at the end of back-table preparation (i.e. pre-isolated normothermic perfusion, *n=4*) and end of ELC (*n=6*).

5.3.11.1 Plasma and bile metabolite extraction, analysis, and processing

5.3.11.1.1 Metabolite extraction

Samples were thawed at 4°C. 400 µL extraction buffer (acetonitrile/methanol/acetone, 8:1:1 ratio) mixture was added to 50 µL samples, then vortexed. The mixture was placed on ice for 30 minutes to precipitate proteins and then centrifuged at 13,000 rpm for 20 minutes at 4°C. 375 µL supernatant was transferred to a new tube and split into two volumes: 187.5 µL for positive mode LC-MS and 187.5

μL for negative mode LC-MS. Metabolites were dried using a Thermo Scientific Savant SNA120 SpeedVac Concentrator and stored at -80°C until LC-MS analysis. Pooled samples were prepared for quality control (QC), which underwent the same sample preparation and analysis as test samples. A pool sample was used for each batch (4).

5.3.11.1.2 Metabolite LC-MS/MS analysis

Chromatographic separation was performed on a Dionex Ultimate 3000 HPLC System (Thermo Fisher Scientific, MA, USA) with a Hypersil Gold C18 column (150 × 2.1 mm, 1.9 μm; Thermo Fisher Scientific) under gradient conditions, using mobile phases A (0.1% formic acid in 2% acetonitrile: water, v/v) and B (0.1% formic acid in 90% acetonitrile: water, v/v). Flow rate was set at 200 μL/min and the column was maintained at 40°C. Extracted metabolites were dissolved in 20 μL solvent A and 5 μL of sample was injected into the column for each run. The gradient started with 1% of solvent B and increased to 90% B in 17 minutes, holding at 90% B for 4 minutes, then 90% solvent B to wash the column for the next sample, and changed to 1% of B for 5 minutes to equilibrate the column.

Mass spectrometry analyses were performed on an Orbitrap Fusion instrument (Thermo Fisher Scientific, MA, USA) fitted with a Heated Electrospray Ionization (HESI) source. Data were acquired in positive and negative modes for each LC-MS analysis. Source parameters for positive ionization mode were set as follows: spray voltage at + 4.2 kV, sheath gas at 35 (arbitrary units), auxiliary gas at 10 (arbitrary units), and sweep gas at 2 (arbitrary units), while for negative ionization mode spray voltage -3.2 kV, sheath gas 40, auxiliary gas 15, and sweep gas 1. The Ion transfer tube temperature was set at 300°C and vaporizer temperature at 300°C. For MS1 acquisition, the data were acquired using the Orbitrap analyser, at a resolution of 120,000 at m/z 200 (FWHM, a spectrum collected at this resolution requires ~0.6 s); maximum injection time was set at 250 ms, automatic gain control (AGC) target at 5.0 x 104, RF lens level at 60%, and the scan range from m/z 70-1000. The MS2 acquisition was performed in Orbitrap with resolution was 30,000 at m/z 200, isolation window of 1.0, scan range ‘normal’, maximum injection time 35 ms. The selected ions were sequentially

fragmented by higher energy collisional dissociation (HCD) with stepped collision energy of 20%, 30%, and 40%. In all cases, one microscan was recorded using dynamic exclusion of 3 seconds and mass tolerance of ± 5 ppm. A targeted exclusion list of background noise with mass tolerance of ± 5 ppm was also included in the method to remove the LC-MS contaminants.

5.3.11.1.3 Metabolite LC-MS/MS data processing and database searching

Raw LC-MS metabolomics data were processed using MS-DIAL5 Desktop Application[281], using ESI(+)-MS/MS from standards+bio+in silico (16,746 unique compounds) for positive mode data and ESI(-)-MS/MS from standards+bio+in silico (15,100 unique compounds) for negative mode data[282]. These databases were accessed/downloaded on February 13, 2024. MS-DIAL5 parameters were set as follows: MS1 centroid tolerance, 0.01Da; minimum peak height, 1,000; preferred ions, [M+H]+1; [M-H]-1; maximum retention time shift, 0.1 min; MS2 centroid mass tolerance of 0.025 Da for library search. Background compounds were removed if the sample to blank area ratio was less than 5. Features identified with mass difference > 5 ppm between the observed mass of a compound and its annotated mass, and compounds with 'not the top hit', 'partial match', 'no match', or 'invalid mass', were also filtered out. Filtered compounds were manually validated by matching fragmentation spectra to those of available standards or to spectra reported in the literature.

5.3.11.2 Plasma protein extraction, digestion, and analysis

5.3.11.2.1 Plasma protein extraction and digestion

Protein quantification was performed by Bradford assay. Equal amounts of protein (300 μg) from the pooled QC and plasma samples were treated with Top14 Abundant Protein Depletion Mini Spin Columns (A36370, Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. The depleted samples were precipitated by 1:6 acetone precipitation overnight at -20°C. The precipitated samples were centrifuged for 15 minutes at 13,000 rpm at 4°C, and the supernatant removed. The resulting pellets were dissolved in 8 M urea and denatured with 50 mM dithiothreitol

by incubation at 60°C for 10 minutes. Samples were alkylated with 100 mM iodoacetamide in the dark at room temperature for 30 minutes. The urea concentration was diluted to 2 M by addition of 50 mM ammonium bicarbonate buffer. Trypsin digestion with mass spectrometry-grade proteases (#90058, Thermo Fisher Scientific, MA, USA) was then performed at 37°C: first at 1:30 mass ratio (trypsin : protein) for 2 hours, then at 1:60 ratio overnight. An end concentration of 1% formic acid was added to quench the enzymatic activation of trypsin, and drop in pH confirmed. The peptide mixture was desalted using Bond Elut C18 columns (#12102001, Agilent Technologies, CA, USA), dried using a Thermo Scientific Savant DNA120 SpeedVac Concentrator, and stored at -80°C until LC-MS analysis.

5.3.11.2.2 Plasma protein LC-MS/MS analysis

The peptide mixture was dissolved in 20 µL buffer A (2% acetonitrile: 98% water with 0.1% formic acid) and separated by Dionex Ultimate 3000 HPLC System connected to a Thermo Orbitrap Fusion mass spectrometer equipped with an online Nanospray Flex™ Ion Source. 4 µL peptide sample was loaded onto Thermo Trap Cartridge (PepMap™ Neo, C18, 100 Å, 300 µm x 5 mm) and eluted from analytical column (bioZen 2.6 µm Peptide XB-C18, Nano Column, 250 x 0.075 mm, Phenomenex Ltd, UK.) by using a 120-minute linear gradient from 5% to 30% of solvent B (90% acetonitrile: 10% water with 0.1% formic acid), followed by column wash and calibration at a flow rate of 400 nL/minute.

The Orbitrap Fusion mass spectrometer was operated in data-dependent acquisition (DDA) mode. The MS1 survey scan of parent ion was from 350 – 1500 m/z, and data were acquired at a high resolution of 120,000 (m/z 200), the AGC target was set to 3×10^5, and the maximum injection time was 100 ms. The second stage of mass spectrometry (MS2) scans was performed on IonTrap at rapid scan rate, with dynamic exclusion, 50 seconds; cycle time, 3 seconds; isolation width, 0.7 m/z and MIPS mode as peptide. Ions with charge states 2 – 7 were sequentially fragmented by collision-induced dissociation (CID) with a fixed collision energy of 35%. For the setting of the Nanospray Flex ion

source, the spray voltage of 2.2 kV was applied on Thermo Scientific Nano Bore steel emitter and ion transfer tube temperature was set at 275°C.

5.3.11.3 Tissue protein extraction, digestion, multiplexing, and analysis

5.3.11.3.1 Tissue protein extraction, digestion, and sample multiplexing

500 µL lysis buffer (0.5 % SDS; 0.1 M TEAB; Halt™ protease/phosphatase inhibitor cocktail (#78440 Thermo)) was added to each sample. Bead homogenisation was performed with Precellys Lysing kits (CK14 2ml) on the Precellys Evolution instrument at 6,500 rpm for 2 sets of 30 seconds with a 15-second break. The mixture was centrifuged at 4°C for 10 minutes at 13,000 rpm. The pelleted cellular debris was discarded and the supernatant was transferred to a new centrifuge tube on ice. Protein concentration was determined by Bradford assay.

Approximately 100 µg of protein from each sample was reduced with 0.5 M TCEP for 1 hour at 60°C and alkylated with 40 mM iodoacetamide for 30 min at room temperature in the dark. After 10-fold dilution using 0.1 M TEAB, protein samples were digested at 37 °C using Pierce Trypsin Protease, MS Grade at 1:30 mass ratio (trypsin : protein) for 2 hours followed by a second step of trypsin at a mass ratio of 1:60 (trypsin : protein) overnight. The reaction was quenched with formic acid. Trypsin enzymatic peptides were dried under vacuum after desalination by SPE Cartridge Bond Elut C18 columns (Agilent Technologies, Santa Clara, CA, USA). The peptides were dissolved with 0.1 M TEAB (Sigma Aldrich, Gillingham, UK) and labelled with isobaric mass tags according to the manufacturer instructions of the 11plex Tandem mass tag (TMT) kit (Thermo Fisher Scientific, Swindon, UK). A pool sample was used to control batch effects between sets of TMT labelling.

5.3.11.3.2 Tissue protein LC-MS/MS analysis

TMT-labelled peptides were pre-fractionated to reduce sample complexity using a 150 mm C18 column on an Agilent 1260 Infinity II high performance liquid chromatography instrument (Agilent Technologies, Santa Clara, CA, USA). Gradient elution was used to collect 27 fractions in 60 min,

using a linear gradient of 2% to 35% acetonitrile at pH 9. These fractions were then dried under vacuum using a Thermo Scientific Savant DNA120 SpeedVac Concentrator and stored at -80°C until LC-MS analysis.

Dried TMT-labelled peptides were resuspended in 20 µL buffer A (2% acetonitrile: 98% water with 0.1% formic acid) and 5 µl of sample was loaded onto Thermo Trap Cartridge (PepMap™ Neo, C18, 100 Å, 300 µm x 5 mm) and eluted from analytical column (bioZen 2.6 µm Peptide XB-C18, Nano Column, 250 x 0.075 mm, Phenomenex Ltd, UK). Online separation was performed using a Dionex Ultimate 3000-high performance liquid phase system, by eluting the peptides with a 65-minute linear gradient: 2 – 25% solvent B (90% ACN, 0.1% FA), 10 minutes; 25 – 50% solvent B, 5 minutes; 50 – 90% solvent B, 8 minutes; column wash with 90% solvent B, followed by 16 minutes column equilibration with solvent A at a flow rate of 400 nL/min.

MS analysis was performed using an Orbitrap Fusion mass spectrometer equipped with a Nanoflex ion source (Thermo Fisher Scientific, Swindon, UK). The parent ions of the peptide were detected and analysed using a high resolution orbitrap with ion source voltage 2.2 kV. MS1 scan range was set at 375 – 1500 m/z, with resolution of 120,000 at m/z 200. MS2 was performed using an ion-trap at rapid scan rate, with the following parameters: charge state 2 – 7, dynamic exclusion of 50 seconds, cycle time 3 seconds, and CID ~35%. Automated Synchronous Precursor Selection for MS3 was used for quantification of TMT11plex and performed on the orbitrap at 30,000 resolutions, with scan range 100-500 m/z, maximum injection time 105 ms and with 65% high-energy collisional dissociation.

5.3.11.4 Plasma and tissue protein LC-MS/MS data processing and database searching

Protein identification was performed using the CHIMERYS 3.0, AI-powered search algorithm (MSAID, Munich, Germany) and MASCOT search engine (version 2.3.2; Matrix Science, London, UK) embedded in Proteome Discoverer 2.2 (Thermo Fisher Scientific, MA, USA). The LC-MS/MS raw data were searched against the *Sus scrofa* database obtained from UniProt (version 2024_03_27 containing 292,143 protein sequences).

Proteome Discoverer parameters were set as follows: MS1 mass tolerance was set to 10 ppm while MS2 mass tolerance was 0.6 Da for IonTrap detection, trypsin was chosen as the enzyme, carbamidomethylation of cystine was selected as fixed modification, and oxidation of methionine, deamidation of asparagine and glutamine were specified as dynamic modifications. For the tissue proteomic analysis, in the Proteome Discoverer settings for TMT modifications, the TMT-11plex reagent was set as a fixed modification on lysine residues and peptide N-termini to ensure accurate quantification across samples. Only unique peptides were used for quantification of proteins. Only proteins with Mascot score ≥ 21 ($p < 0.05$), at least one unique peptide and that unique peptide was identified with two peptide-spectrum matches (PSMs), were accepted and included in further analysis.

5.3.11.5 Proteomic and metabolic statistical analysis

Porcine proteins were mapped to human genes using the UniProt mapping tool. Protein and metabolite abundance data were filtered to remove analytes present in less than 10% samples. For the plasma (but not tissue) studies, missing data were imputed using random forest. Batches were quantile normalised, batch effect corrected (ComBat algorithm), and log2 transformed. To calculate the differential abundance, an empirical bayes moderated t-statistic was calculated (LIMMA). For plasma metabolites and proteins, the treatment group : time interaction (intervention relative to control group) and effect of time for control samples were evaluated. For bile metabolites, bile samples pre-connection and at 16-hours were compared to perfusate and plasma samples at the corresponding time points. For tissue proteins, end-ELC samples were compared to pre-NMP samples. A false discovery rate < 5% using the Benjamini–Hochberg method was considered significant.

Principal component analysis was performed as follows:

- Plasma proteomics and metabolomics: common analytes with p-value < 0.05 in both treatment group : time and control time analyses.
- Tissue proteomics: proteins with p-value < 0.05 in end-ELC vs pre-NMP analysis.
- Bile metabolomics: metabolites with p-value < 0.05 in 16-hour bile samples.

Volcano plots highlight analytes with adjusted p-value < 0.05 (< 0.01 in bile metabolome volcano plot) and absolute logFC > mean logFC + standard deviation as significant.

Heatmaps were produced from the top 50 differentially-expressed analytes as follows:

- Plasma proteomics: adjusted p-value < 0.05 in treatment group : time and/or control time analyses and p-value < 0.05 in the other analysis.
- Plasma metabolomics: adjusted p-value < 0.05 in treatment group : time or control time analyses.
- Tissue proteomics: adjusted p-value < 0.05 in end-ELC vs pre-NMP analysis.
- Bile metabolomics: adjusted p-value < 0.05 in bile at 16 hours vs plasma at 16 hours (upregulated metabolites only).

Proteomic pathway analysis was performed using Reactome[283]. Metabolomic pathway analysis was performed for metabolites with p-value < 0.05 using the SMPDB library on web-based tool MetaboAnalyst version 6.0[284]. R packages ggplot2 and pheatmap were used for graphical presentation.

5.3.12 Statistical analysis

Parametric and non-parametric data are summarised using mean (range) and median (range), respectively. Values related to extra-corporeal liver vascular flows, bile production, and bile glucose are expressed as mean ± standard deviation. For variables with multiple time points per experiment, statistical significance between the control and intervention group was assessed using two-way repeated measures anova. For variables with a single value per experiment, statistical significance was assessed using two-tailed unpaired Student's t-test (parametric data) or Mann Whitney U test (non-parametric data). A p-value < 0.05 was considered statistically significant.

Statistical analysis of the metabolomic and proteomic data is described in *5.3.11.5* above.

5.4 Results

5.4.1 Donor liver retrieval, isolated normothermic perfusion phase, and acute liver failure model

Livers were retrieved from terminally anaesthetised donor pigs (*n = 6*). The liver donor pigs were exsanguinated to provide blood for the normothermic perfusion circuit prior to *in situ* cold perfusion, with a mean exsanguination time (effective warm ischaemic time) of 8 (range 6 – 10) minutes. The mean cold ischaemic time prior to reperfusion on the *metra* was 75 (55 – 99) minutes. The mean isolated normothermic perfusion phase duration was 302 (254 – 378) minutes (*figure 29)*.

Ex situ haemofiltration normalised perfusate potassium and reduced perfusate ACT to the pre-connection target within 5 hours (*figure 30)*. Attainment of functional criteria (perfusate lactate < 2.8 mmol/L; pH > 7.2; alanine transaminase < 6000 IU/L; evidence of glucose consumption; portal vein flow >500 mL/minute) was confirmed prior to commencing ELC for 5/6 donor livers. For one liver, functional data were only available for glucose consumption and portal vein flow (*table 12*).

ALF was induced in 12 patient pigs by hepatic inflow devascularisation (hepatic artery and portal vein ligation; porto-caval anastomosis). The mean porto-caval anastomosis time was 13 (9 – 17) minutes. Inflow devascularisation resulted in consistent liver injury based on biochemical liver function. Effective devascularisation was confirmed at the experiment end for all pigs by post-mortem examination and liver histological assessment.

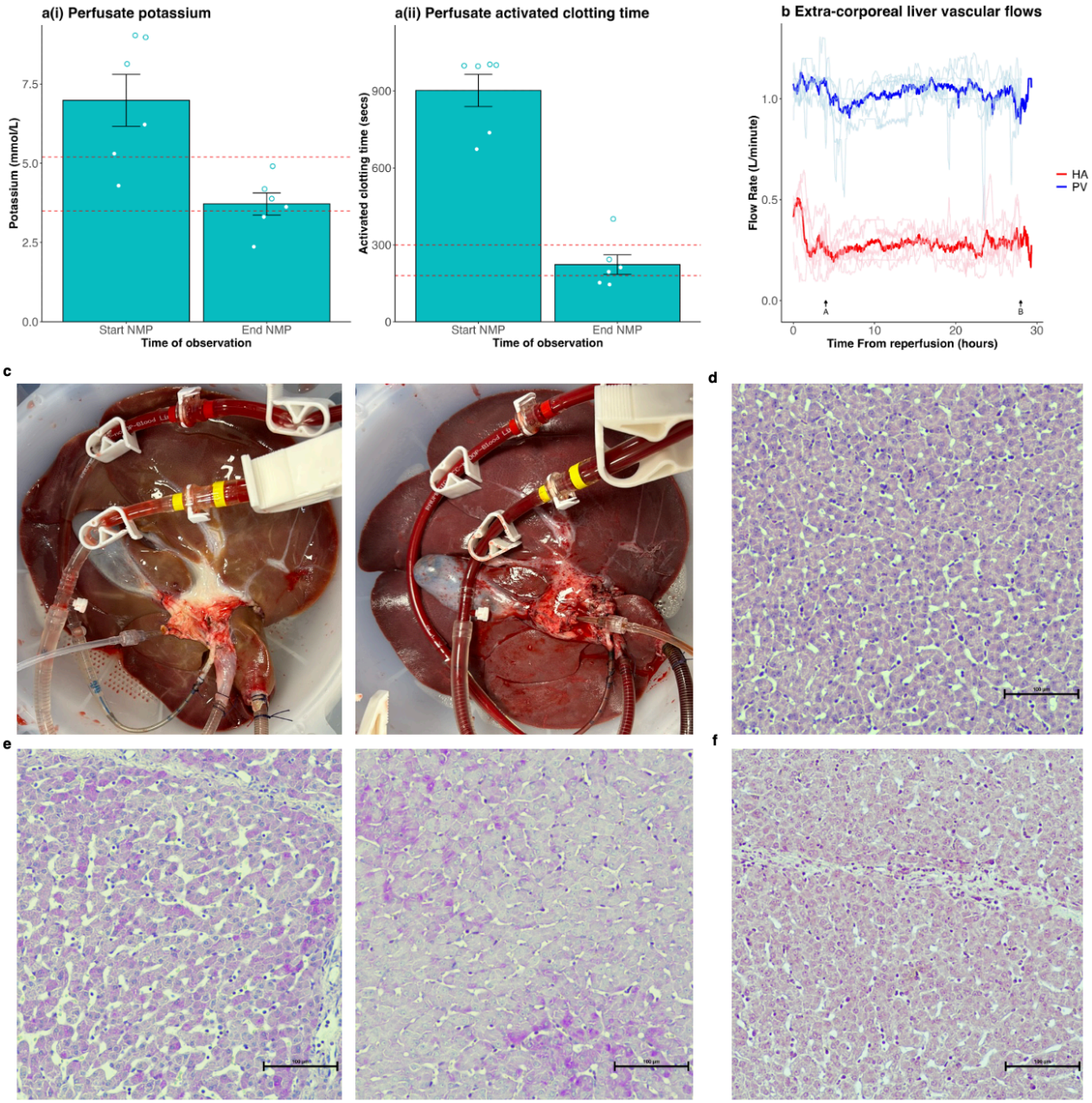


Figure 30: Extra-corporeal liver perfusion during isolated normothermic perfusion phase (NMP) and extra-corporeal liver cross-circulation (ELC).

a: Perfusate potassium and activated clotting time during isolated NMP phase (mean ± s.e.m., individual studies, *n=6*). 'Start NMP' timepoint refers to the perfusate sample prior to liver reperfusion. 'End NMP' refers to final perfusate sample prior to commencing ELC (254 – 378 minutes from start of NMP). b. Hepatic artery and portal vein flows of extra-corporeal liver during isolated NMP phase (pre-A) and ELC phase (A to B) (mean, individual studies). HA: hepatic artery; PV: portal vein. (*n=6*) c: Macroscopic appearance of extra-corporeal liver prior to commencing isolated NMP phase (left) and at end of ELC (right). d: End-ELC extra-corporeal liver biopsy stained with haematoxylin and eosin. e: pre-NMP (left) and end-ELC (right) extra-corporeal liver biopsy stained with periodic-acid Schiff. f: end-ELC extra-corporeal liver biopsy stained with Perl's.

Study	Lactate (mmol/L)	pH	ALT (IU/L)	Glucose (mmol/L)
1	2.21	7.4	23	8.2
2	0.88	7.5	29	5.2
3	N/A	N/A	N/A	3.7
4	<0.3	7.46	29	3.4
5	0.3	7.44	30	4.6
6	0.3	7.44	33	4.7

Table 12: end of isolated normothermic perfusion phase functional assessment by perfusate biochemistry. ALT: alanine transaminase.

5.4.2 Functional hepatic replacement

The target blood exchange rate between patient and *metra*-ELC system (400mL/minute) was achieved in all cases. The extra-corporeal livers maintained homogeneous parenchymal perfusion (*figure 30*), with physiological hepatic artery (280 ± 160mL/minute), portal vein (1030 ± 130mL/minute) flows (*figure 30*), and well-preserved microscopic appearance (*figure 30, table 13*). All extra-corporeal livers produced bile throughout ELC (10.3 ± 3.8 mL/hour), with consistently low bile glucose (0.1 ± 0.2 mmol/L).

ELC provided effective replacement of hepatic detoxification, excretory, synthetic, and metabolic regulatory function (*figures 31, 33*). Blood glucose regulation during ELC was supported by partial mobilisation of extra-corporeal liver glycogen (*figure 30, table 13*).

Replacement of hepatic function by ELC was associated with improvement in clinical markers of ALF: haemodynamic stability; hepatic encephalopathy; renal function. 5/6 pigs treated with ELC survived until 25 hours post-devascularisation, compared to 2/6 control pigs (*figures 32, 33)*.

Figures 31 and *32* present the data for the last common timepoint with data available for all pigs, whereas *figure 33* presents the data for the final timepoint available for each pig.

Liver	Patient pig native liver (*n = 12*)		Extra-corporeal liver (*n = 6*)	
Time	**Pre-devascularisation**	**End of study**	**Pre-NMP**	**End-ELC**
Necrosis	0.2 (0-2)	2.8 (2-4)	0.3 (0-1)	1.3 (0-2)
Sinusoidal congestion	1.0 (0-2)	3.5 (0-4)	0.8 (0-1)	1.7 (1-2)
Vacuolisation/ballooning	1.5 (0-3)	0.1 (0-1)	1.5 (0-2)	0.7 (0-2)
Neutrophil infiltration	1.3 (0-3)	3.0 (2-4)	1.3 (0-2)	3.5 (2-4)
Cholestasis	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Hepatocyte glycogen content	2.4 (0-3)	0.4 (0-3)	2.8 (2-3)	2.0 (0-3)
Perl's stain	0.3 (0-2)	0.8 (0-2)	0.3 (0-1)	1.0 (0-2)

Table 13: Histological scoring of patient pig native liver (*n=12*) at baseline (pre-devascularisation) and end of study, and extra-corporeal liver (*n=6*) pre-isolated normothermic perfusion (NMP) and at end of extra-corporeal liver cross-circulation (ELC) on haematoxylin and eosin, periodic-acid Schiff, and Perl's staining (mean (range)).
Key: Necrosis: 0: none; 1: single-cell; 2: <30%; 3: 30-60%; 4: >60%. Remainder of measures: 0: none; 1: minimal; 2: mild; 3: moderate; 4: severe.

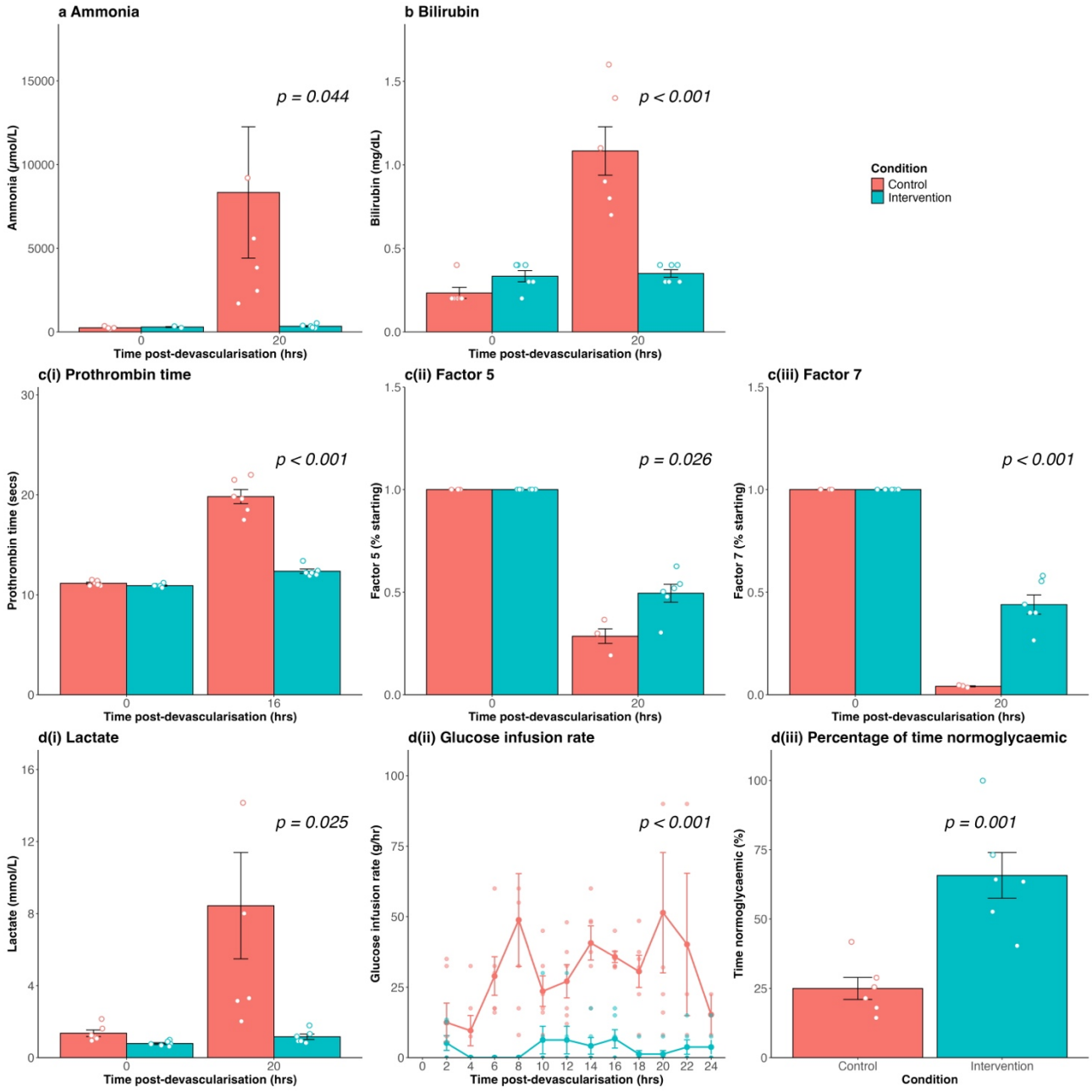


Figure 31: Impact of extra-corporeal liver cross-circulation on biochemical hallmarks of acute liver failure.
a: Ammonia (f-statistic 5.478; Df 1). b: Bilirubin (f-statistic 49.848; Df 1). c Prothrombin time (f-statistic 317.721; Df 1), factor V (t-statistic -3.137; Df 4.9654) and factor VII (t-statistic -8.4753; Df 5.0532). d: i) Lactate (f-statistic 7.123; Df 1) ii) glucose infusion rate during study (f-statistic 75.257; Df 1) iii) percentage of time normoglycaemic (blood glucose 3.5-6.0 mmol/L) during study (t-statistic 4.4531; Df 10). Time 0 is pre-hepatic artery ligation. a – d(i): second time point is the last common time point with results available for all pigs (final study values for ammonia, bilirubin, prothrombin time, and lactate are presented in *figure 33*). Mean ± s.e.m. and individual studies presented. Outliers > 1.5 x (mean + s.e.m.) removed from graphical presentation. Statistical analysis to compare control (*n=6*; factor V, VII: *n=3*) and intervention (*n=6*) groups by two-way repeated measures ANOVA (a-d(ii)) or two-tailed unpaired Student's t-test (d(iii)).

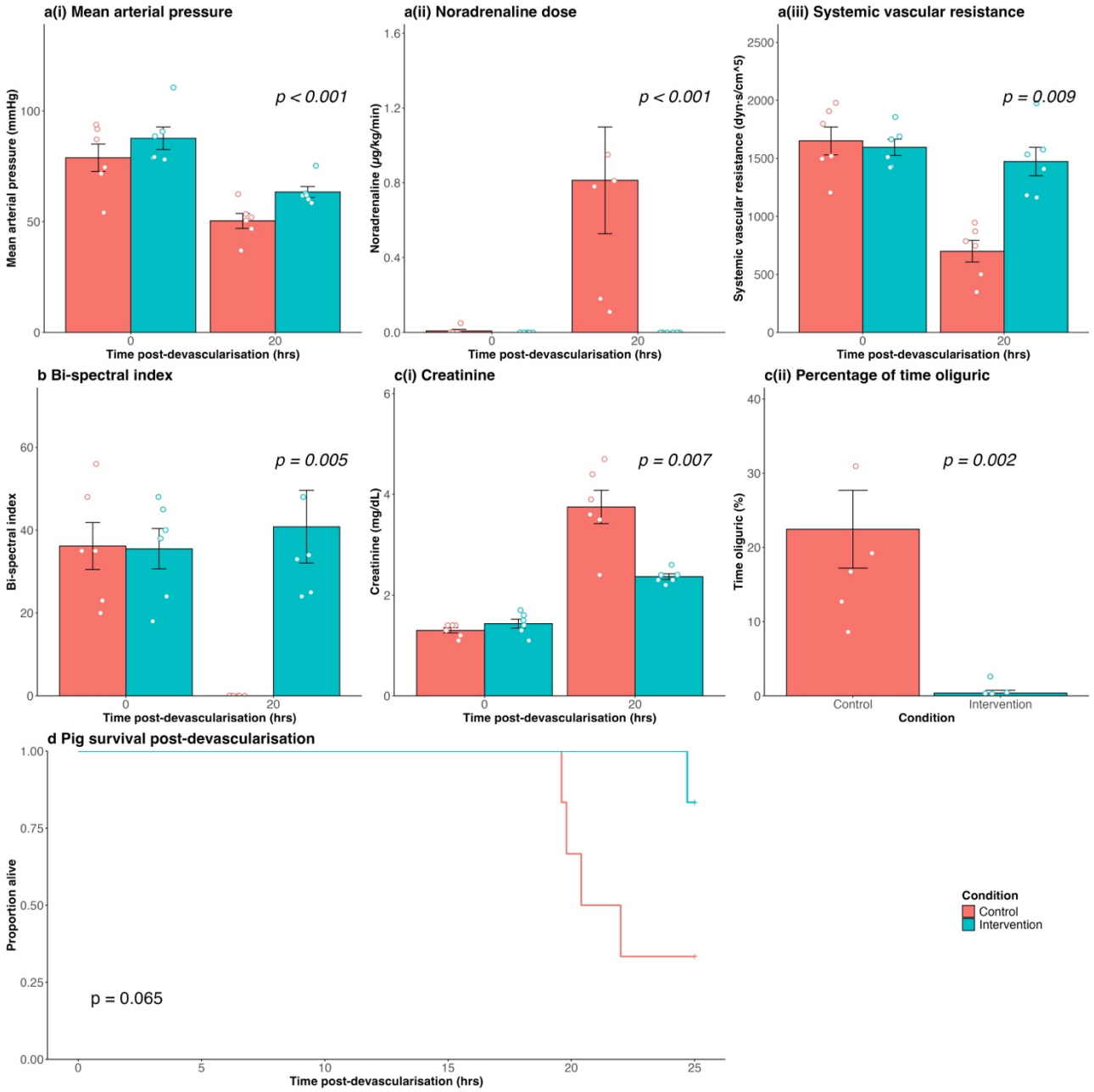


Figure 32: Impact of extra-corporeal liver cross-circulation (ELC) on clinical hallmarks of acute liver failure. a i) Mean arterial pressure, ii) noradrenaline dose requirement (f-statistic 31.407; Df 1), iii) systemic vascular resistance. b) Bi-spectral index (f-statistic 15.100; Df 1). c i) Creatinine (f-statistic 11.991; Df 1), ii) percentage of time oliguric (i.e. urine output < 0.5 mL/kg/hr) during study (t-statistic 4.1960; Df 10). d) Pig survival during study (χ^2 3.398719; Df 1). Time 0 is pre-hepatic artery ligation. a(i) – c(i): second time point is the last common time point with results available for all pigs (final study values for mean arterial pressure, noradrenaline dose requirement, systemic vascular resistance, bi-spectral index, and creatinine are presented in *figure 33*). a – c: Mean ± s.e.m. and individual studies presented. Outliers > 1.5 x (mean + s.e.m.) removed from graphical presentation. Statistical analysis to compare control (*n=6*) and intervention (*n=6*) groups by two-way repeated measures ANOVA (a – c(i)), two-tailed unpaired Student's t-test (c(ii)) or log-rank (d).

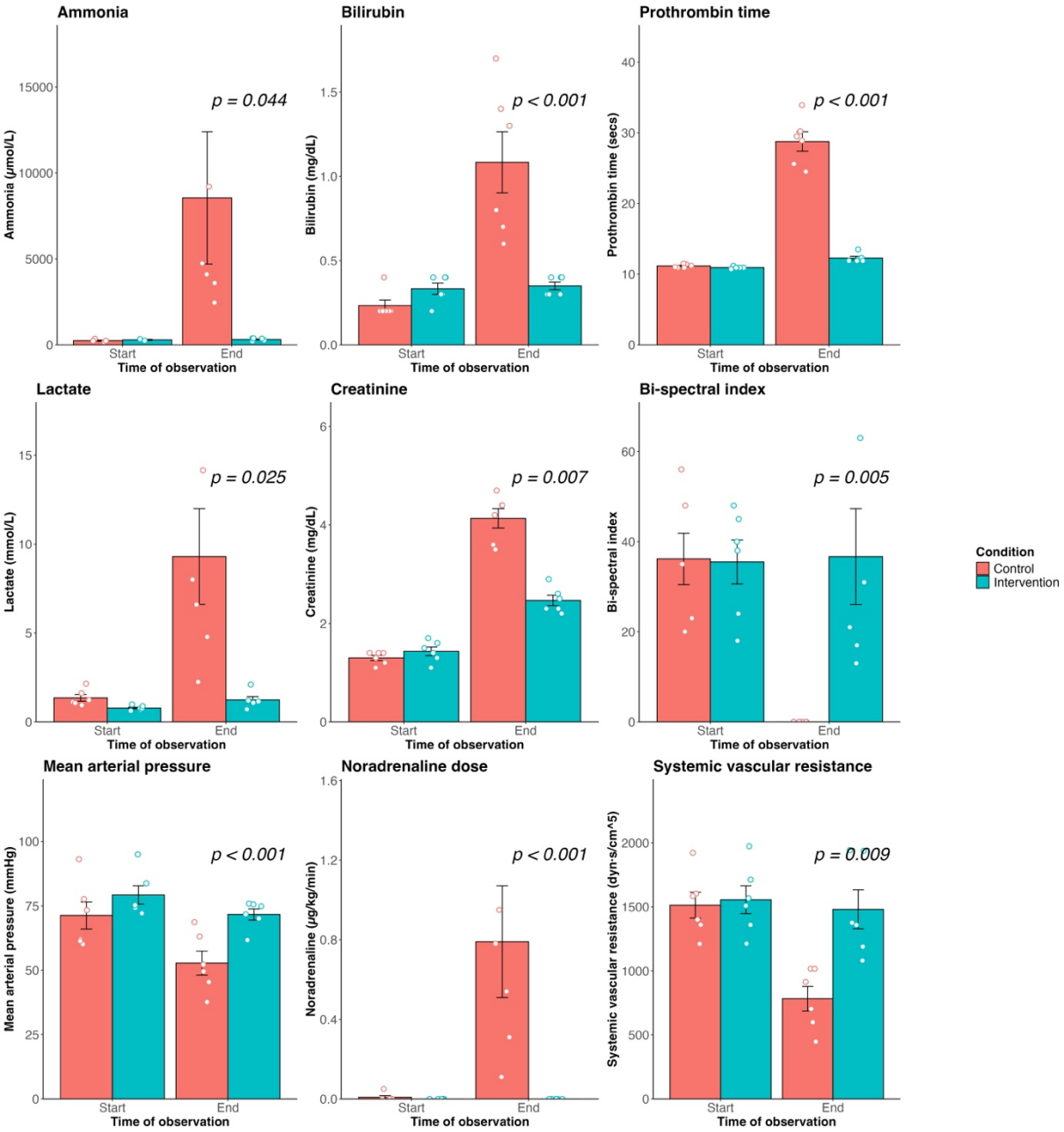


Figure 33: Impact of ELC on biochemical and clinical hallmarks of acute liver failure at start and end of study period. Ammonia (f-statistic 5.478; Df 1). Bilirubin (f-statistic 49.848; Df 1). Prothrombin time (f-statistic 317.721; Df 1). Lactate (f-statistic 7.123; Df 1). Creatinine (f-statistic 11.991; Df 1). Bi-spectral index (f-statistic 15.100; Df 1). Mean arterial pressure. Noradrenaline requirement (f-statistic 31.407; Df 1). Systemic vascular resistance. Mean ± s.e.m. and individual studies presented. Outliers > 1.5 x (mean + s.e.m.) removed from graphical presentation. Statistical analysis to compare control (*n=6*) and intervention (*n=6*) groups by two-way repeated measures ANOVA. Start of observation is pre-hepatic artery ligation. End of observation is the final sampling timepoint, closest hour: 25 hours post-devascularisation for pigs surviving to this point (control: *n=2*; intervention: *n=6*), or closest hour to time-of-death (20 hours: control, *n=3*; 22 hours: control, *n=1*).

5.4.3 Immunological impact

ELC was associated with a reduction in circulating DAMPs: cell-free DNA, extra-cellular histones, and uric acid. Endotoxin was reduced but this effect did not reach statistical significance (*figure 34*). ELC was associated with lower plasma interleukin-6, and no significant difference in TNFα, interleukin-1β, and interleukin-10 (*figure 34*).

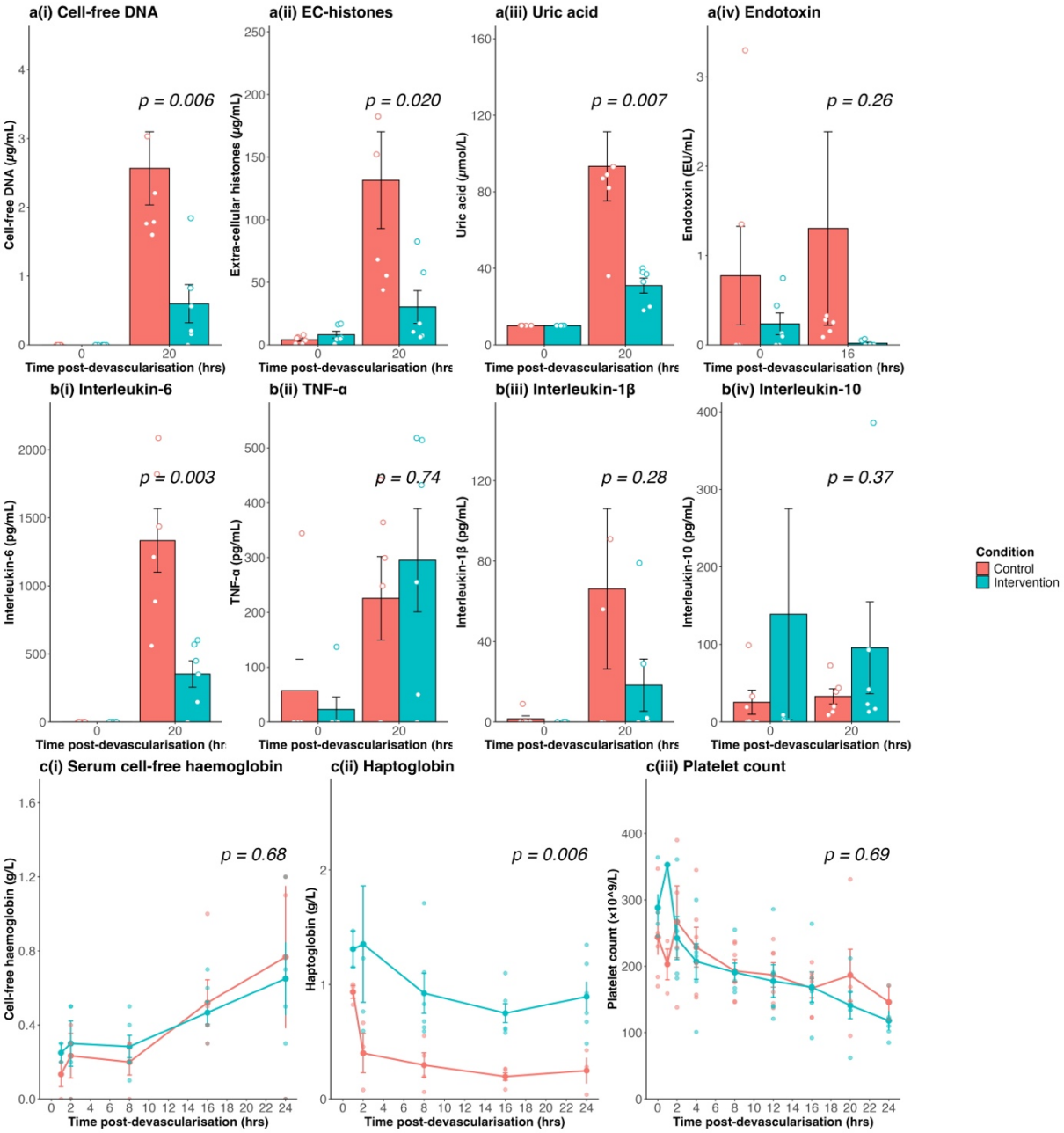


Figure 34: Impact of extra-corporeal liver cross-circulation (ELC) on inflammatory component of acute liver failure and evaluation of potential complications of ELC.
a) i) Cell-free DNA (f-statistic 11.74; Df 1) ii) extra-cellular histones (f-statistic 8.137; Df 1) iii) uric acid (f-statistic 11.34; Df 1) and iv) endotoxin (f-statistic 1.408; Df 1). b i) Interleukin-6 (f-statistic 15.26; Df 1), ii) TNF-a (f-statistic 0.119; Df 1) iii) interleukin-1b (f-statistic 1.298; Df 1) and iv) interleukin-10 (f-statistic 0.874; Df 1). c i) Serum cell-free haemoglobin (f-statistic 0.178; Df 1), ii) haptoglobin (f-statistic 14.120; Df 1), and iii) platelet count (f-statistic 0.177; Df 1) during study. Time 0 is pre-hepatic artery ligation. (i) – (iv): second time point is the final common time point with results available for all pigs. Mean ± s.e.m. and individual studies presented. Outliers > 1.5 x (mean + s.e.m.) removed from graphical presentation. Statistical analysis to compare control (*n=6*) and intervention (*n=6*) groups by two-way repeated measures ANOVA. EC histones: extra-cellular histones. TNF-a: Tumour necrosis factor-alpha.

5.4.4 Plasma proteome and metabolome impact

Principal component analysis demonstrated separation between the 20-hour post-devascularisation plasma proteome and metabolome of the control and intervention groups. The intervention group clustered more closely with the healthy baseline (0-hour) samples than the control group (*figure 35*). ELC impacted the plasma proteome broadly across domains relevant to ALF/ACLF pathophysiology (*table 9*), with upregulation of liver-derived plasma proteins related to complement (C9, C4BPB, C4A, C1QC), nutrient regulation (RBP4, SEPP1), acute phase response (CRP, HP), and apolipoprotein metabolism (APOC3). Adaptive changes occurred in plasma proteins associated with oxidative stress response (SOD1, PON1, CTH, CBS, GGCT, PRDX6), immune regulation (MIF, SLPI, FETUB), metabolism (ASL, CNDP2, QPRT, PCBD1, TPI1, ACADM, GLUD1), chaperone proteins (CLU, HSPA9, HSP90B1), and autophagy (CTSL, PSMA5). Hepatocyte damage markers (e.g. LDHA, BHMT) increased over time in both control and intervention pigs (*figures 35, 36, 37*). ELC altered the plasma metabolome of ALF (*table 10*) by modulating bile acid metabolism (5alpha-cholestanol), sphingolipid/choline metabolism (sphingosine 1-phosphate, sphingomyelins, phosphoryl choline), pentose phosphate pathway (ribose 5-phosphate), β-oxidation (palmitic acid), ω-oxidation (suberic acid, 3-methyladipic acid)), leucine degradation/ketogenesis (3-hydroxymethylglutaric acid), and oxidative stress/nutrient handling (beta-carotene). Both control and intervention pigs demonstrated plasma metabolite changes related to ketogenesis (acetoacetate), proteolysis (trimethylated lysine), and lysine metabolism (pipecolic acid). There was no significant difference in plasma 6-keto-prostacyclin $F_{1\alpha}$ (the longer-lived metabolite of epoprostenol) between the treatment groups ($p = 0.14$) (*figures 35, 38, 39)*.

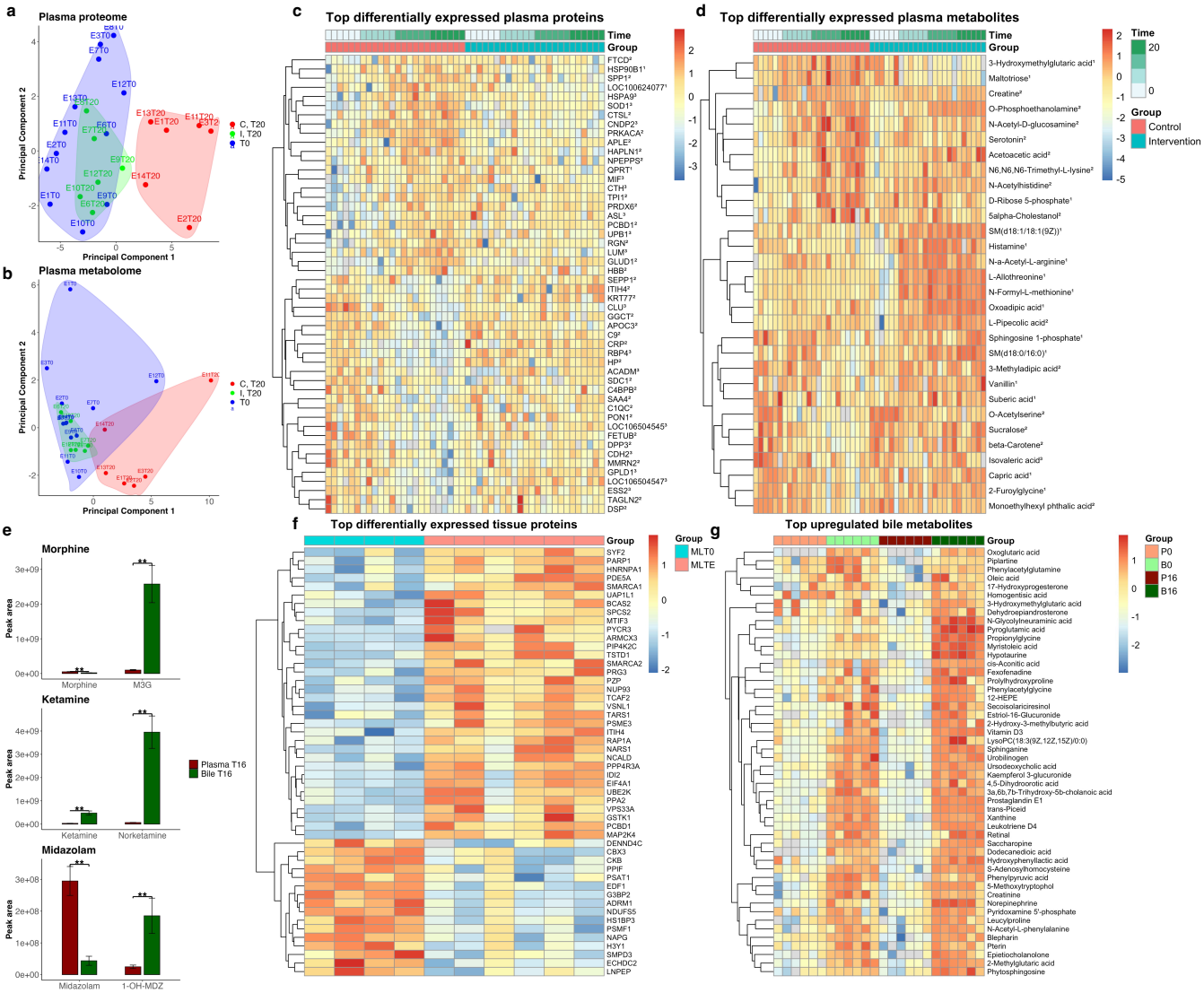


Figure 35: Patient pig plasma proteomic and metabolomic analyses; extra-corporeal liver tissue proteomic and bile metabolomic analyses.

a: Patient pig plasma proteome principal component analysis (common proteins with $p < 0.05$ in treatment group: time and control time analyses). b: Patient pig plasma metabolome principal component analysis (common metabolites with $p < 0.05$ in treatment group: time and control time analyses). C,T20: Control group at 20 hours; I, T20: intervention group at 20 hours; T0: both groups pre-devascularisation. c. Patient pig plasma proteome heatmap: adjusted $p < 0.05$ in treatment group : time and/or control time analyses and $p < 0.05$ in the other analysis (top 50 analytes). (*n=6* per timepoint per group). [1]treatment : time analysis adj. $p < 0.05$ + control time analysis $p < 0.05$; [2]control time analysis adjusted $p < 0.05$ + treatment : time analysis $p < 0.05$. [3]treatment : time analysis and control time analysis adjusted $p < 0.05$. d. Patient pig plasma metabolome heatmap: adjusted p-value < 0.05 in treatment group : time or control time analyses. (*n=6* per timepoint per group). [1]treatment : time analysis adjusted $p < 0.05$; [2]control time analysis adjusted $p < 0.05$. [3]treatment : time analysis and control time analysis adjusted $p < 0.05$. e: anaesthetic parent drug (morphine, ketamine, midazolam) and metabolite (morphine-3-glucuronide (M3G), norketamine, 1-hydroxy-midazolam (1-OH-MDZ)) concentrations in intervention patient pig plasma (*n=6*) and extra-corporeal liver bile (*n=6*) at 16 hours. Mean ± s.e.m. presented. **p < 0.01. f: Extra-corporeal liver tissue proteome heatmap: adjusted p-value < 0.05 in end-ELC (MLTE, *n=6*) vs pre-NMP (MLT0, *n=4*) analysis (top 50 analytes). g: Extra-corporeal liver bile metabolome heatmap: adjusted p-value < 0.05 in extra-corporeal liver bile at 16 hours vs patient pig plasma at 16 hours (top 50 upregulated analytes) (P0: end-isolated NMP perfusate (*n=6*); B0: end-isolated NMP bile (*n=6*); P16: 16-hour plasma (*n=6*); B16: 16-hour bile (*n=6*)). Differential analyte abundance calculated using empirical bayes moderated t-statistic with false discovery rate determined using Benjamini–Hochberg method (a – d, f, g). Statistical analysis to compare plasma and bile drug and drug metabolite concentrations by two-tailed unpaired Student's t-test (e).

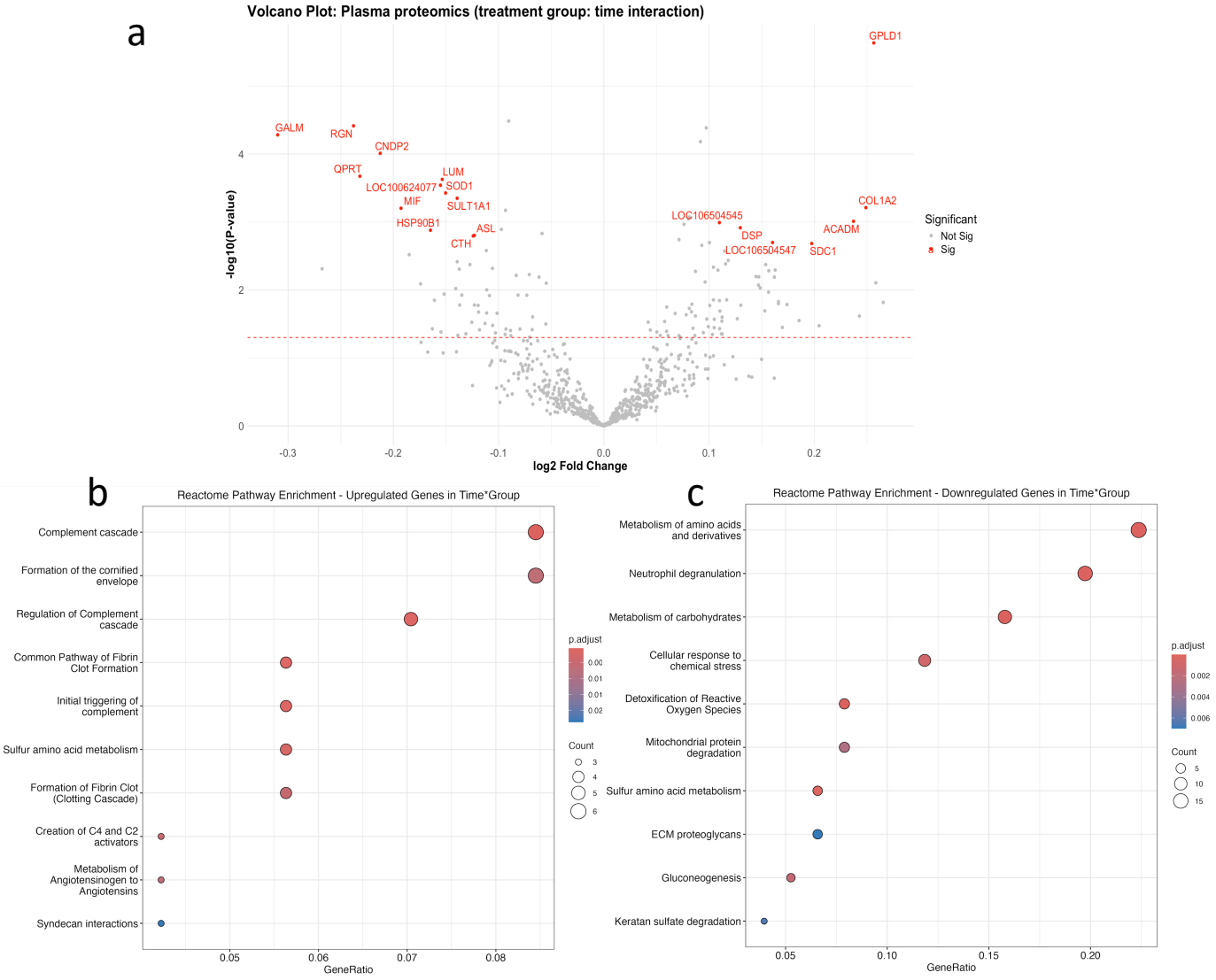


Figure 36: Patient pig plasma proteomics treatment group : time analysis (intervention relative to control group). Differential analyte abundance calculated using empirical bayes moderated t-statistic with false discovery rate determined using Benjamini–Hochberg method. (*n=6* per treatment group per time point). a) Volcano plot: analytes with adjusted p-value < 0.05 and absolute logFC > mean logFC + standard deviation identified as significant. b) Reactome pathway enrichment analysis (upregulated pathways) c) Reactome pathway enrichment analysis (downregulated pathways). FC: fold change.

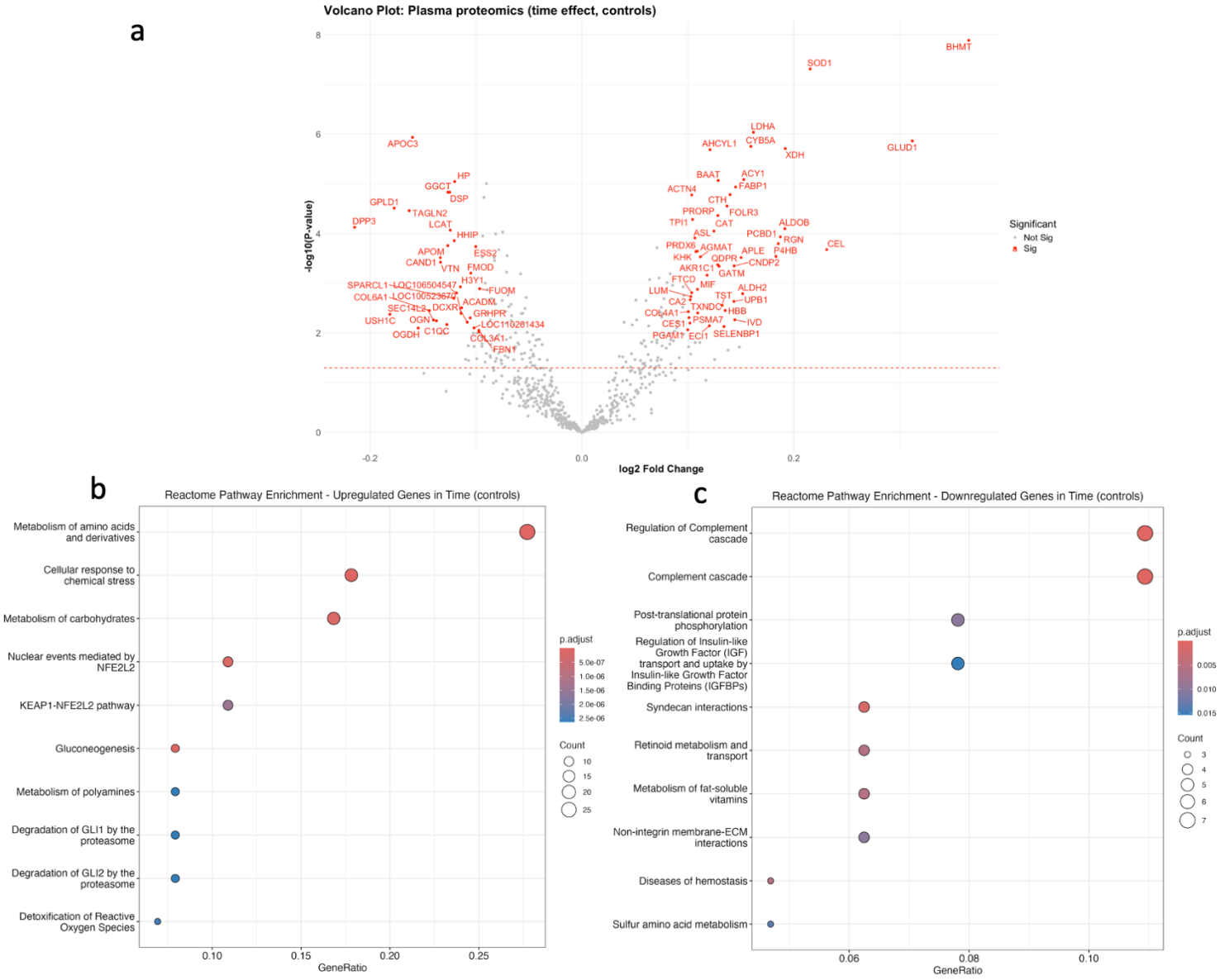


Figure 37: Control patient pigs plasma proteomics time effect analysis.
Differential analyte abundance calculated using empirical bayes moderated t-statistic with false discovery rate determined using Benjamini–Hochberg method. (*n=6* per time point). a) Volcano plot: analytes with adjusted p-value < 0.05 and absolute logFC > mean logFC + standard deviation identified as significant. b) Reactome pathway enrichment analysis (upregulated pathways) c) Reactome pathway enrichment analysis (downregulated pathways). FC: fold change.



Figure 38: Patient pig plasma metabolomics treatment group : time analysis (intervention relative to control group).
Differential analyte abundance calculated using empirical bayes moderated t-statistic with false discovery rate determined using Benjamini–Hochberg method. (*n=6* per treatment group per time point). a) Volcano plot: analytes with adjusted p-value < 0.05 and absolute logFC > mean logFC + standard deviation identified as significant. b) SMPDB pathway enrichment analysis. FC: fold change.

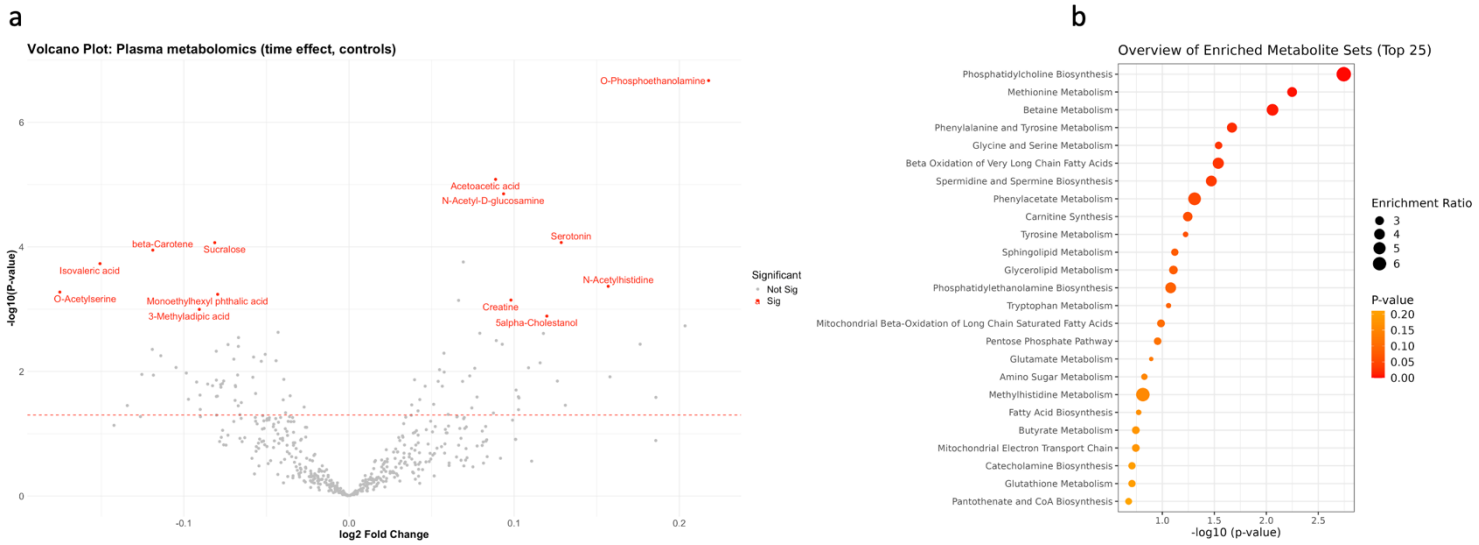


Figure 39: Control patient pigs plasma metabolomics time effect analysis.
Differential analyte abundance calculated using empirical bayes moderated t-statistic with false discovery rate determined using Benjamini–Hochberg method. (*n=6* per time point). a) Volcano plot: analytes with adjusted p-value < 0.05 and absolute logFC > mean logFC + standard deviation identified as significant. b) SMPDB pathway enrichment analysis. FC: fold change.

5.4.5 Extra-corporeal liver tissue proteome and bile metabolome

Between the end of cold storage and end of ELC, the tissue proteome of the extra-corporeal livers demonstrated upregulation of synthetic activity (DNA/RNA processing (NUP93, HNRNPA1, SYF2, PPP4R3A), transcription (BCAS2, SMARCA2, SMARCA1), translation (NARS1, EIF4A1), protein processing/folding (UAP1L1, TMX3), vesicle activity (VPS33A)), oxidative stress response (GSTK1, TSTD1), unfolded protein response/autophagy (UBE2K, PSME3), cell signalling (PARP1, PIP4K2C, SPCS2, NCALD, MAP2K4, PDE5A, VSNL1), and regulation of inflammation (ITIH4). Additional changes occurred in metabolic pathways involving fatty acids/lipids (ECHDC2), amino acids/proteins (PCBD1, PYCR3, PSAT1), sphingomyelins/sphingolipids (SPMD3), amino-sugars (NPL), and oxidative phosphorylation/mitochondrial function (NDUFS5, CKB, PPA2, MTIF2) (*figures 35, 40*). Extra-corporeal liver bile was enriched with bilirubin derivatives (urobilinogen), bile acids (ursodeoxycholic acid; 3α,6β,7β-trihydroxy-5β-cholanoic acid), phospholipids (lysoPC(18:3(9Z,12Z,15Z)/0:0)), fatty acids (myristoleic acid; oleic acid; dodecanedioic acid), and excretory metabolites (estriol-16-glucuronide, kaempferol 3-glucuronide). The extra-corporeal livers actively excreted metabolites of the intravenous anaesthetic agents morphine (morphine-3-glucuronide), midazolam (1-hydroxy-midazolam), and ketamine (norketamine). The parent drugs morphine and midazolam were excluded from bile, whereas ketamine was actively excreted (albeit less significantly than its metabolite) (*figures 35, 41*).

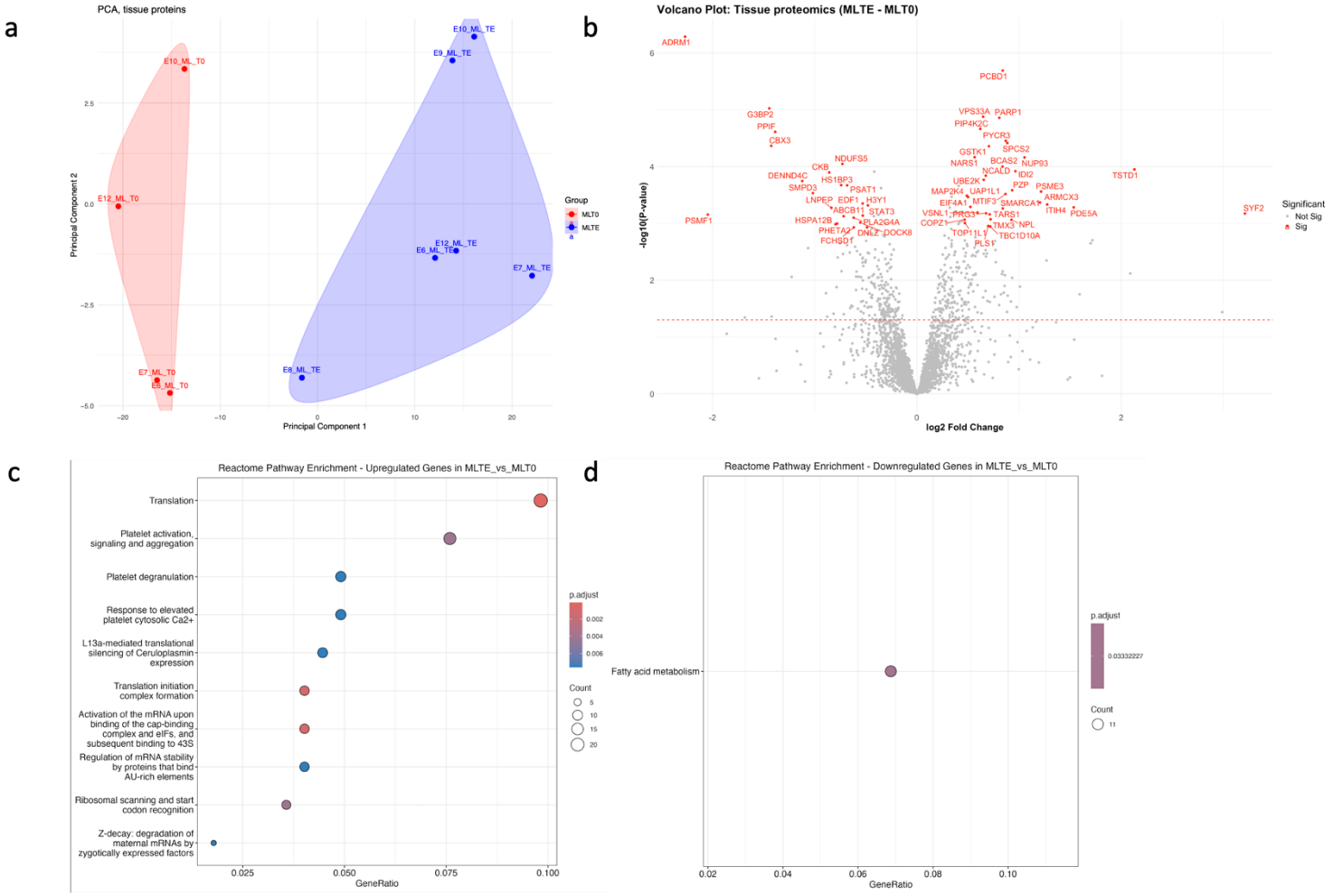


Figure 40: Extra-corporeal liver tissue proteomics analysis of extra-corporeal liver at end of extra-corporeal liver cross-circulation (ELC) (MLTE, *n=6*) *versus* pre-isolated normothermic perfusion (NMP) (MLT0, *n=4*).
Differential analyte abundance calculated using empirical bayes moderated t-statistic with false discovery rate determined using Benjamini–Hochberg method. a) Extra-corporeal liver tissue proteome principal component analysis (PCA) (proteins with $p < 0.05$ in end-ELC (MLTE) vs pre-NMP (MLT0) analysis). b) Volcano plot: analytes with adjusted p-value < 0.05 and absolute logFC > mean logFC + standard deviation identified as significant. c) Reactome pathway enrichment analysis (upregulated pathways) d) Reactome pathway enrichment analysis (downregulated pathways). FC: fold change.

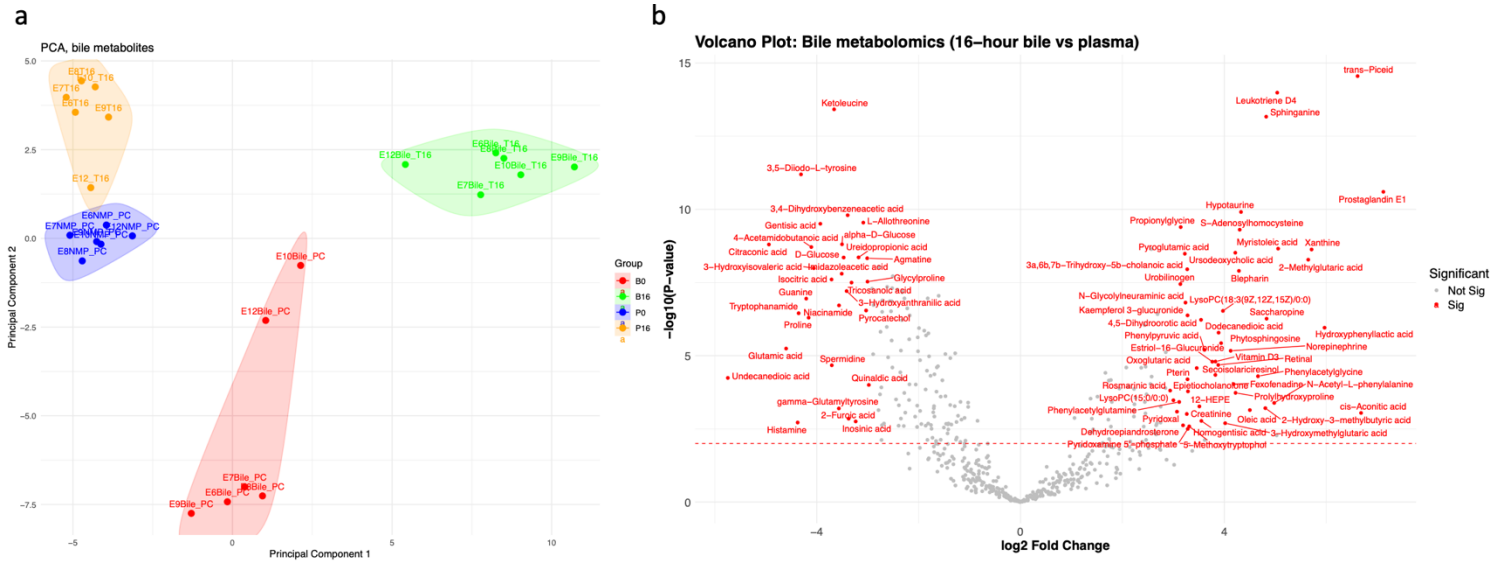


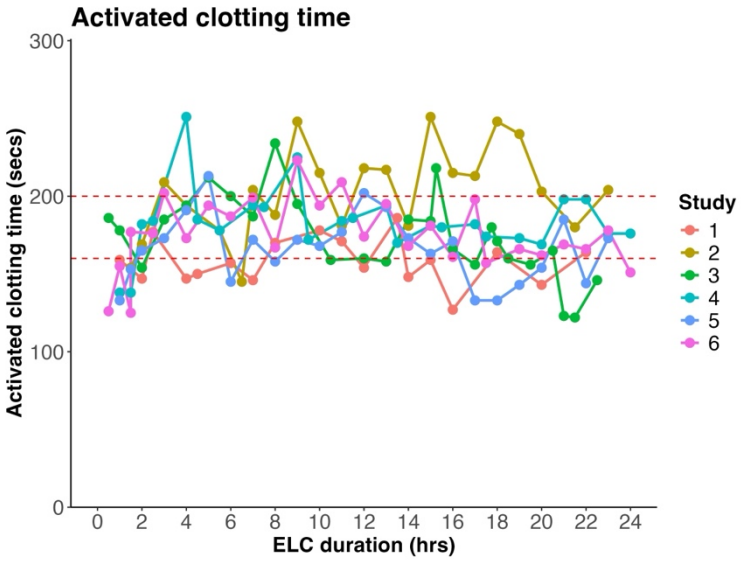
Figure 41: Extra-corporeal liver bile metabolomics analysis comparing bile samples at 16 hours of extra-corporeal liver cross-circulation (ELC) and end of isolated normothermic perfusion (NMP) with corresponding plasma and perfusate samples.
Differential analyte abundance calculated using empirical bayes moderated t-statistic with false discovery rate determined using Benjamini–Hochberg method. (*n=6* per sample type per timepoint). a) Extra-corporeal liver bile metabolome principal component analysis (PCA) (metabolites with $p < 0.05$ in 16-hour bile samples) (P0: end of isolated NMP perfusate; B0: end of isolated NMP bile; P16: 16-hour post-devascularisation plasma; B16: 16-hour post-devascularisation bile). b) Volcano plot: analytes with adjusted p-value < 0.01 and absolute logFC > mean logFC + standard deviation identified as significant. FC: fold change.

5.4.6 Evaluation of potential complications

Serum cell-free haemoglobin did not differ between the groups and haptoglobin remained higher in the intervention group (*figure 34*). Perl's staining of the extra-corporeal liver did not demonstrate substantial iron deposition (*figure 30, table 13*). Platelet counts declined in both groups, without significant difference between groups. The mean 24-hour platelet count in the intervention group was 118 (85 – 171) x 10^9/L (*figure 34*).

During ELC, a median of 1.75 (0.5 – 4.0) units of leuco-depleted blood were administered to the *metra* to maintain the portal reservoir 50 – 90% full. The control group required no blood transfusions. There was no significant difference in haemoglobin between the intervention and control groups (final haemoglobin, controls: 8.6 (7.2 – 10.4) g/dL; intervention: 9.5 (6.9 – 10.6) g/dL,

p=0.92, f-statistic 0.011, Df 1). In the intervention group, the mean hourly heparin dose was 2,871 (2,438 – 3,667) IU/hour (*figure 42*); the control group did not receive anticoagulation.



Study	Mean hourly heparin (IU/hr)	Mean hourly heparin (IU/kg/hr)
1	2438	34.8
2	3667	71.9
3	2774	51.4
4	2468	45.7
5	2864	50.2
6	3015	51.1
Mean	**2871**	**50.9**

Figure 42: Pig activated clotting time (ACT) during extra-corporeal liver cross-circulation (ELC).
Horizontal dashed lines indicate the target ACT range: 160 – 200 seconds. Individual studies presented. Table presents the mean hourly heparin dose used during ELC in the intervention cases.

5.5 Discussion

The *metra*-ELC system effectively supported hepatic function in a porcine ALF model. The protocol ensured safe connection by correcting perfusate biochemistry and confirming liver function prior to recipient connection. Improvement in clinically-relevant prognostic variables was associated with wide-ranging adaptive changes in the plasma proteome and metabolome. Previously reported complications of extra-corporeal systems (haemolysis and severe thrombocytopenia) were not observed.

The potential advantage of ELC over artificial and bio-artificial liver support systems lies in the use of a whole liver maintained under close-to physiological conditions. The extra-corporeal liver responds broadly across the range of normal hepatic function to regulate the plasma proteome and metabolome. ELC modulates changes in the levels of plasma proteins and metabolites driven by the pathophysiology of ALF/ACLF, related to complement[254-257], inflammation[93,255], oxidative

stress[93,255,256], nutrient regulation[258,259], apolipoprotein metabolism[255-257,259-261], chaperone proteins[254,259], sphingolipid/sphingomyelin metabolism[89,262-264], pentose phosphate pathway[72,262,265], fatty acid metabolism[72,262,266], bile acid metabolism[89,262,267,268], and ketogenesis[269]. Following native liver devascularisation, the plasma proteome and metabolome of the intervention group resembled the baseline, healthy samples more closely than the control group. Improvements in the plasma proteome and metabolome were underpinned by co-ordinated adaptations of the extra-corporeal liver tissue proteome characterised by recovery from ischaemia-reperfusion injury, response to oxidative stress, metabolic reprogramming, and synthetic function.

Failure of hepatic detoxification and excretion is responsible for prominent clinical manifestations of ALF/ACLF. ELC normalised ammonia and bilirubin levels. Bile enrichment with drug metabolites suggests intact hepatocyte glucuronidation, cytochrome P450, and transport mechanisms, whilst low bile glucose indicates intact cholangiocyte function[97]. Evidence of preserved hepatic synthetic function provided by the plasma proteomic data is confirmed by direct assays of factors V, VII, and haptoglobin. Refractory hypoglycaemia is a late manifestation of ALF/ACLF. Control pigs required significant glucose infusion and demonstrated erratic blood glucose control. ELC-treated pigs required minimal glucose supplementation, supported by appropriate mobilisation of hepatocyte glycogen from the extra-corporeal liver during the fasting period of the experiment. This, in combination with the plasma metabolomic data, suggests preservation of the liver's central role in metabolic regulation. ALF/ACLF are characterised by increased susceptibility to infection associated with loss of hepatic immunologic and reticulo-endothelial function[270,285,286]. ELC increased circulating liver-derived immune proteins including complement and c-reactive peptide, and was associated with reduced levels of secretory leukocyte protease inhibitor (SLPI) implicated in the immune paresis of ALF[287]. Endotoxin levels were reduced but this was not statistically significant.

Regarding the systemic inflammatory response of ALF driven by DAMPs released from dying hepatocytes[270], ELC was associated with lower levels of plasma cell-free DNA, extra-cellular histones, and uric acid. This may be due to restoration of an intact hepatic reticulo-endothelial/immunological system: liver sinusoidal endothelial cells and Kupffer cells play an important role in clearance of DAMPs and PAMPs[270,285,286]. Although the degree of hepatocyte

necrosis (in the native livers) was the same in both groups, it is possible that more severe multi-organ failure in control pigs increased the total DAMP burden.

Ischaemia-reperfusion (including during *ex situ* normothermic perfusion[225]) and extra-corporeal membrane oxygenation[288] are associated with pro-inflammatory cytokine release. Rather than increasing inflammatory markers, ELC reduced levels of interleukin-6 and markers of oxidative stress (e.g. superoxide dismutase 1 (SOD1)). By removing cytokines such as interleukin-6 from perfusate (*see chapter 3*), *ex situ* haemofiltration prior to connection may minimise the patient's initial exposure to cytokines on commencing ELC. During ELC, the effect of the extra-corporeal liver on circulating DAMPs and restoration of hepatic immune regulation may explain the less inflammatory environment.

Control pigs developed a hyperdynamic circulation characteristic of the systemic inflammatory response of ALF, and demonstrated a consistent and profound decrease in BIS. BIS (a measure of electro-encephalogram activity under general anaesthesia) correlates with hepatic encephalopathy grade in chronic liver disease[289,290] and ALF[291,292], and has been validated in porcine anaesthesia[293]. ELC improved cardiovascular, cerebral, and renal status - predictors of mortality in ALF[43] and ACLF[2].

Artificial systems do not replicate the integration of hepatic function with a patient's physiology that is provided by ELC. Plasma exchange, the only therapeutic intervention shown to improve survival in ALF[86], replaces a patient's plasma with allogenic plasma during a therapeutic session. Whilst this may transiently improve the plasma proteome, it is limited by deterioration between sessions[93]. Replacement with 'off-the-shelf' plasma does not permit homeostatic response to changes in the patient's physiology, as is achieved by a metabolically active liver.

Bio-artificial liver support systems, comprising hollow-fibre devices containing hepatocytes, lack critical liver architecture and non-hepatocyte cells. Whilst specific hepatocyte function (e.g. ammonia detoxification) may be preserved, many of the roles of a liver (e.g. excretory, reticulo-endothelial, and immunological function) are not. In a porcine ALF model, a bio-artificial liver worsened conjugated bile acid accumulation due to lack of biliary excretion and further depleted plasma sphingomyelin[89]. This contrasts with ELC, in which bile acids are excreted in the bile and plasma sphingomyelin levels

are improved. Indeed, for ELC with human recipients, bile acids excreted by the extra-corporeal liver will be replaced by enteral administration of ursodeoxycholic acid (see *chapters 7* and *8*).

The only infusion administered to the *metra* perfusate during ELC was epoprostenol (Flolan, a synthetic prostacyclin) at 8.33 μg/hour (139 ng/min). *In vivo*, prostacyclin is synthesised by endothelial and vascular smooth muscle cells. It acts in a paracrine manner to promote vasodilatation and inhibit platelet aggregation, opposing the action of thromboxane A_2[294]. This is a standard infusion administered to the *metra* circuit during normothermic preservation to support the microcirculation of the donor liver[95]. It is used clinically as a treatment for pulmonary arterial hypertension at a starting dose of 2 ng/kg/min, which is incrementally increased according to effect and tolerance. If epoprostenol infused into the *metra* perfusate during ELC were to reach the recipient in significant amounts, this could impact their haemodynamic status and platelet function.

The half-life of Flolan in plasma is 2.7 minutes[295]. During ELC, Flolan is administered to the portal reservoir. To estimate the dose that the patient is exposed to, consider the dose over one minute (assuming donor liver vascular flows equivalent to the mean values from this experiment and an exchange rate of 400 mL/minute): 139 ng Flolan is delivered to the 1 L of blood (mean portal flow per minute) that flows through the portal reservoir, before passing via the donor liver into the IVC outflow (mean: 1.3 L/minute). The IVC outflow next passes via the centrifugal pump and oxygenator to either the hepatic artery line, portal reservoir, or ELC bypass line: 400 mL/minute (30.8%) is exchanged with the recipient and 900 mL/minute (69.2%) passes through the liver a second time. Since the total blood volume of the *metra* circuit is about 1.5 L, it takes 1.15 minutes ($\frac{1.5}{1.3}$) for blood to complete one circuit of the *metra*, and therefore for Flolan entering the portal reservoir to reach the pre-reservoir bypass fork for the first time. For the 30.8% of the blood that is exchanged with the recipient, it takes a further 18 seconds (0.3 minutes) (118 mL single bypass line volume at 400 mL/minute) to reach the recipient; 30.8% of the original dose therefore reaches the patient 1.45 minutes ($1.15 + 0.3$) after administration. The remaining 69.2% completes a further loop of the *metra* circuit before reaching the bypass fork a second time; this process continues until the hypothetical 1 minute dose has been cleared. Computing the sum of this infinite geometric series, the recipient is

exposed to approximately 44% of the original dose: an effective dose 61 ng/min, or 0.87 ng/kg/min for a 70 kg individual (*figure 43*). This is less than half of the starting dose for pulmonary arterial hypertension[295].

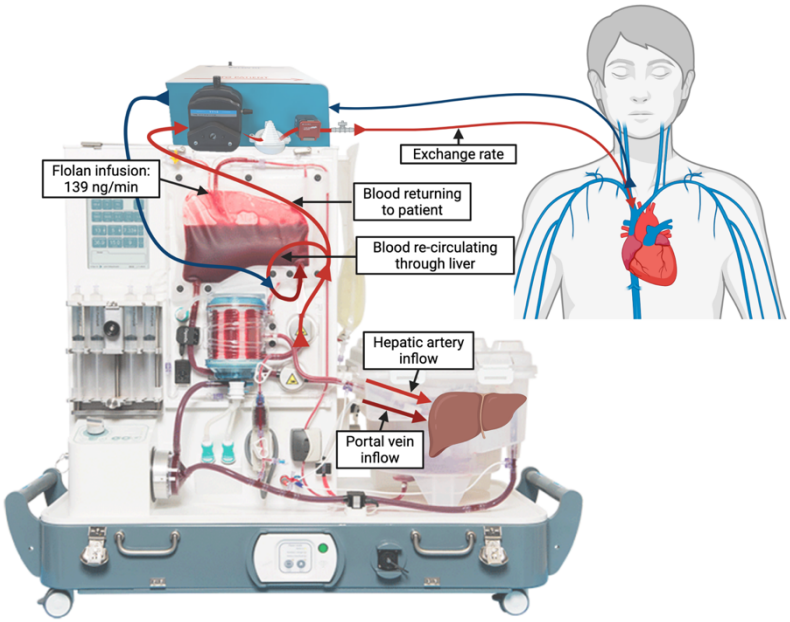


Figure 43: Epoprostenol pharmacokinetics with fractional blood exchange.
Flolan is infused into the portal reservoir at a rate of 139 ng/min. It passes along the portal line to the liver, after which it flows through the IVC line to the centrifugal pump and through the oxygenator. After the oxygenator, the blood either flows through the hepatic artery line directly back to the liver or towards the portal reservoir and the exchange bypass fork. The time taken for Flolan to reach the exchange bypass fork for the first time is approximately the time for blood to complete one circuit of the metra: 1.15 minutes (total blood volume of the metra (1.5 L) divided by total blood flow through the *metra* per minute (1.3 L)). From there, the blood that is sent to the recipient takes a further 0.3 minutes to pass along the exchange bypass line and reach the recipient's central venous system (i.e. 1.45 minutes (1.15 + 0.3) from the point of infusion). The rest of the blood recirculates back to the portal reservoir.

On each pass through the liver, the fraction of blood that is sent to the recipient equals $\frac{exchange\ rate}{hepatic\ artery\ flow + portal\ vein\ flow}$. The fraction that recirculates through the liver equals $1 - \frac{exchange\ rate}{hepatic\ artery\ flow + portal\ vein\ flow}$. Using the mean values from this experiment and an exchange rate of 400 mL/minute, the fraction sent to the recipient is 0.308 ($\frac{400}{300+1000}$) and the fraction that re-circulates is 0.692. The fraction re-circulating exactly twice equals $0.692 \times 0.308 = 0.213$. The fraction that recirculates through the liver *n* times, f_n, equals $0.308 \times 0.692^{n-1}$.

The half-life of Flolan, $t_{1/2}$, is 2.7 mins. The elimination rate, *k*, equals $\frac{ln\,2}{2.7} = 0.2567/min$. The fraction of epoprostenol remaining after time, *t* (mins), is $e^{-0.2567.t}$.

The amount of epoprostenol delivered to the recipient after one pass equals $139 \times 0.308 \times e^{-0.2567 \times 1.45}$. The amount delivered after *n* passes equals $139 \times 0.308 \times 0.692^{n-1} \times e^{-0.2567 \times (1.15.n+0.3)}$. The total dose delivered to the recipient is the sum of this series between *n* = 1 and *n* = infinity (i.e. modelling infinite passes). This computes as:

$139 \times 0.308 \times e^{-0.2567 \times 1.45} \times \frac{1}{1-0.692 \times e^{-0.2567 \times 1.15}} = 61\ ng/min$ (i.e. 44% of the starting dose).

In the experiment described in *chapter 7*, on two occasions the site of epoprostenol infusion was moved from the portal reservoir to the hepatic artery inflow line to increase the effective concentration delivered to the arterial tree of the donor liver. This approximately halves the time taken for the epoprostenol dose to reach the bypass fork for the first time (from 1.15 mins to 0.58 mins). After the first pass, the pharmacokinetic model returns to the scenario described above, with all subsequent loops taking 1.15 minutes. The amount of epoprostenol delivered after *n* passes then equals:

$139 \times 0.308 \times 0.692^{n-1} \times e^{-0.2567 \times (0.58+0.3+1.15 \times (n-1))}$.

The sum of this infinite series is:

$139 \times 0.308 \times e^{-0.2567 \times 0.3} \times e^{-0.2567 \times 0.58} \times \frac{1}{1-0.692 \times e^{-0.2567 \times 1.15}} = 70\ ng/min$ (i.e. 51% of the starting dose).

Due to the short half-life of epoprostenol, pharmacokinetic analysis must rely on measurement of the primary inactive metabolite of prostacyclin (6-keto-prostacyclin $F_{1\alpha}$), which is formed by spontaneous hydration; the half-life of 6-keto-prostacyclin $F_{1\alpha}$ is 13.2 minutes[296,297]. Plasma metabolomic analysis in this study did not detect prostacyclin/epoprostenol in either treatment group. 6-keto-prostacyclin $F_{1\alpha}$ was detected in both groups, without significant difference between the groups. This, combined with the absence of haemodynamic instability on commencing ELC and significantly lower vasopressor requirement in the intervention group, suggests that recipients were not exposed to substantial amounts of epoprostenol. The local platelet inhibitory action of epoprostenol within the portal reservoir may explain why clotting of the extra-corporeal circuit was not observed even at lower ACT values (the lowest recorded ACT value was 122 seconds), and could support further lowering of the ACT target in the future (see *chapter 8*).

The reasons for selecting hepatic inflow devascularisation as the model of ALF are explained in *5.1*. However, ischaemia is not a typical clinical cause of ALF; paracetamol overdose is the most common aetiology in the UK[43]. Toxin-induced porcine ALF models exist but carry significant disadvantages including off-target toxic effects (e.g. paracetamol-induced methaemoglobinaemia due to metabolic differences between pigs and humans[298]), a prolonged and unpredictable prodrome prior to developing ALF associated with potential animal suffering (e.g. D-galactosamine[275]), and use of hazardous substances (e.g. carbon tetrachloride[277]) (*table 11*). Established porcine models of ACLF have not been described. The length of time to develop cirrhosis makes such models impractical. They would necessarily be associated with considerable animal suffering as chronic liver failure must be established before acute decompensation is triggered to reflect the clinical scenario.

Hepatic inflow devascularisation generates a significantly accelerated model of fulminant liver failure due to the immediate complete cessation of native hepatic function; previous studies have reported mean survival times of 10 – 11 hours for control pigs[273,274,278]. Although our intensive medical management protocol extended this survival time, the rapid course meant that we needed to commence ELC 1-hour post-devascularisation. In the clinical setting, ELC will be started in the context of established liver failure with existing physiological compromise (e.g. haemodynamic

instability). This represents an important limitation to the direct translation of the findings of this study to clinical ELC.

Due to a combination of this accelerated model and the requirement for general anaesthesia throughout the study, the maximum ELC duration was pre-defined as 24 hours. More prolonged general anaesthesia in the pig is associated with substantial anaesthetic complications unrelated to liver failure (e.g. respiratory compromise[299]) that do not reflect critical care in the human setting. Longer durations of anaesthesia also carry considerable logistical and financial implications that would have limited the practicality of the experiment. ELC durations greater than 24 hours are likely required for consistent clinical benefit (*chapter 9*). The demonstration in this study of well-preserved extra-corporeal liver function, haemodynamic parameters, and microscopic appearance at 24 hours suggest that longer periods of support are possible. Due to the limitations of the porcine model, the *metra*-ELC system was tested for longer durations (up to 84 hours) in the human decedent model, which more closely reflects the clinical setting (*chapter 7*).

Although the magnitude and consistency of the observed effect allowed for demonstration of statistical significance across a broad range of markers, this must be interpreted in light of the small sample size of the study: 6 in the intervention group and 6 in the control group. ELC was evaluated in a single model of liver failure (i.e. hepatic devascularisation) in pigs of the same age, sex, weight, and breed. There is substantial heterogeneity in the natural history of clinical ALF and ACLF depending upon aetiology, demographics, and underlying co-morbidities that is not captured in this study.

This experiment tested ELC using allografts. For allo-ELC to be a viable therapy in the clinical setting, this approach would depend upon the use of declined donor livers. The donor livers in this experiment experienced an effective warm ischaemic injury during the exsanguination period. The length of this (8 (6 – 10) minutes) is close to the target warm ischaemia time (from cessation of circulation to *in situ* cold aortic perfusion) in a clinical Donation after Circulatory Death organ retrieval (i.e. 10 minutes). However, this period does not include functional warm ischaemia during the dying process that is common in the clinical setting. In this experiment, donors were healthy pigs, rather than the extended criteria donors (e.g. raised body mass index, increased age) associated with declined grafts. These criteria are difficult to replicate in an animal model: a realistic model of hepatic

steatosis in pigs is hard to achieve and the livers of aged Large White x Landrace pigs are too large for perfusions devices designed for humans. The short cold ischaemia time in this model reflects a 'device-to-donor'[95] rather than 'back-to-base'[300] approach to liver normothermic perfusion. The perfusate used in the isolated normothermic perfusion phase comprised autologous leuco-depleted blood, rather than the combination of allogenic packed red cells and 5% human albumin solution that will be used when ELC is performed clinically, because a centrifuge capable of generating packed red cells was not available within the animal facility.

Though this model did not directly assess the effect of ELC using extended criteria grafts, when such grafts are used for ELC rigorous functional assessment will be conducted during the isolated normothermic perfusion phase (see *chapters 4, 8*) before proceeding to ELC. The level of function should therefore be comparable to that of the livers used in this study. The unanswered question is how many potential declined livers are rejected on these functional grounds and, more pertinently, how many patients are able to commence ELC using declined allografts in a timely manner. The latter incorporates wider feasibility considerations such as how frequently donor families consent for liver donation specifically for ELC. These factors will be explored in the phase-1 clinical trial described in *chapter 8*.

In summary, this experiment demonstrates that the *metra*-ELC system using allografts provides effective hepatic support in a large animal model. Replacing whole liver function by ELC may address the underlying pathophysiology of ALF more comprehensively than artificial and bio-artificial systems.

5.6 Declaration

I designed and ran the study; performed the liver perfusions, ELC, and sample collection (with support from the Transplant Research Fellows and OrganOx clinical specialists included under *Acknowledgements*); data analysis; and interpretation.

Recipient pig surgery was performed by Professor Peter Friend and the Transplant Research Fellows. Recipient pig anaesthesia and medical management were performed by Professor Eddie Clutton and

Dr Stephen Greenhalgh, academic veterinary anaesthetists at the Large Animal Research and Imaging Facility, University of Edinburgh.

I performed all laboratory analysis except: cell-free DNA and extra-cellular histones were measured at the University of Liverpool; uric acid was measured at the Clinical Biochemistry Laboratory, Oxford University Hospitals; factors V and VII were measured at the Coagulation Laboratory, Oxford University Hospitals; histological staining was performed by the Oxford Centre for Histopathology Research; mass spectrometry was led by Dr Sadr Shaheed.

6 *Ex situ* isolated xenoperfusion of transgenic pig livers with human whole blood

6.1 Background

The two potential sources of livers for ELC are declined human liver allografts and xenografts. The incidence of ALF is comparable to the number of declined human livers[32,45-47], although not all declined livers will be suitable or provide sufficient function for ELC and availability will be unpredictable. However, the incidence of ACLF greatly exceeds the total number of declined livers[1,59-63]: for ELC to become a realistic therapy for patients with ACLF, a different/additional source of donor organs would be needed. Xenografts may prove a more plentiful and reliable source of grafts for ELC.

Previous attempts at ELC, prior to 2000, used mainly wild-type xenografts. Whilst there were promising examples of prolonged ELC[102,108], the majority of cross-circulations were too short (median: 5 hours) for consistent clinical benefit[29,109-111]. The only series (*n = 2*) reporting ELC using transgenic pig livers (h*CD55*.h*CD59*) was limited to durations of 6.5 – 10 hours[103].

The development of CRISPR-cas9 genetic engineering has enabled generation of porcine donors with multiple genetic edits to increase compatibility with humans, described in *1.11.1.1*. The latest generation of edits has enabled survival of renal xenografts in non-human primates for multiple years[107] and clinical cases for several months[197,198]. Non-human primate liver xenotransplant studies have so far been limited to using grafts with relatively minimal genetic edits (mainly α-Gal knock-out only)[199,200,202,207-209]; the longest survival for an orthotopic xenotransplant is 29 days[203]. There is a single case report of auxiliary heterotopic implantation of a pig liver with multiple genetic edits (triple glycan knock-out; h*CD46*.h*CD55*.h*THBD*) into a human decedent for 10 days[205]. *Chapters 6* and *7* will assess the potential of pig livers with the latest generation genetic edits to be used for ELC ('xeno-ELC').

For ELC to provide consistent clinical benefit, the perfused donor livers would need to function for multiple days. Potential obstacles to clinical translation of liver xenografts include: characterisation of

the interaction with the human immune, coagulation, and complement systems; liver xenograft-associated erythrophagocytosis and thrombocytopenia; and potential drug metabolic pathway differences between pigs and humans. Non-human primate[199-203] and human decedent[301] studies provide valuable information regarding the *in vivo* behaviour of xenografts. However, such experiments are expensive, logistically challenging, and, in the case of decedent studies, infrequent and unpredictable. Differentiating xenograft from native liver function in auxiliary transplant experiments[205] is challenging. Sequential tissue sampling (e.g. to investigate rapid-onset xenograft-associated thrombocytopenia) would be associated with a high risk of uncontrollable haemorrhage of the graft.

Ex situ isolated whole blood xenoperfusion provides a platform for preliminary exploration of the potential for transgenic pig livers to be used for ELC. It also enables assessment, with refinement if necessary, of liver perfusion technology and techniques optimised for human livers, when applied to porcine xenografts.

6.2 Aims

This study consists of two parts. In part 1, the primary aim was to evaluate the viability of transgenic porcine livers perfused *ex situ* with pooled human whole blood over 48 hours ($n = 5$). The secondary aims were to assess: (i) erythrophagocytosis; (ii) drug metabolism; (iii) perfusate potassium concentration; (iv) perfusate insulin concentration.

In part 2, the mechanism of porcine liver xenograft-associated thrombocytopenia was investigated through additional short-duration xenoperfusions ($n = 4$).

6.3 Methods

6.3.1 Transgenic porcine liver generation

Porcine livers were procured from transgenic Yucatan minipig clones. The genetic alterations can be grouped into three categories[107]:

i. Triple glycan knock-out to prevent hyperacute rejection: functional knock-out of alpha-1,3-galactosyltransferase (α1,3GT), cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH), and beta-1,4-N-acetyl-galactosaminyltransferase 2/-like (β4GalNT2/β4GalNT2L), resulting in undetectable endogenous levels of galactose-α-1,3-galactose (α-Gal), N-glycolylneuraminic acid (Neu5Gc), and sialyl-dimeric antigen (SDa) glycan molecules, respectively.

ii. Hemizygous insertion of 15S transgenic construct to address incompatibilities between porcine and human coagulation, complement, and immune systems. The inserted transgenes (*PROCR*, *THBD*, *TNFAIP3*, *HMOX1*, *CD46*, *CD55*, *CD47*) express the proteins EPCR, TM, A20, HO1, CD46, CD55, and CD47.

iii. Functional inactivation of porcine endogenous retrovirus *pol* gene to prevent zoonotic retroviral transmission.

This study includes livers from porcine donors that have either undergone triple glycan knock-out and seven transgene insertion (3KO.7TG, designated EGEN-5734) or all three categories of genetic modification (3KO.7TG.RI, designated EGEN-5784). Experimental protocols involving cloning and production of porcine donors were approved by the eGenesis-WI and Exemplar (Oxford, IA, USA) Institutional Animal Care and Use Committees (IACUCs).

6.3.2 Porcine liver retrieval and backbench preparation

Pig livers were retrieved from anaesthetised Yucatan minipigs and prepared, per *2.1.2* and *2.2*. Experimental protocols involving porcine liver retrieval were approved by the eGenesis-WI or Accuro farms IACUCs (depending on site of study).

6.3.3 Porcine liver xenoperfusion

The porcine livers underwent normothermic perfusion on an OrganOx *metra*[4,95]. The *metra* was primed with 3 – 4 units of O-type human whole blood from different donors. The following

medications were administered to the primed circuit: 500 mg meropenem, 50 mg caspofungin, 10,000 IU heparin, and 10 mL 10% calcium gluconate. The following medications were infused to the circuit during perfusion, per the *metra* instructions for use[4]: heparin (833 IU/hour), epoprostenol (8.33 µg/hour), insulin (6.67 IU/hour), and sodium taurocholate (bile salts, 150 mg/hour). Additional units of human whole blood were administered during perfusion to maintain the portal reservoir approximately 70% full, if required.

To support 48-hour xenoperfusion (xenoperfusions 1 – 5 (part 1)), the standard *metra* circuit was modified to enable continuous *ex situ* haemofiltration as described in *chapter 3 (figure 44)*. The dialysate was NxStage PureFlow RFP-404. Continuous *ex situ* haemofiltration supported prolonged perfusion by maintaining perfusate electrolyte concentrations at close-to physiological levels and preventing accumulation of metabolic waste (e.g. urea). *Ex situ* haemofiltration was not used during the short-duration xenoperfusions (xenoperfusions 6 – 9 (part 2)) that investigated the mechanism of xenograft-associated thrombocytopenia.

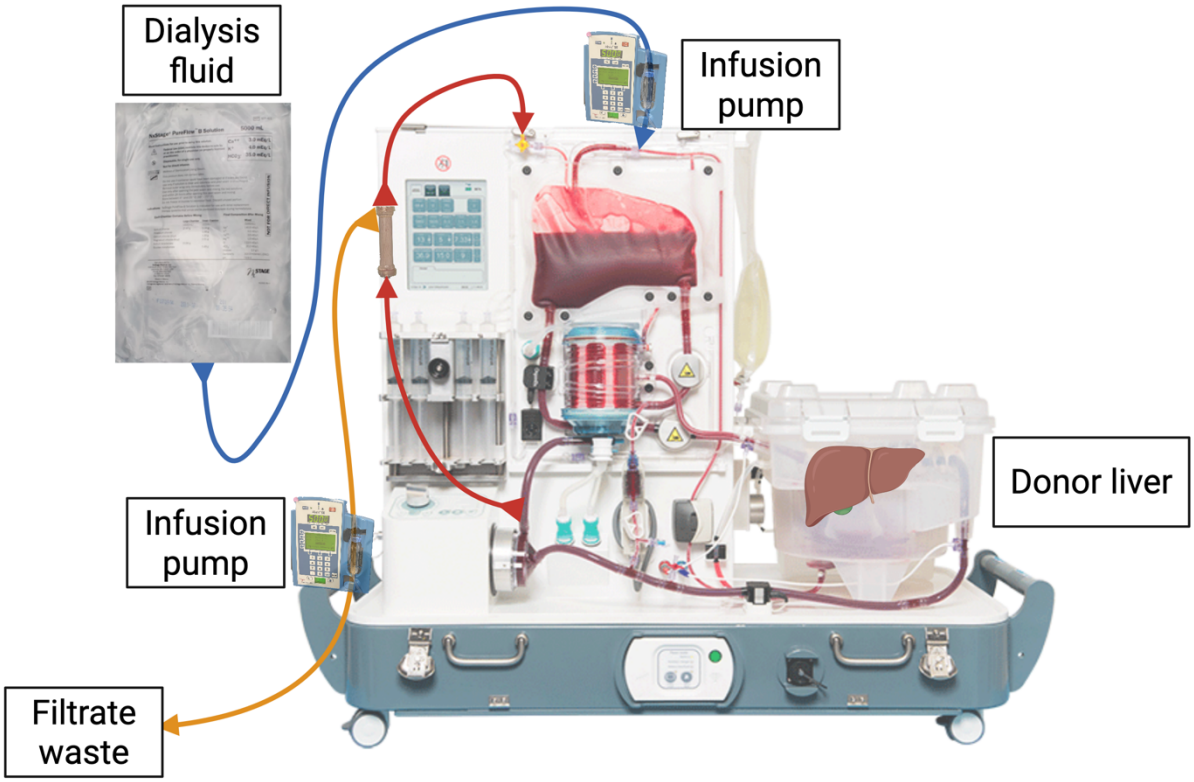


Figure 44: *metra* perfusion circuit modified to enable *ex situ* haemofiltration during prolonged perfusion. *Ex situ* haemofiltration was enabled by insertion of a tubing loop containing a DP03 paediatric haemoconcentrator (Medica, Modena, Italy) between centrifugal pump and portal reservoir, in parallel to the main perfusion circuit. Filtrate was drawn from the haemoconcentrator using an Alaris SE 7130 infusion pump (BD, Wokingham, UK), and discarded. Dialysate (NxStage PureFlow RFP-404) was infused into the portal reservoir using a second Alaris SE 7130 pump at a rate equal to the rate of filtrate removal.

6.3.4 48-hour xenoperfusion to assess xenograft viability (xenoperfusions 1 – 5 (part 1))

To assess xenograft viability, macroscopic appearance, bile production, and perfusate samples for iSTAT CG4+, Chem8+ cartridges, liver function tests (SST, IDEXX BioAnalytics (Grafton, MA, USA)), and complete blood count (EDTA, IDEXX BioAnalytics (Grafton, MA, USA)) were assessed pre-xenoperfusion and at 1, 2, 6, 12, 24, 36, and 48 hours during xenoperfusion. For xenoperfusions 4 and 5, 48-hour bile pH and bile glucose were tested using iSTAT CG4+ and Chem8+ cartridges. The *metra* recorded (every second): hepatic artery flow; portal vein flow; inferior vena cava flow; hepatic artery pressure; inferior vena cava pressure; and perfusate pO_2; pCO_2; pH using a shunt sensor (Terumo). Values are presented as mean ± standard deviation.

Urea production was calculated from perfusate urea concentration over time, taking account of urea clearance by *ex situ* haemofiltration. Glucose consumption was calculated from the perfusate glucose concentration over time, taking account of glucose administration by parenteral nutrition[4], dialysate infusion, and clearance by *ex situ* haemofiltration. Urea production and glucose consumption were calculated at 12, 24, 36, and 48 hours.

Perfusate insulin concentrations were measured pre-reperfusion (*n = 4*) and during the first 24 hours of xenoperfusion (at 1 (*n = 1*), 6 (*n = 2*), 12 (*n = 3*), and 24 (*n = 1*) hours) by ELISA (10-1113-01, Mercodia, Uppsala, Sweden (at eGenesis, Cambridge, MA, USA)). For reference, these are compared to fasting human serum insulin concentrations using samples from decedents 1 – 4 (*n = 9*, *chapter 7*).

Biopsies of the xenograft were taken at the end of back-table preparation (prior to starting xenoperfusion) and after 48 hours xenoperfusion. Pre-xenoperfusion biopsies were peripheral wedge biopsies. The 48-hour biopsies for xenoperfusions 1 – 3 were peripheral wedge biopsies, whilst those for xenoperfusions 4 and 5 were central biopsies. For xenoperfusion 4, central biopsies were taken from each lobe of the liver.

6.3.4.1 Xenograft cytochrome P450 activity (xenoperfusions 4 and 5)

During xenoperfusions 4 and 5, the cytochrome P450 (CYP450) activity of the xenograft was assessed by measuring clearance of midazolam, omeprazole, and metoprolol. In humans, these drugs are metabolised by the CYP450 enzymes CYP3A4, CYP2C19, and CYP2D6, respectively. This was performed 4 times for each of xenoperfusion 4 and 5, separated by at least 1 hour. Runs 1 and 2 were conducted between hours 6 and 8 of xenoperfusion; runs 3 and 4 were conducted between hours 24 and 28 of xenoperfusion. Immediately prior to drug administration, *ex situ* haemofiltration was paused and a baseline perfusate sample was taken from the inferior vena cava line. A cocktail containing the three drugs was administered to the inferior vena cava line. The amount of each drug administered to the circuit was as follows (xenoperfusion 4, 5): midazolam: 0.15 mg, 0.34 mg; omeprazole: 0.525 mg, 0.60 mg; metoprolol: 0.15 mg, 0.34 mg. Perfusate samples were taken from the inferior vena cava line at the following timepoints (minutes): 1, 2, 4, 8, 15 (xenoperfusion 5, additionally: 20, 30). *Ex situ* haemofiltration was resumed once sample collection was completed.

Frozen perfusate plasma aliquots were shipped on dry ice to an analytical lab (Meadowhawk Biolabs, Marlborough, MA, USA) for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of the parent drug concentrations in the perfusate over time, in order to determine the xenograft intrinsic clearance of each drug. Samples were processed using a protein precipitation extraction method. Liquid chromatography (LC) and mass spectrometry (MS) conditions, as well as internal standards, for the analysis are listed in *table 14*.

A curve of calibration standards for each drug was generated. High, mid, and low concentrations of each drug were run in triplicate against the curve for QC. Mean and standard deviation (SD) of the triplicates were used to determine percent coefficient of variance (%CV). The acceptance criterion was set at ± 20% CV; however, individual samples that fell outside of this were still included in the statistical analysis. Calibration standards and QC samples were run alongside test samples on a 96-well plate. An aliquot of the formulated drug cocktail from each pharmacokinetic run was analysed in triplicate to determine the percent of nominal concentration of each drug within the cocktail.

Intrinsic clearance of each drug by the xenograft (per kg liver tissue) was calculated using the half-life ($t_{1/2}$) method. Half-life was calculated from the elimination rate constant (k), based on the gradient of the linear regression trendline of the $\log_{10}$(perfusate IVC concentration) *versus* time plot for each

replicate run of each drug. The linear section of the elimination profile was determined by inspection. $t_{1/2}$ was calculated as follows: $k = gradient \times -2.3$; $t_{1/2} = \frac{0.693}{k}$.

The volume of distribution (V_d) (perfusate volume (1.5 L) + the vascular/extracellular space of the xenograft) was estimated as 1.7 L for xenoperfusion 4 and 1.8 L for xenoperfusion (the difference being due to differences in the masses of the xenografts). Clearance (Cl) of each drug was calculated using: $Cl\ (\mathrm{L/min}) = 0.693 \times \frac{V_d}{t_{1/2}}$.

The intrinsic clearance (Cl_{int}) of each drug was then estimated based on the equation (Q_{IVC}: inferior vena cava flow rate; f_u: total fraction of drug unbound in plasma of human whole blood (midazolam: 0.02; omeprazole: 0.05; metoprolol: 0.90[302])): $Cl_{int}\ (\mathrm{L/min}) = \frac{Q_{IVC} \times Cl}{Q_{IVC} \times f_u}$.

Cl_{int} was then normalised per kilogram xenograft mass.

LC Conditions:			Time (min)	% MPB
Column ID & Dimensions:	Acquity UPLC BEH C18 1.7µm; 2.1x50mm		Initial	15.0
Column Temperature (°C)	55		0.25	15.0
Flow Rate (mL/min):	0.800		1.25	45.0
Mobile Phase A:	100:0.1 Water: Formic Acid		1.30	95.0
Mobile Phase B:	100:0.1 Acetonitrile: Formic Acid		1.50	95.0
			1.55	15.0
			1.85	15.0
MS Conditions:				
MS/MS:	API 7500			
Ionization Method:	Electrospray			
Positive/Negative Ion:	Positive Ion			
Resolution	Unit			
Source Temperature (°C)	450			
Acquisition Parameters:		**Q1 (amu)**	**Q3 (amu)**	
Analyte(s):	Midazolam	326.1	291.1	
	Omeprazole	346.1	198.1	
	Metoprolol	268.2	116.1	
Internal Standard(s):	Midazolam-d4	330.2	295.1	
	Omeprazole-d3	349.1	198.0	
	Metoprolol-d6	274.3	122.2	

Table 14: liquid chromatography and mass spectrometry sample analysis conditions.
LC = liquid chromatography; min = minute; MPB = mobile phase B; MS = mass spectrometry.

6.3.5 Short-duration xenoperfusion to assess liver xenograft-associated thrombocytopenia (xenoperfusions 6 – 9 (part 2))

Xenoperfusions up to 6 hours were performed, under 3 different conditions: (i) control: standard normothermic liver perfusion on the *metra*, (ii) caplacizumab[303] pre-treatment: 22 mg caplacizumab (von-Willebrand factor (vWF) inhibitor) (Sanofi, Paris, France) administered to perfusate prior to xenograft reperfusion, and (iii) pegcetacoplan[304] pre-treatment: 1080 mg pegcetacoplan (complement C3 inhibitor) (Apellis Pharmaceuticals, Waltham, MA, USA) administered to perfusate prior to xenograft reperfusion. Perfusion viability was assessed in the same manner as xenoperfusions 1 – 5. Perfusate (EDTA) samples were taken for manual measurement of perfusate platelet count (IDEXX BioAnalytics (Grafton, MA, USA)) pre-xenoperfusion and at following timepoints (minutes): 5, 10,

15, 30, 45, 60, 120, 240. The absolute platelet count was haematocrit-corrected to account for haemodilution upon reperfusion due to washout of organ preservation solution and 5% albumin solution from the graft, and expressed as a percentage of the starting value. Perfusate samples were taken to measure levels of human and porcine vWF using ELISAs (human: ab223864, Abcam, Cambridge, UK; porcine: CSB-E09395p, Cusabio, Houston, TX, USA (at eGenesis, Cambridge, MA, USA)).

Xenograft biopsies were taken pre-reperfusion and throughout xenoperfusion for histology, immunohistochemistry, and electron microscopy analysis.

One wild-type porcine liver retrieved from an anaesthetised Large White x Landrace pig underwent standard normothermic perfusion on the *metra* with autologous whole blood (per *2.1.2, 2.2, 2.3*). Perfusate (EDTA) samples were taken for automated measurement of perfusate platelet count on the Sysmex XN20 pre-reperfusion and at the following timepoints (minutes): 20, 40, 60, 120, 240.

6.3.6 Histology, immunohistochemistry, and electron microscopy

In part 1, biopsies were formalin-fixed, paraffin-embedded, and stained with H&E. In part 2, biopsies were split for (i) formalin; (ii) electron microscopy. Formalin-fixed specimens were paraffin-embedded for: (i) H&E staining; (ii) immunohistochemistry: platelet markers (CD41, CD61, CD62p) and vWF (Applied Pathology Systems, Shrewsbury, MA, USA). Scanning and transmission electron microscopy were performed at University of Massachusetts, MA, USA.

6.3.6.1 Transmission electron microscopy

Liver tissue samples were fixed in 2.5% glutaraldehyde in cacodylate buffer (overnight at 4°C), washed three times in fresh 0.1 M cacodylate buffer and post fixed in 1% osmium tetroxide for 1 hour at room temperature. Following a second series of washes in fresh buffer, the liver tissue samples were dehydrated in a graded series of ethanol concentrations to 100% (2X), then transferred to propylene oxide (2X) and then into a 50:50 (v/v) of epoxy resin/propylene oxide and allowed to infiltrate overnight. The next day, the liver tissue samples were transferred through five changes of

fresh epoxy resin and finally placed in Beem capsule moulds and polymerised at 60°C for 48 hours. Once polymerised, the embedded liver samples were trimmed and sectioned. The sections were collected on copper support grids and further contrasted with lead citrate and uranyl acetate prior to imaging on a ThermoFisher/FEI Tecnai 12 G2, and the images were recorded using a Gatan RIO 9 digital camera system at 120 kV accelerating voltage.

6.3.6.2 Bulk frozen-fracture scanning electron microscopy

Liver tissue samples were fixed in 2.5% glutaraldehyde in cacodylate buffer (overnight at 4°C), washed three times in fresh 0.1 M cacodylate buffer and post fixed in 1% osmium tetroxide for 1 hour at room temperature. Following a second series of washes in fresh buffer, the liver tissue samples were dehydrated in a graded series of ethanol concentrations up to 95% ethanol. The partially dehydrated tissue was then quickly immersed in liquid nitrogen until the boiling ceased and placed on a pre-cooled steel block and fractured using a cooled knife. The fractured pieces were collected and thawed in 100% ethanol and critical point dried in a CO_2 CPD system (Autosamdri-815, Tousimis Research Corporation). Once dried, the pieces were mounted on aluminium stubs with silver conductive paste and sputter coated with Au/Pd (approximately 4 nm), (Cressington 208 HR Sputter Coater, Ted Pella) and examined on an ThermoFisher/FEI, Quanta 200 FESEM at 10 kV.

6.3.7 Statistical analysis

Outcomes are reported individually for each study. Where appropriate, parametric and non-parametric data are summarised using mean ± standard deviation and median (range), respectively. In part 1, there is no comparator group. In part 2, the sample size per group is too small to allow statistical comparison.

6.4 Results

6.4.1 48-hour xenoperfusion to assess xenograft viability (xenoperfusions 1 – 5 (part 1))

Five 48-hour xenoperfusions of transgenic porcine livers were performed with pooled human whole blood (3KO.7TG.RI: *n = 4*; 3KO.7TG: *n = 1*). Perfusion was electively terminated at 48 hours in all cases. The livers weighed 1.37 ± 0.44 kg. Cold ischaemic times were 77 ± 4 minutes.

The livers maintained homogeneous parenchymal perfusion with physiological hepatic artery (0.27 ± 0.11 L/minute) and portal vein (0.97 ± 0.04 L/minute) flows. Perfusate ALT levels remained low and within normothermic perfusion transplantability criteria[3] (*figure 45*).

The livers demonstrated hepatic function throughout xenoperfusion, with lactate clearance, glucose consumption, urea production, and bile production. Perfusate albumin level was maintained throughout perfusion (*figure 46*). For xenoperfusions 4 and 5, 48-hour bile chemistry was evaluated, which showed bile glucose < 1.1 mmol/L and bile pH 7.65 – 7.68.

Perfusate haematocrit decreased mildly during xenoperfusion with the 48-hour haematocrit 83 ± 16 % of the starting value (*figure 46*). A median of 1 unit (range: 0 – 2) of whole blood was administered to the circuit during perfusion to maintain the portal reservoir filling height. On one occasion (xenoperfusion 5) 1 unit of A-type packed red cells was inadvertently administered, resulting in acute haemolysis due to pre-existing anti-A antibodies in the O-type whole blood-based perfusate. Transient mottling of the liver was observed, which recovered within 6 hours. The perfusion dynamics and functional parameters of the liver were otherwise maintained, but the final perfusate haematocrit was the lowest of the series (62.2% of the starting value).

Ex situ haemofiltration maintained the perfusate potassium concentration close to the physiological range; severe hyperkalaemia was prevented (*figure 46*). The perfusate insulin concentration during xenoperfusion was supra-physiological, and substantially exceeded the fasting human serum level (*figure 47*).

Histological analysis demonstrated generally preserved microscopic appearance. Necrosis was absent-to-mild in all perfusions except one. However, intra-parenchymal haemorrhage was observed, which was usually mild and focal (*table 15*, *figure 48*).

Intrinsic xenograft clearance of midazolam was 16.14 ± 2.83 L/min/kg and 10.85 ± 2.24 L/min/kg for xenoperfusions 4 and 5, respectively. Intrinsic xenograft clearance of omeprazole was 2.36 ± 0.87

L/min/kg and 1.89 ± 0.76 L/min/kg for xenoperfusions 4 and 5, respectively. The concentration of metoprolol achieved in the perfusate exceeded the lower limit of quantification in only one run of xenoperfusion 4; it was therefore not possible to reliably calculate intrinsic xenograft clearance of metoprolol in this study (*figure 49*).

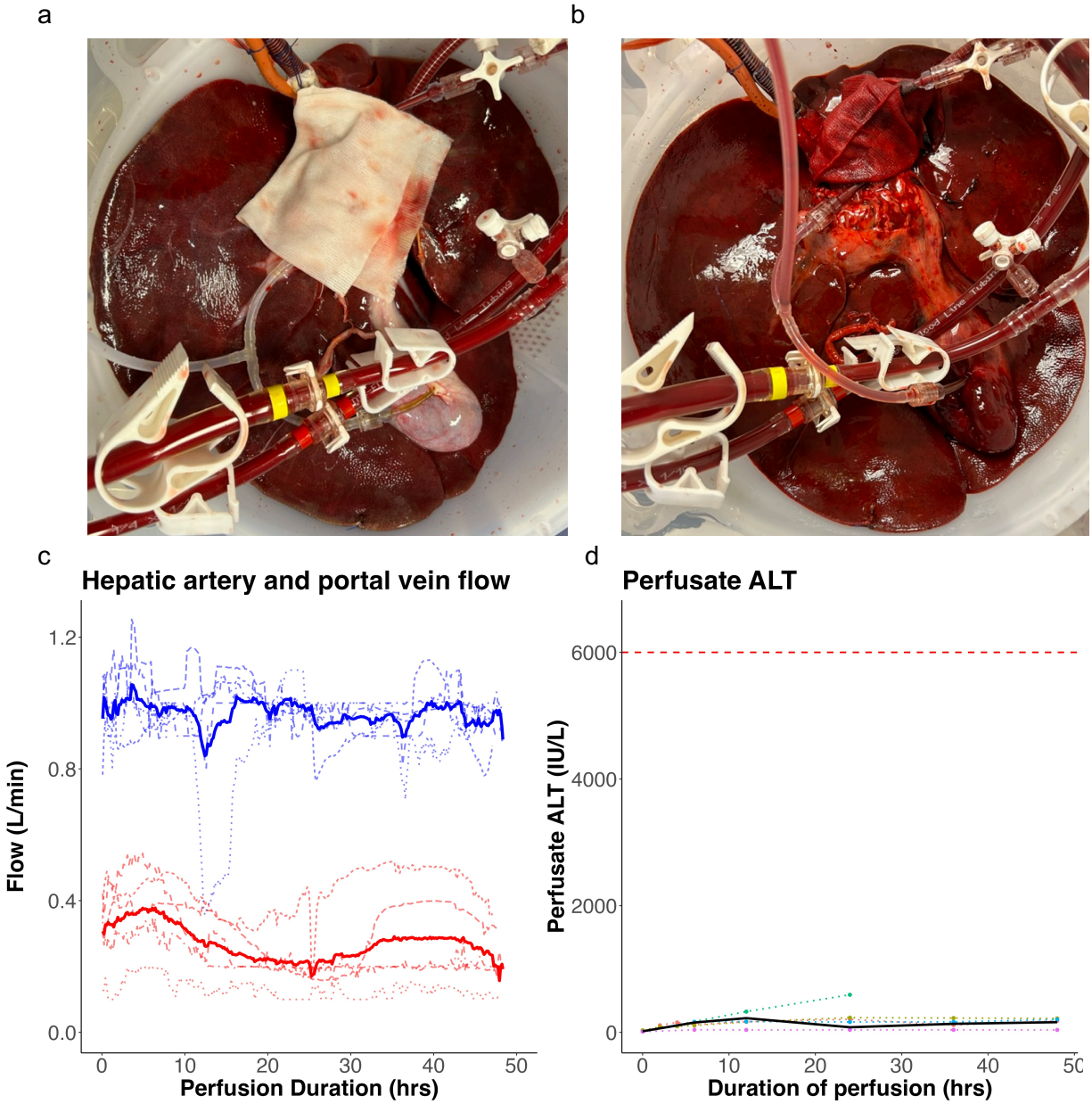


Figure 45: 48-hour xenoperfusion of transgenic porcine livers with human whole blood: perfusion parameters (xenoperfusions 1 – 5 (part 1)).
a: macroscopic appearance of xenograft immediately post-reperfusion. b: macroscopic appearance of xenograft after 48 hours xenoperfusion. c: xenograft hepatic artery (red) and portal vein (blue) flows during xenoperfusion. d: perfusate alanine transaminase (ALT) levels during xenoperfusion. Horizontal red dashed line is the threshold for liver transplantability (6,000 IU/L), per the Watson et al. normothermic perfusion criteria[3]. Dashed lines: individual xenoperfusions; solid line: mean values. Mean and individual studies presented.

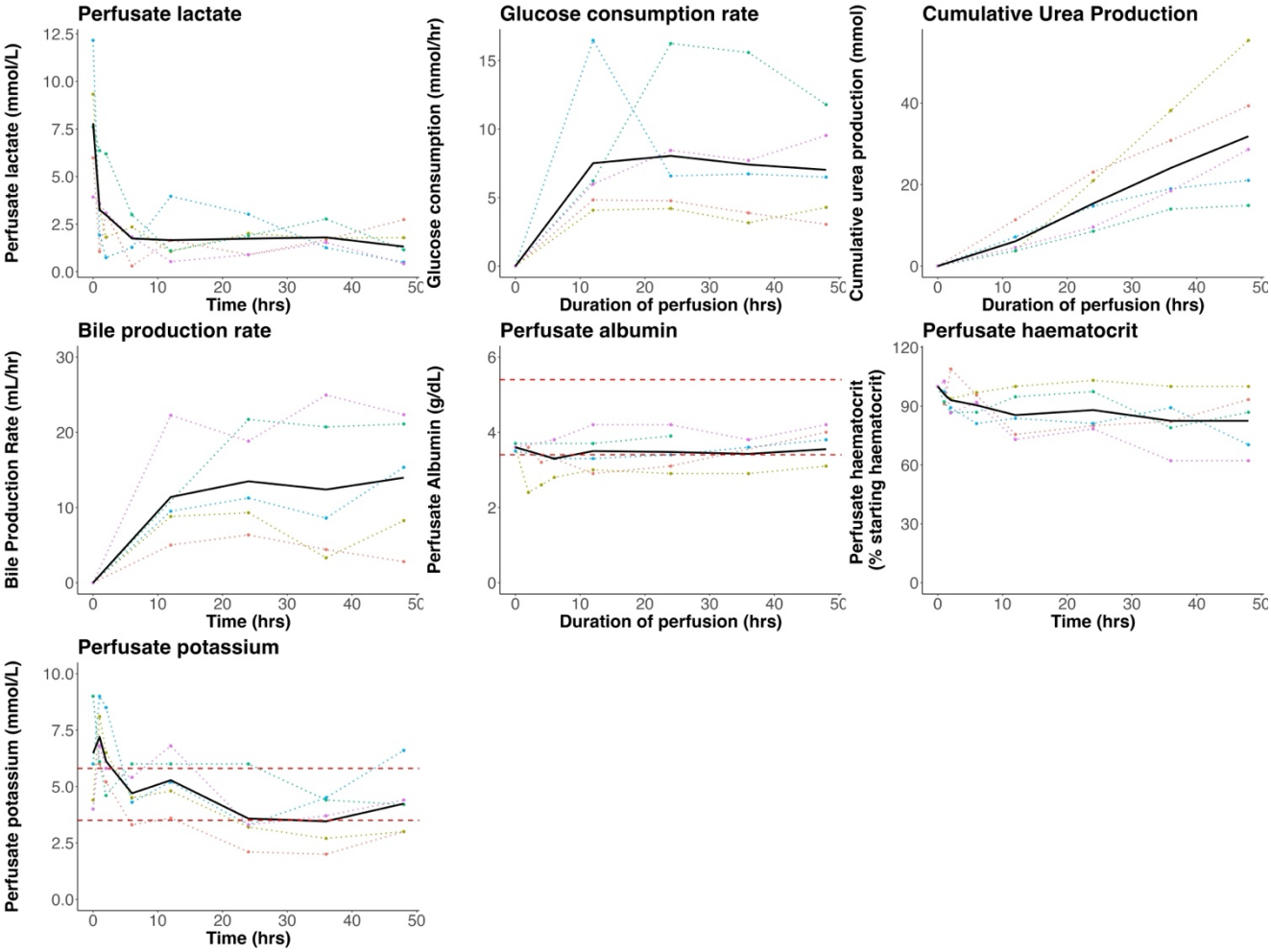


Figure 46: 48-hour xenoperfusion of transgenic porcine livers with human whole blood: perfusate parameters (xenoperfusions 1 – 5 (part 1)).
Perfusate lactate during xenoperfusion. Glucose consumption rate by xenograft during xenoperfusion. Cumulative urea produced by xenograft during xenoperfusion. Bile production rate by xenograft during xenoperfusion. Perfusate albumin during xenoperfusion. Perfusate haematocrit (percentage of starting haematocrit) during xenoperfusion. Perfusate potassium during xenoperfusion. Dashed lines: individual xenoperfusions; solid black line: mean values. Horizontal red dashed lines: physiological range of albumin and potassium in human blood. Mean and individual studies presented.

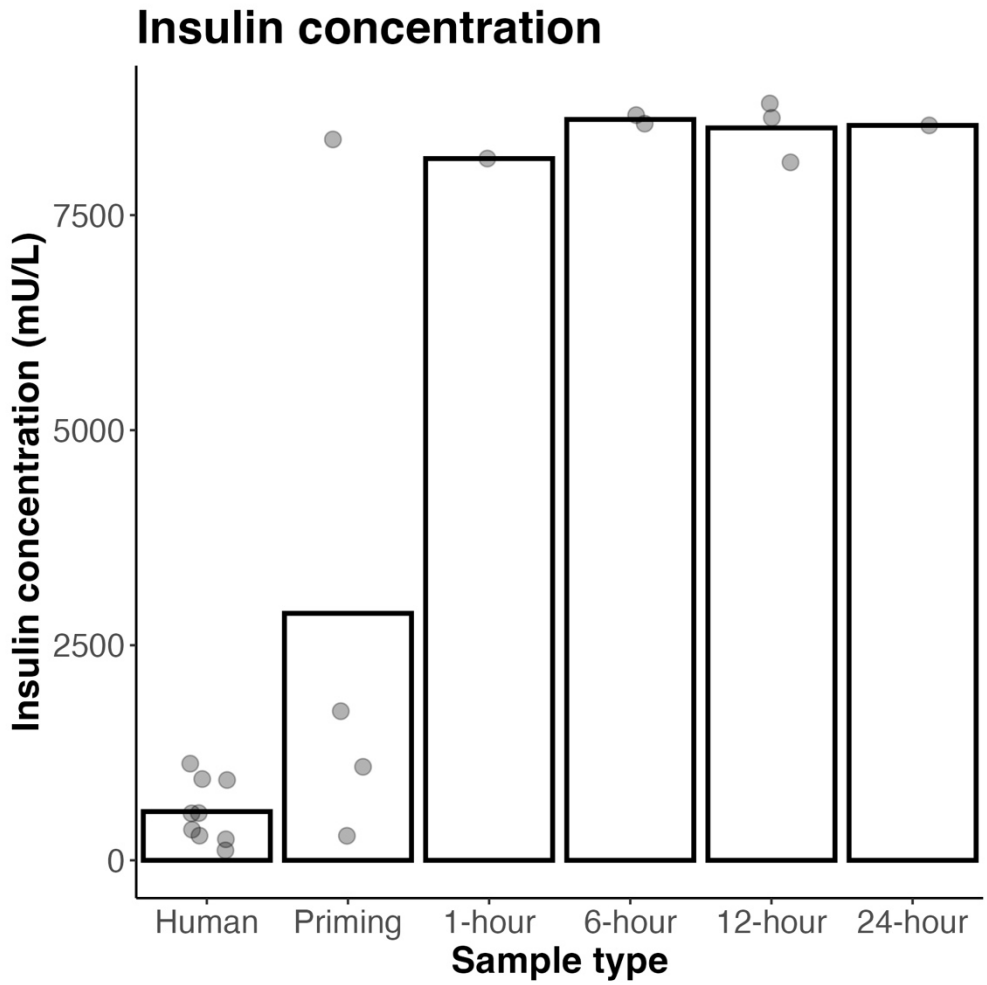


Figure 47: perfusate insulin concentration during xenoperfusion.
Pre-reperfusion ('priming') and over the first 24 hours of xenoperfusion using standard *metra* insulin dosing[4] ('1-, 6-, 12-, 24-hour'), compared to fasting human serum insulin concentration (decedents 1 – 4 (*chapter 7*)). Mean and individual studies presented.

Xenoperfusion (lobe)	Timepoint (hours)	Hepatocyte necrosis	Congestion/haemorrhage	Inflammatory infiltrate
1	**0**	0	0	0
	48	0	2m	0
2	**0**	0	0	0
	48	3m	3e	0
3	**0**	0	0	1m
	48	2m	2m	0
4	**0**	0	0	0
Left medial	**48**	0	2m	0
Left lateral	**48**	0	2m	0
Right medial	**48**	3	4m	0
Right lateral	**48**	1	2m	0
Caudate	**48**	0	2m	0
5	**0**	0	0	0
	48	0	0	0

Table 15: histological analysis of haematoxylin and eosin-stained biopsies from pre-xenoperfusion (end of back-table preparation) and after 48 hours of perfusion for xenoperfusions 1 – 5 (part 1).
Pre-xenoperfusion biopsies were peripheral wedge biopsies. 48-hour biopsies were peripheral wedge biopsies for xenoperfusions 1 – 3, and central biopsies for xenoperfusions 4 and 5. For xenoperfusion 4, a central biopsy was taken from each lobe. Severity: 0: absent; 1: minimal; 2: mild; 3: moderate; 4: marked; 5: severe. Distribution: f: focal; m: multifocal; d: diffuse; e: locally extensive; w: widespread.

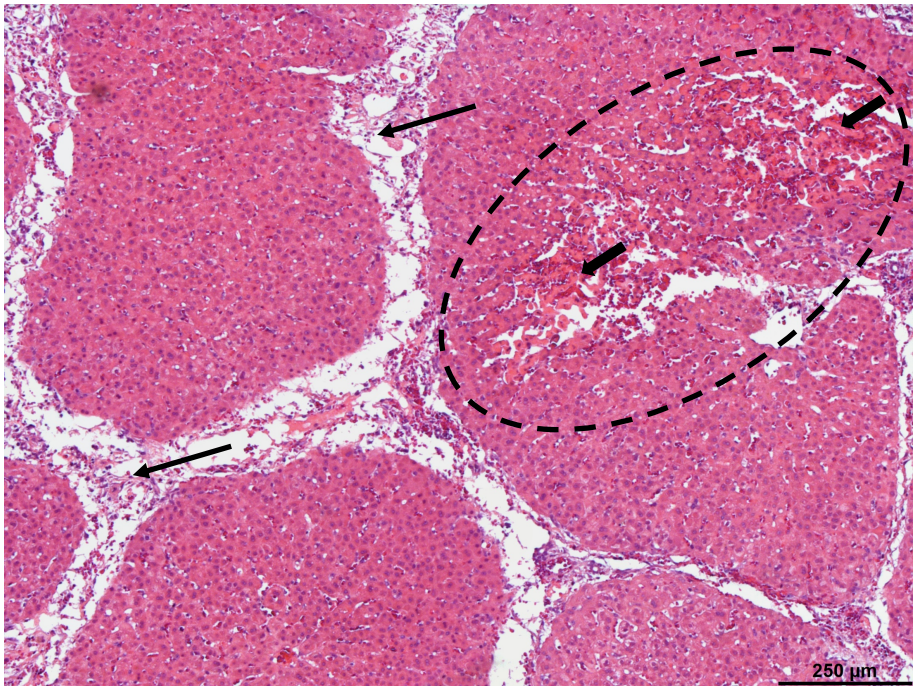


Figure 48: 48-hour peripheral wedge biopsy from xenoperfusion 3 demonstrating focus of congestion/haemorrhage (dashed circle), hepatocyte necrosis (thick arrows), and widening of inter-lobular septa (thin arrows).

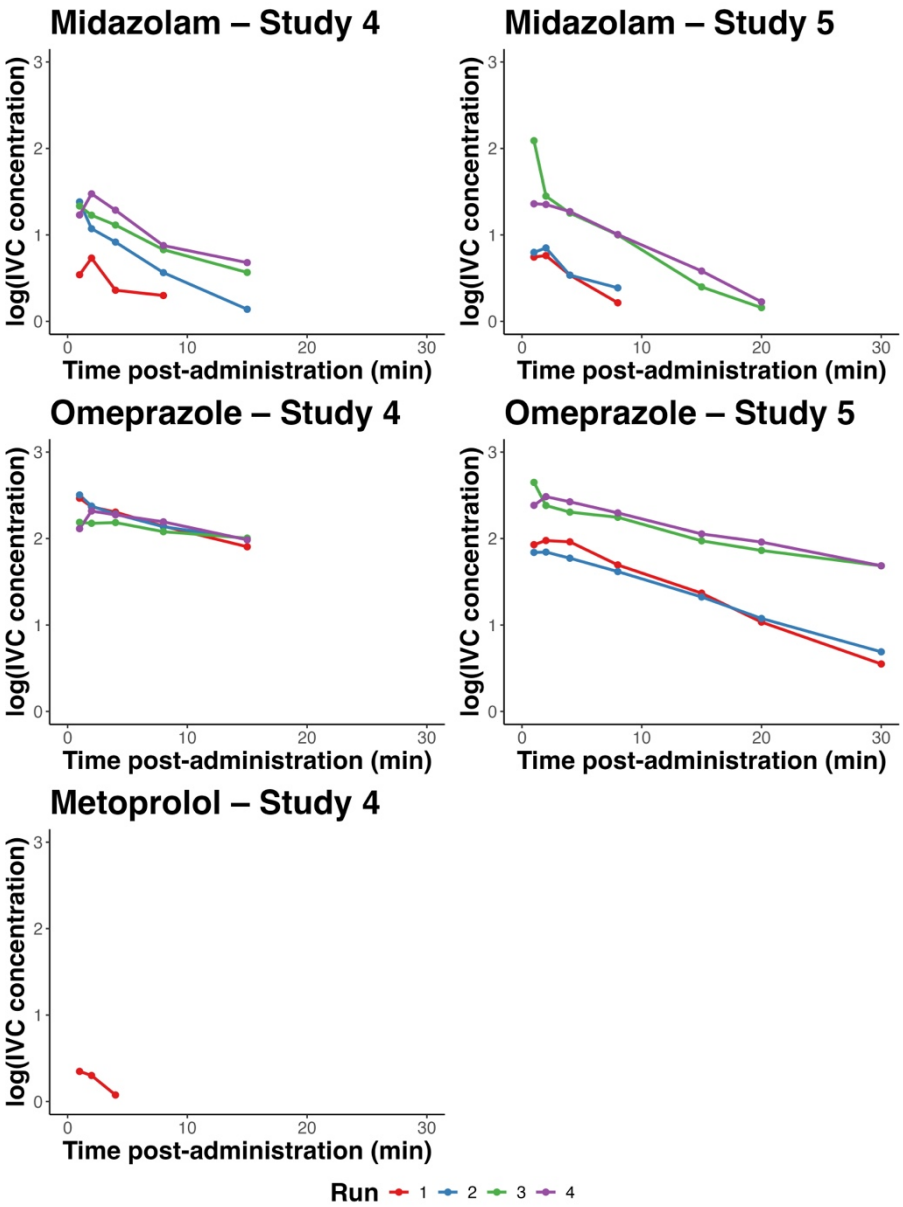


Figure 49: clearance of midazolam, omeprazole, and metoprolol administered to perfusate during xenoperfusions 4 and 5 to assess activity of xenograft cytochrome P450 enzymes CYP3A4, CYP2C19, and CYP2D6, respectively.
Log_{10}(perfusate IVC concentration (ng/mL)) plotted against time post-administration for each drug. Perfusate concentrations of metoprolol within the range of detection of the assay were only achieved during run 4 of xenoperfusion 4. IVC: inferior vena cava. Individual runs presented.

6.4.2 Short-duration xenoperfusion to assess liver xenograft-associated thrombocytopenia (xenoperfusions 6 – 9 (part 2))

Four short-duration (4 – 6 hour) xenoperfusions of 3KO.7TG.RI porcine livers were performed with pooled human whole blood. The livers weighed 1.21 ± 0.18 kg. All livers demonstrated perfusion viability, assessed according to: hepatic artery flow, portal vein flow; macroscopic and microscopic appearance; lactate clearance, perfusate ALT, perfusate pH, bile production, bile glucose, and bile pH[3] (*table 16*).

Control and pegcetacoplan pre-treated xenoperfusions demonstrated a rapid, profound decline in perfusate platelet count. Caplacizumab pre-treatment delayed the onset of thrombocytopenia, demonstrating a similar rate of decline to wild-type pig liver perfusion with autologous blood (*figure 50*).

In all xenoperfusions, prior to commencing normothermic perfusion, immunohistochemistry demonstrated that endothelial expression of vWF was limited to the endothelium of the central and portal veins, with no sinusoidal endothelial vWF expression; no platelets were present within the hepatic sinusoids. Within 15 minutes, widespread sinusoidal endothelial vWF expression was observed and there was evidence of platelet aggregation within hepatic sinusoids. This progressed over the first hour (*figure 51, table 17*). The perfusate human vWF concentration decreased one-hour post-reperfusion, whilst the perfusate porcine vWF concentration increased (albeit to a lesser degree) (*figure 51*).

In all xenoperfusions, scanning electron microscopy demonstrated aggregation of activated platelets on the sinusoidal endothelium after 15 minutes (*figure 51*). Transmission electron microscopy showed aggregation of masses of platelets within the sinusoids, associated with clot material (*figure 51*). No evidence of platelet phagocytosis by Kupffer cells or sinusoidal endothelial cells was observed.

Xenoperfusion	2-hr lactate (mmol/L)	2-hr pH	4-hr ALT	Bile pH	Bile glucose (mmol/L)	Bile production (mL/hr)	HA mean flow (L/min)	PV mean flow (L/min)
6 (vWFi)	2.25*	7.24	266	N/A	N/A	9	0.18	0.71
7 (vWFi)	1.28*	7.32	80	>8.2	<1.1	15	0.29	1.02
8 (C3i)	2.77	7.22	67	8.16	<1.1	15	0.23	0.87
9 (control)	2.29	7.22	65	8.08	<1.1	13	0.32	1.01

Table 16: perfusion viability criteria for xenoperfusions 6 – 9 (part 2).
Viability assessed according Watson et al. normothermic liver perfusion transplantability criteria[3]. Bile chemistry not assessed for xenoperfusion 6. vWFi: perfusate pre-treated with 22 mg caplacizumab. C3i: perfusate pre-treated with 1080 mg pegcetacoplan. ALT: alanine aminotransaminase. HA: hepatic artery. PV: portal vein. *interpolated values.

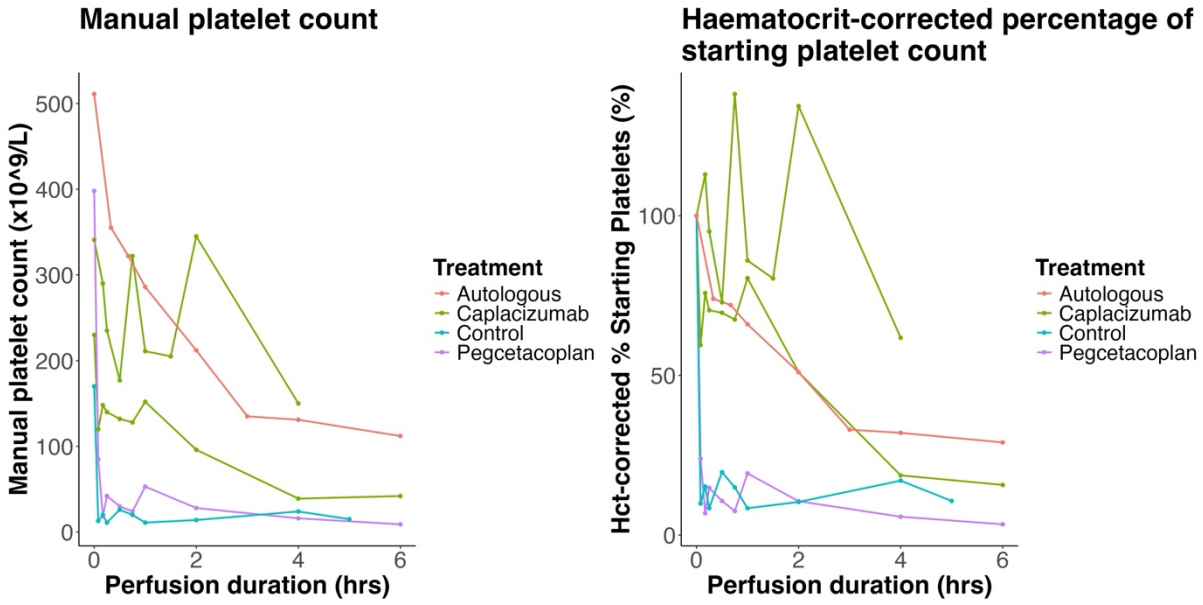


Figure 50: perfusate platelet count during short-duration normothermic perfusion under different conditions to assess mechanism of xenograft-associated thrombocytopenia (xenoperfusions 6 – 9; autologous perfusion 1 (part 2)).
Left: absolute manual platelet count during xenoperfusion. Right: manual platelet count expressed as a percentage of the starting platelet count and corrected for haemodilution upon initial reperfusion. Perfusate platelet count during autologous perfusion was automated. Individual studies presented.

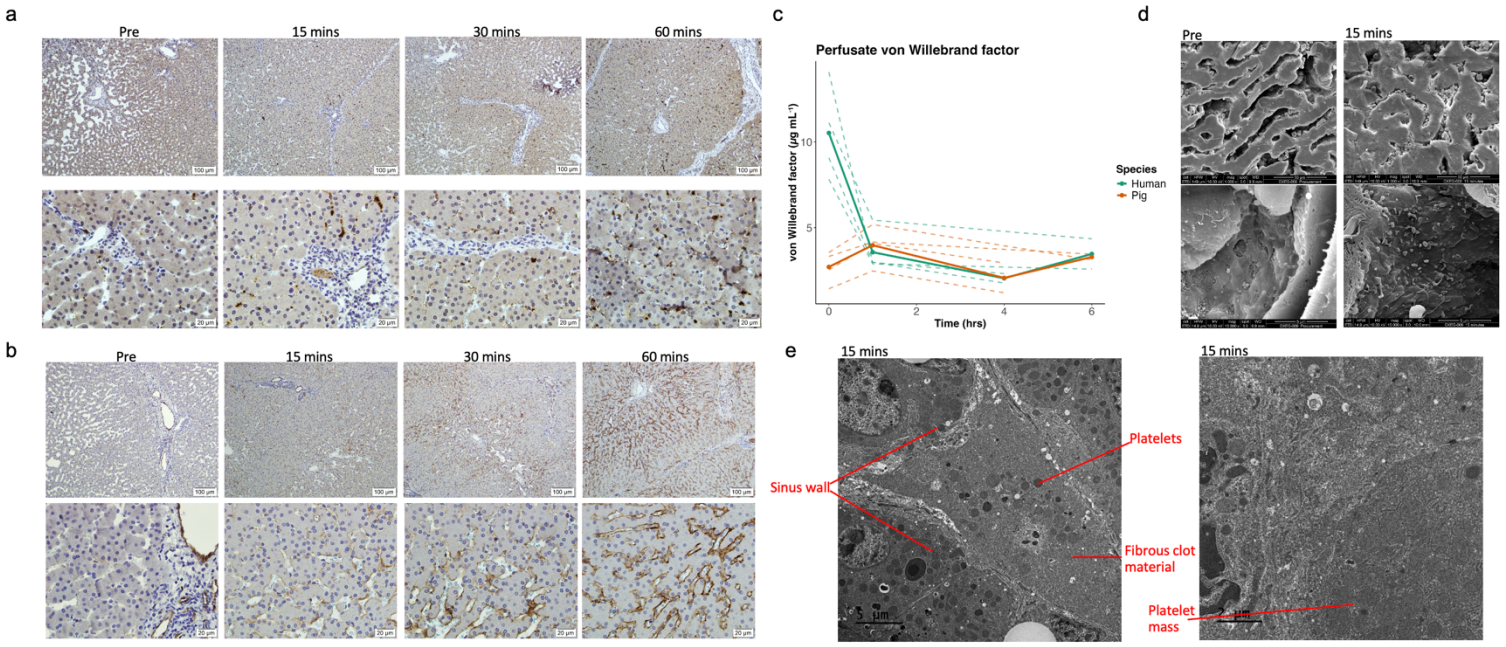


Figure 51: xenograft immunohistochemistry, electron microscopy, and perfusate von Willebrand factor (vWF) levels during short-duration xenoperfusion.
a: xenograft CD41 (platelet) staining on peripheral wedge biopsies taken pre-reperfusion and at 15, 30, and 60 minutes post-reperfusion with human whole blood (xenoperfusion 9 (control)). b: xenograft vWF staining on peripheral wedge biopsies taken pre-reperfusion and at 15, 30, and 60 minutes post-reperfusion with human whole blood (xenoperfusion 9 (control)). c: perfusate vWF levels during xenoperfusions 6 – 9 measured using anti-human and anti-porcine vWF antibody-based ELISAs (mean, individual studies; pre & 1-hour: $n = 4$, 4-hour: $n = 2$, 6-hour: $n = 2$)). d: scanning electron microscopy images of xenograft biopsies taken pre-reperfusion and 15 minutes post-reperfusion demonstrating platelet aggregation on the sinusoidal endothelial wall (xenoperfusion 9 (control)). e: transmission electron microscopy images of xenograft biopsies taken 15 minutes post-reperfusion demonstrating aggregation of platelets within the sinusoid associated with clot material (*left*) and as a mass of platelets (*right*) (xenoperfusion 9 (control)).

Xenoperfusion	Time (mins)	CD41	CD61	CD62p	vWF
6 (vWFi)	**0**	0	1	0	0
	10	1	1	0	2
	15	1	1	0	1
	30	2	1	1	2
	45	3	2	2	4
	60	3	2	2	4
	120	4	2	2	4
	240	5	3	3	3
7 (vWFi)	**0**	1	1	0	0
	60	3	2	2	4
	240	3	2	2	4
8 (C3i)	**0**	1	1	0	0
	15	4	3	3	5
	60	5	4	3	5
	240	5	4	3	5
9 (control)	**0**	1	1	0	0
	15	2	1	0	2
	30	2	1	1	3
	60	2	1	1	4
	120	2	1	1	5
	240	2	1	1	5

Table 17: immunohistochemical analysis of thrombocytopenia-related markers from xenograft biopsies (xenoperfusions 6 – 9 (part 2)).
Scoring: CD41, CD61, CD62p: 0: absent; 1: minimal (< 5% cell/area or faint staining); 2: mild (6 – 25% cells stained); 3: moderate (26 – 50% stained); 4: marked (51 – 75% stained); 5: severe (diffusely stained in > 75% cells or area). vWF: same scale, based specifically on sinusoidal staining (all portal and central vein endothelial cells stained positive). vWF: von Willebrand factor. vWFi: caplacizumab pre-treatment. C3i: pegcetacoplan pre-treatment.

6.5 Discussion

This study evaluated *ex situ* isolated whole blood xenoperfusion of porcine livers with triple glycan knock-out, seven human transgene insertion ± porcine endogenous retrovirus inactivation. Viable xenoperfusion was maintained until elective termination at 48 hours in all five livers, with evidence of preserved hepatic metabolic, detoxification, and excretory function. Significant erythrophagocytosis of human red cells by the pig livers was not observed. The pig livers demonstrated evidence of CYP450 activity comparable to human CYP3A4 and CYP2C19. An additional four short-duration xenoperfusions suggested that thrombocytopenia associated with porcine liver xenografting may be vWF-mediated.

48-hour xenoperfusion is substantially longer than recent whole blood isolated xenoperfusion studies using porcine livers with multiple gene edits. Pig livers with single (α-Gal) glycan knockout and five human transgene (h*PROCR*.h*THBD*.h*HMOX1*.h*CD46*.h*CD47*) insertion were perfused for 856 ± 61 minutes[305]; double (α-Gal; Neu5Gc) glycan knockout with *CD46* transgene insertion pig livers were perfused for 297 ± 77 minutes[306]; and α-Gal knockout.h*CD46* pig livers with humanised vWF were perfused up to 12 hours[307]. This contrasts with reports of longer isolated xenoperfusions up to 72 hours of wild-type[308] and single genetic edit (h*CD55*) pig livers in the past[309-311]. Our study refutes the suggestion that multiple gene edits could increase pig liver susceptibility to ischaemia-reperfusion injury or, counter-intuitively, reduce compatibility with human xenoperfusion.

Although prolonged isolated xenoperfusion of wild-type and single gene-edited pig livers has been reported, these studies demonstrated significant erythrophagocytosis of human red cells by the xenografts. The 48-hour haematocrit in these studies was 40 – 55% of the starting values[308-311], with the 72-hour haematocrit in one study 2% of the starting value[308]. The hypothesised mechanism is that sialoadhesin (a lectin) on porcine Kupffer cells binds N-acetylneuraminic acid (Neu5Ac) on human red cells, followed by phagocytosis[308,311]. Pig red blood cells express Neu5Gc rather than Neu5Ac. CMAH functional inactivation, and the resultant Neu5Gc knock-out, mean that such transgenic pig red cells express Neu5Ac (like humans). This has been associated with reduced erythrophagocytosis in isolated xenoperfusions up to 8 hours[306]. In our study using triple (including Neu5Gc) glycan knock-out pig livers, the mean 48-hour haematocrit was 83% of the starting value. This supports the proposed mechanism and extends evidence of the protective effect of Neu5Gc knock-out to perfusions up to 48 hours. This effect cannot be tested in non-human Old World primate xenotransplant studies because, like pigs, their red cells express Neu5Gc[33,174].

The liver metabolises a range of drugs and toxins via the CYP450 enzyme system. This experiment demonstrates that Yucatan pig livers possess CYP450 activity comparable to human CYP3A4 and CYP2C19. The intrinsic hepatic clearances of midazolam and omeprazole by the xenografts (midazolam: 10.85 – 16.14 L/min/kg liver mass; omeprazole: 1.89 – 2.36 L/min/kg liver mass) were similar to equivalent human values experimentally determined in microsome and hepatocyte-based

assays[312] (midazolam: 6.3 – 17.5 L/min/kg liver mass; omeprazole: 0.8 – 1.9 L/min/kg liver mass (assuming an average human liver mass of 1.5 kg[313])). Whilst there was evidence of clearance of metoprolol (CYP2D6), the rate could not be reliably calculated in this study. 30% of clinical drugs are metabolised by CYP3A4/5 and 7% are metabolised by CYP2C19. A further 20% are metabolised by CYP2D6[314]. Although it is reassuring that the xenografts demonstrate CYP450 activity comparable to a human liver, it is not possible to directly extrapolate quantitative pharmacokinetics from this study: during *ex situ* isolated xenoperfusion, livers lack the usual neuro-hormonal stimuli that influence CYP450 activity. Careful titration ± monitoring of drug levels will be required for drugs with a narrow therapeutic index when administered in the presence of a xenograft (particularly given the variable contribution by the native liver in the case of ELC or auxiliary transplantation). Furthermore, this study measured disappearance of the parent drug but did not characterise the metabolites generated. The detailed pathways by which xenografts metabolise common human drugs remain to be determined.

Using the standard *metra* insulin dosing regimen[4] during normothermic perfusion resulted in supra-physiological concentrations of insulin in the perfusate. This is not unexpected as the standard infusion rate is 6.67 IU actrapid per hour and this is the rationale for reducing the insulin infusion dose for steatotic liver perfusion in the DeFat trial[315]. In this study, despite additional insulin clearance by *ex situ* haemofiltration (see *chapter 3*), the mean perfusate insulin concentration (8,500 mU/L) substantially exceeded the mean fasting human serum concentration (570 mU/L). In the experiment described in *chapter 3*, the mean perfusate insulin level during normothermic perfusion of slaughterhouse pig livers with *ex situ* haemofiltration was similar (5,250 mU/L). In the context of ELC, after a period of isolated normothermic perfusion, the perfusate is infused into the recipient: 1.5 L of perfusate is typically infused over the first 20 minutes. Excessive insulin administration could induce hypoglycaemia. The perfusate insulin load becomes diluted in the total blood volume of the system (i.e. recipient blood volume (5 L) + perfusate volume (1.5L)): a perfusate insulin concentration of 8,500 mU/L (total amount = 1.5 x 8,500 mU/L = 12,750 mU/L) would be expected to increment the overall effective blood concentration of the recipient by about 2,000 mU/L over 20 minutes. To avoid

excessive insulin load, the insulin infusion dose was reduced in the decedent study (see *7.3.5, 7.4.1, 7.5*).

Porcine liver xenografts have been consistently associated with rapid-onset, severe thrombocytopenia in non-human primate studies[199,200,202,203]. This was also observed in the recent case report of heterotopic auxiliary xenotransplant in the human decedent model[205]. In our control xenoperfusion study, rapid clearance of circulating platelets occurred within the first 5 minutes. Various mechanisms have been postulated:

i. Increased O-linked glycosylation of porcine vWF results in constitutive, rather than stress-induced, interaction with glycoprotein Ib-IX-V receptor (GP1bR) on human platelets[316]. Addition of the humanised vWF genetic edit to Gal-KO.h*CD46* pig livers delayed onset of thrombocytopenia during isolated *ex situ* xenoperfusion[307].

ii. Porcine liver sinusoidal endothelial cells phagocytose human platelets *in vitro*[317], which may be mediated by endothelial asialoglycoprotein receptor-1 (ASGR-1) binding galactose β1-4 N-acetyl glucosamine. The latter is more highly expressed on human than porcine platelets[318]. Uptake of human platelets by porcine sinusoidal endothelial cells occurs within 15 – 30 minutes in a Kreb's solution-based perfusion model[319]. In this model, genetic modification to knock-out ASGR-1 delayed platelet clearance[320].

iii. Porcine Kupffer cells phagocytose human platelets *in vitro*, which may be mediated by MAC-1 binding β-N-acetyl d glucosamine – also more highly expressed on human compared to porcine platelets[318,321].

iv. Human platelets contain α-granules that contain IgG and IgM against major porcine glycans (α-Gal, Neu5Gc, SDa). *In vitro* experiments suggest that α-granule-derived antibodies may bind wild-type pig endothelial cells, which could then tether human platelets via their Fc receptors[322]. However, the results of *chapters 6* and *7* suggest that a potential implication of this (that triple glycan knock-out would reduce xenograft-associated thrombocytopenia[322]) appears not to be the case.

The findings of this study provide tentative support for the hypothesised vWF-mediated mechanism: liver sinusoidal endothelial cells, activated by ischaemia-reperfusion injury, express (porcine) vWF, which binds human platelet GP1bR more avidly than human vWF, triggering platelet aggregation. This appears to be attenuated by pre-treatment with caplacizumab – a humanised, bivalent variable-domain-only immunoglobulin fragment against the A1 (GP1bR-binding) domain of vWF, which is used clinically as a therapy for acquired thrombotic thrombocytopenic purpura (TTP)[303]. Caplacizumab is known to interact with porcine vWF[323]. On the assumption that it has a lower affinity for porcine than human vWF, a perfusate caplacizumab concentration in excess of the plasma concentration achieved by the clinical TTP regimen (11 mg daily) was targeted. It is not certain whether the delayed platelet clearance observed in the caplacizumab-treated perfusions is because the phenomenon is driven by vWF molecular incompatibility, or because caplacizumab inhibits platelet aggregation downstream of the primary trigger. The immunohistochemistry in our analysis does not distinguish whether vWF expressed on the sinusoidal wall is derived from the pig liver or human plasma/platelets. Within the first hour of xenoperfusion, the perfusate concentration of human vWF decreased (possibly due to consumption within platelet aggregates), whilst the perfusate concentration of porcine vWF increased (potentially due to release by xenograft endothelial cells). The immunohistochemical and electron microscopic images appeared similar between the different xenoperfusion treatment groups, possibly explained by the challenge of differentiating quantitative differences using these methods. Immunohistochemical and electron microscopic analysis of the autologous wild-type pig liver perfusion were not available for comparison.

The xenoperfusion model is an *ex situ* model in which a porcine liver is exposed to human whole blood. However, the rest of human physiology is absent. The lack of normal homeostatic mechanisms (e.g. electrolyte management; hormonal control) renders physiological maintenance of an organ more challenging *ex situ* than *in vivo*. Absence of intact immune (e.g. secondary lymphoid tissue) and haematological/coagulation (e.g. spleen) systems limits extrapolation to *in vivo* xenografting. Platelet activation by exposure to the artificial surfaces and high shear stress associated with an extra-corporeal perfusion system complicates interpretation of the thrombocytopenia mechanism[280]. Part 2 of this study was an exploratory pilot limited by a small sample size: the goal was to screen potential

thrombocytopenia mechanisms, with a constrained number of transgenic porcine donors, to guide therapeutic testing in the decedent studies (*chapter 7*). The findings require validation in larger numbers and *in vivo*.

In conclusion, this study demonstrates that 3KO.7TG.RI transgenic porcine livers can be viably perfused with human whole blood for 48 hours, with maintenance of xenograft metabolic function. Adaptation of this perfusion system to enable extra-corporeal cross-circulation using xenografts may provide temporary hepatic support for patients with ALF or ACLF. Several factors remain to be fully characterised – in particular, the mechanism of xenograft-associated thrombocytopenia, the immune response to the xenograft, and physiological compatibility in a human. These must be assessed *in vivo*: the decedent model offers a potential pathway between *ex situ* xenoperfusion and clinical translation.

6.6 Declaration

I designed and ran the study; performed the liver perfusions, sample collection, and point-of-care testing (with support from the Transplant Research Fellows and OrganOx clinical specialists included under *Acknowledgements*); data analysis; and interpretation.

Recipient pig surgery was performed by Professor Peter Friend and the Transplant Research Fellows. Recipient pig anaesthesia was performed by the veterinary staff at eGenesis-WI. Laboratory, histological, immunohistochemical, and electron microscopy analyses were performed in the USA, as specified in the Methods section.

7 Extra-corporeal liver cross-circulation using transgenic porcine xenografts in the decedent model

7.1 Background

As described in *chapter 1*, pig kidney and heart to non-human primate xenotransplant studies over the past three decades have resulted in meaningful prolongation of xenograft survival due, in particular, to advances in genetic engineering of porcine donors, immunosuppression regimens, and understanding of xenobiology[162,324-327]. However, findings in the non-human primate model have not translated directly to clinical xenotransplants: replication of the xenograft edits, immunosuppression, and organ preservation technique from a non-human primate cardiac xenotransplant study with 100% 8-month survival[328] was associated with less than 2-month survival in two expanded-access clinical cases[195]. Though partly related to the pre-transplant clinical status of the recipients, several aspects limit translation from non-human primate to clinical xenotransplant.

Genetic edits of porcine donors are designed to increase compatibility with humans rather than non-human primates. Knock-out of CMAH to prevent pig organs expressing Neu5Gc avoids pre-formed antibodies in humans (who lack CMAH). By contrast, this may increase immunogenicity in non-human primates (e.g. baboons, macaques) that express CMAH[*174*]. Likewise, insertion of human-specific complement, coagulation, and immune regulatory genes will be less effective in non-human primate recipients[327]. Clinically-approved therapies in humans may either be ineffective (e.g. complement inhibitors) or not feasible (e.g. plasma exchange) in the non-human primate model, whilst the drugs used in non-human primate studies may not be clinically approved or effective (e.g. non-human primate-specific anti-CD40, anti-CD154, anti-CD28[329,330] monoclonal antibodies)[301].

The decedent model offers a potential pathway between non-human primate and clinical xenotransplant studies: human-specific genetic edits are tested in the context of human immunology and physiology; clinically-approved drugs and therapies can be used; investigational drugs can be trialled in the setting of human pharmacokinetics; frequent, in-depth sampling (beyond what is possible in a living recipient) facilitates detailed analysis of, for example, the xeno-immune response[301].

The decedent model tests interventions in humans who have been declared dead by neurologic criteria and ruled out as potential organ donors[185,186]; informed family consent is provided for whole body donation for research[301]. The decedent is maintained in an intensive care unit or operating theatre. The duration of the decedent study should be the shortest that will answer the intended scientific question. This process has been developed in discussion with ethicists, legal experts, religious leaders, and the transplant community (including transplant professionals and patient groups)[301,331]. The FDA and institutional review boards do not oversee decedent studies; their purview is limited to living humans in clinical trials. Instead, a decedent study-specific oversight committee reviews the protocol and monitors the study[301].

There are several ethical considerations related to the decedent model. Consent is provided by a proxy (next-of-kin) rather than the individual. This contrasts with, for example, whole-body donation for medical education, in which consent is typically provided by the individual. The informed consent process must be robust with clear, explicit communication; adequate time to consider the decision; and without duress (financial or emotional). The decedent model relies on societal trust of the researchers, clinicians, and institutions involved. This demands rigorous oversight, transparency, communications of results, respectful treatment of those involved, and protection against exploitation. The use of the model must be proportionate to the potential for scientific and medical advancement.

The pathophysiology of brain-death is an important component of the decedent model. Rapidly increasing intra-cranial pressure can induce haemodynamic instability and myocardial depression[332], although this is frequently transient[333]. Hypothermia, vasoplegia, central diabetes insipidus, disseminated intravascular coagulation, hypothalamic-pituitary axis dysregulation, hyperglycaemia, and a systemic inflammatory state may occur; intensive management includes temperature management, vasopressin, heparin, thyroxine, fludrocortisone, insulin, methylprednisolone, antibiotics, electrolyte control, and therapeutic bronchoscopy[301,334]. In the setting of organ donation for allotransplantation after brain-death, donors are typically managed for 2 – 3 days prior to donation[301]. However, with modern intensive donor management, increasing the duration between brain-death and organ donation may improve graft outcomes[335-338]. Although the effect of brain-death is a limitation of the decedent model, best practice donor management can mitigate its impact.

Additional limitations of the model include the low number of cases, unpredictability of when decedents will be available, high costs, and the relatively short duration compared to the intended graft survival for kidney and heart xenografts.

A number of decedent xenotransplants using transgenic porcine donors have been performed in recent years, described in *1.11.1.2*: (i) α-Gal knock-out thymo-kidneys for 54 hours (*n = 2*) and 61 days (*n = 1*)[33,187,192]; (ii) 10GE kidneys for 3 (*n = 2*) and 7 (*n = 1*) days[188-191]; (iii) 10GE hearts for 66 hours (*n = 2*)[193]; (iv) heterotopic auxiliary triple glycan knock-out, h*CD46*.h*CD55*.h*THBD* liver xenotransplant for 10 days (*n = 1*)[205]. The decedent model is particularly suitable for evaluating ELC for several reasons: (i) the intended clinical duration of ELC (days – (possibly) weeks) is much shorter than the target survival for kidney and heart xenografts (months – years), and more compatible with a feasible duration over which to maintain a decedent; (ii) adequate modelling of the perfusion dynamics of the ELC system requires a human-sized recipient (vessel size, blood volume, organ flows), making non-human primate experiments not feasible; (iii) the ELC intervention comprises a novel medical device and protocol that should be tested in the clinical environment prior to treating patients.

In this study, the decedent model was used to evaluate xeno-ELC using the *metra*-ELC system with latest-generation genetically-modified pig livers.

7.2 Aims

The primary aim was to determine the feasibility of ELC using transgenic pig livers in human decedents with intact native livers (*n = 4*) by assessing: (i) impact of xeno-ELC on the clinical and biochemical status of the decedent; (ii) xenograft perfusion dynamics and function; (iii) immunological response to the xenograft; (iv) zoonotic transmission.

The secondary aim was to assess functional hepatic replacement by xeno-ELC with a decedent following native hepatectomy (*n = 1*).

7.3 Methods

7.3.1 Study design

Four decedents underwent ELC with a total of five transgenic 3KO.7TG.RI porcine livers. Decedents 1 – 3 each underwent ELC with single porcine xenografts ('ELC 1 – 3'). Decedent 4 underwent two consecutive sessions of ELC with two xenografts ('ELC 4 and 5'). 20.8 hours into the second ELC session, decedent 4 underwent hepatectomy with porto-caval anastomosis followed by a further 48 hours of ELC. After discontinuation of ELC, decedent 4 was observed with continued critical care support until cessation of circulation (*figure 52*).

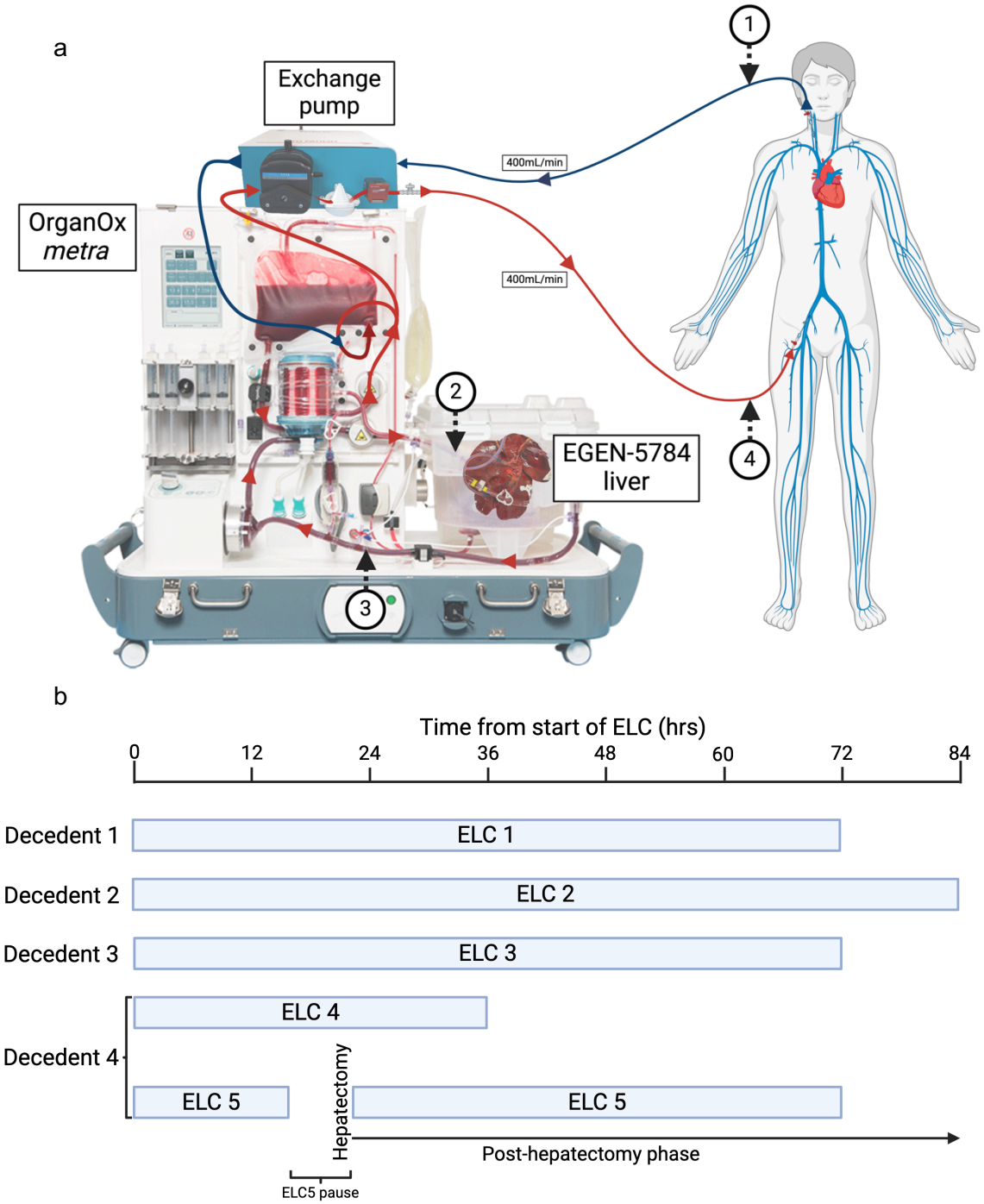


Figure 52: *metra*-ELC system and ELC durations.
a: *metra*-ELC system. Numbered sites refer to sites of blood sampling and flow measurement for the HepQuant test: 1: decedent to *metra*-ELC blood flow; 2: xenograft (EGEN-5784) portal vein inflow; 3: xenograft inferior vena cava outflow; 4: *metra*-ELC to decedent blood flow.
b: ELC durations for ELC 1 – 5. ELC 4 and 5 occurred consecutively in the same decedent with a 5.25 hour gap. After 16.1 hours, ELC 5 was temporarily paused for 6.2 hours for platelet transfusion followed by hepatectomy. Hepatectomy was completed at 20.8 hours. ELC 5 was resumed 1.5 hours post-hepatectomy and continued for a further 48 hours. Decedent 4 underwent a total of 100 hours of ELC (36 hours ELC 4 + 16 hours ELC 5 pre-hepatectomy + 48 hours ELC 5 post-hepatectomy). ELC: extra-corporeal liver cross-circulation.

7.3.2 Authorisation and study oversight

The investigators confirmed with the University of Pennsylvania institutional review board that studies involving decedents fall outside its purview. The Governing Board of the Gift of Life Donor Program (GLDP, Philadelphia, PA, USA) approved the study protocol, the authorisation form that described the study for next-of-kin, and identified the potential decedents. eGenesis (Cambridge, MA, USA) convened a Medical Oversight Committee, which comprised a hepatologist, an abdominal transplant surgeon with ethics expertise, an infectious diseases specialist, an abdominal surgeon (nominated by the Gift of Life Donor Program), a donor family member (nominated by the Gift of Life Donor Program), and representatives from eGenesis. The Committee approved the protocol and reviewed data from the decedent studies. The decedents' next-of-kin provided written authorisation for the study protocol.

7.3.3 Decedent selection and maintenance

The decedents' bodies were transferred to the Gift of Life Donor Program Donor Care Centre at the Hospital of the University of Pennsylvania where critical care support was continued according to the Gift of Life Donor Program Clinical Manual. The four decedents were a 79-year-old male (decedent 1, 84 kg), a 74-year-old female (decedent 2, 112 kg), a 57-year-old male (decedent 3, 85 kg), and an 80-year-old female (decedent 4, 51 kg). All were declared brain dead and eligible for the study only after the Gift of Life Donor Program had exhausted transplant organ placement efforts. Decedent 3 was found to have liver cirrhosis at pre-trial surgical exploration. The blood-types of decedents 1 – 4 were A+, O+, O+, and B+, respectively.

7.3.4 Porcine organ donors

Five porcine livers (designated EGEN-5784) were procured from transgenic 3KO.7TG.RI Yucatan minipig clones (see *6.3.1*), provided by eGenesis. The porcine donors (designated ESUS-1784) were transported, via high-efficiency particulate air (HEPA) filtered van, from the eGenesis research donor

facility (Mt. Horeb, WI, USA) to Accuro Farms (Southbridge, MA, USA). Upon arrival, the porcine donors were housed in a HEPA filtered trailer designated specifically for eGenesis donors until selection for organ procurement.

The porcine donors were selected based on weight, overall health, results of clinical testing (haematology, biochemistry, coagulation), and viral pathogen testing.

Porcine donors were determined to be in good health based on biweekly clinical pathology testing (IDEXX, Grafton, MA, USA), which showed no clinically significant findings and parameters within normal limits. Porcine viral pathogen testing (UMN, Veterinary Diagnostic Laboratory, St. Paul, MN, USA) included porcine cytomegalovirus (pCMV), porcine lymphotropic herpes virus (PLHV), hepatitis E virus (HEV), and porcine circovirus 3 (PCV3). Porcine viral pathogen testing was performed monthly and at the time of procurement. Porcine donors tested negative for all viruses at all timepoints. The porcine donor ages, body, and liver weights are in *table 18*.

ELC	Porcine Donor ID	Species	Sex	Age (months)	Body Weight (kg)	Liver Weight (kg)
1	A9338	Yucatan	F	15	84.7	1.3
2	22129	Yucatan	F	24	95.1	2.0
3	A9334	Yucatan	F	22	108.7	1.6
4	23055	Yucatan	F	24	76.7	1.14
5	22134	Yucatan	F	34	101.6	1.41

Table 18: porcine donor identification, age, body and liver weights.
ELC: extra-corporeal liver cross-circulation. ID – identification. F – female.

7.3.5 Porcine liver retrieval, preparation, isolated normothermic perfusion phase, and extra-corporeal liver cross-circulation

Porcine liver retrieval and preparation occurred at Accuro Farms (Southbridge, MA, USA), and are described in *2.1.2* and *2.2*. The isolated normothermic perfusion phase and *metra*-ELC protocol are described in *2.3*, *2.4*, and *2.5*.

During the isolated normothermic perfusion phase, the anticoagulation and insulin regimen differed from that undertaken in the experiment of *chapter 5*. The perfusate comprised packed human red cells (3 – 4 units) and 500 mL 5% human albumin solution, rather than autologous leuco-depleted blood.

The lack of starting perfusate coagulation factors or platelets meant that the perfusate ACT declined more gradually during this phase than in *chapter 5*. Although livers synthesise clotting factors during isolated normothermic perfusion[100], this is too slow to substantially affect ACT over a few hours. In these cases, it is not feasible to target a specific ACT range prior to connection. Due to the anticipated longer duration of isolated normothermic perfusion compared to *chapter 5*, insulin infusion was included during this phase to support graft metabolic function.

For decedents 1 – 3, standard *metra* heparin (10,000 IU bolus and 833 IU/hour infusion) dosing for normothermic liver preservation was used (see *2.3*). For decedent 4, the total heparin load of the perfusate was rationalised to a single starting bolus of 5,000 IU and no infusion. The perfusate ACT was monitored hourly, with additional 1,000 IU administered if ACT ≤ 200 seconds. Based on the elevated perfusate insulin levels detected during *ex situ* isolated xenoperfusion, for decedents 1 – 3 the insulin infusion dose was reduced to one-half of the standard *metra* dose (i.e. 3.33 IU/hour) and, for decedent 4, to one-fifth (i.e. 1.33 IU/hour) (see *6.5*)[4]. In all cases, the standard *metra* epoprostenol infusion (8.33 μg/hour) was used and bile salt infusion was omitted.

Ex situ haemofiltration of the perfusate (at a rate of 999 mL/hour) commenced 60 minutes prior to liver reperfusion. The porcine liver was reperfused on the *metra* as per the IFU[4]. Following reperfusion, the haemofiltration rate was reduced to 500 mL/hour. The porcine liver was monitored for 1 hour at Accuro Farms to ensure adequate vascular flows and haemostasis before transferring to a speciality van supplied by Gift of Life. During the isolated normothermic perfusion phase, the metabolic activity, electrolytes, haematocrit, and ACT were monitored using iSTAT CG4+, Chem8+, and ACTk cartridges (Abbott).

For ELC 5, the porcine liver was transported from Accuro Farms to the Hospital of the University of Pennsylvania under static cold storage in UW solution, before commencing the isolated normothermic perfusion phase.

On arrival at the Gift of Life Donor Care Centre at the Hospital of the University of Pennsylvania, perfusate parameters were confirmed to be safe for connection to the decedent. The *ex situ*

haemofiltration circuit was disconnected. The insulin and heparin (if present) infusions were terminated. The epoprostenol infusion was continued throughout ELC.

An Edwards OptiSite arterial perfusion cannula (16 Fr x 24 cm) (Edwards Lifesciences, Irvine, Ca, USA) was placed in the right internal jugular vein. A Maquet Arterial HLS cannula (15 Fr x 15 cm) (Maquet Cardiopulmonary GmbH, Rastatt, Germany) was placed in the femoral vein. Blood circulated from the internal jugular vein to the *metra*; blood from the *metra* was returned to the decedent via the femoral vein.

Following administration of the pre-connection medications to the decedent (intravenous 500 mg methylprednisolone, 2,500 IU heparin, 1 g cefazolin), ELC was commenced at a rate of 50 mL/minute. This was increased in increments of 50 mL/minute every 10 minutes to a maximum of 400 mL/minute (*figure 53*). During ELC, a heparin infusion targeted an ACT of 160 – 200 seconds, bile salts excreted in the xenograft bile were replaced using enteral ursodeoxycholic acid (4 mg/kg 8-hourly), and anti-microbial prophylaxis was 1 g cefazolin 8-hourly.

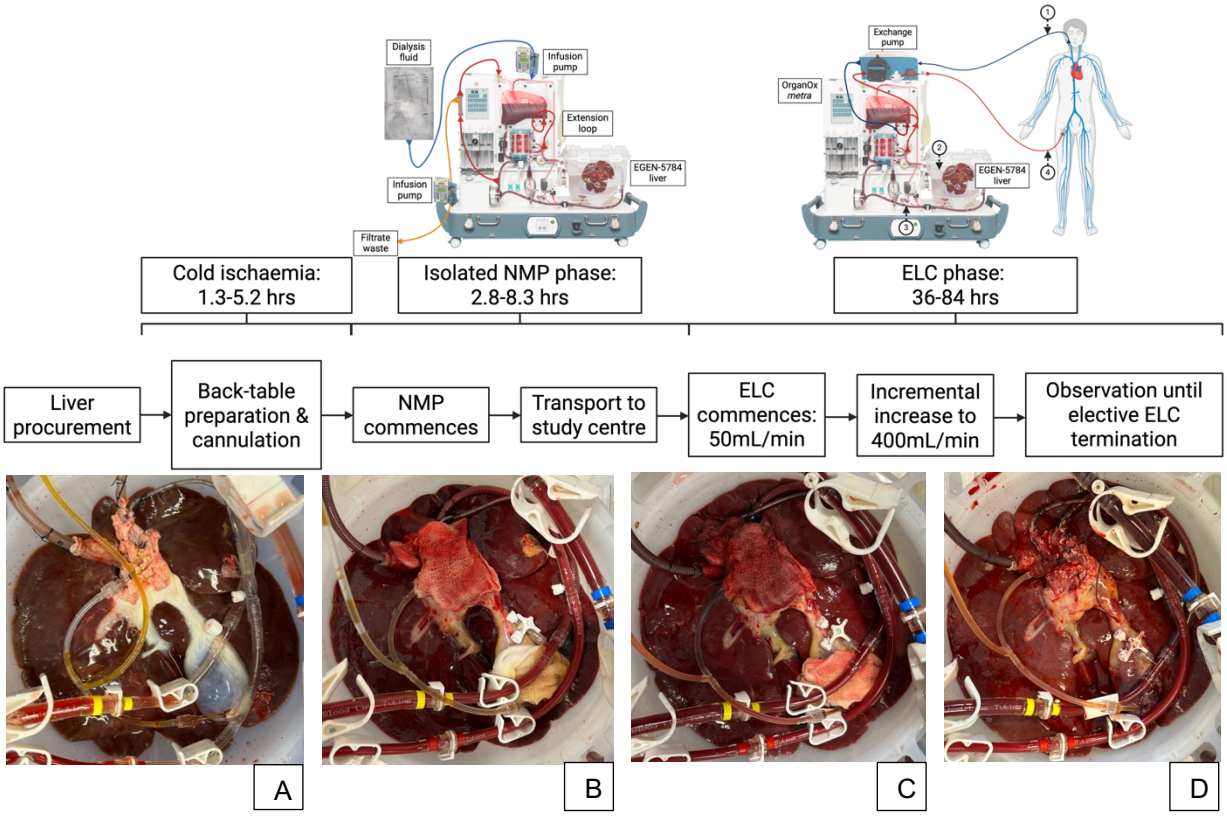


Figure 53: progress of the transgenic porcine livers through the study, demonstrating the 3 main phases: cold ischaemia; isolated normothermic machine perfusion; and extra-corporeal cross-circulation.
Images demonstrate the macroscopic appearance of the xenograft (EGEN-5784) during ELC 2 at: A: end of cold ischemia; B: end of isolated NMP; C: 40 hours ELC; D: 84 hours ELC. NMP: normothermic machine perfusion. ELC: extra-corporeal cross-circulation.

7.3.6 Sample analysis

The decedents underwent arterial blood gas, liver function, renal function, haematological, and coagulation blood tests (Hospital of the University of Pennsylvania, PA, USA) prior to ELC and 4 – 6 hourly during ELC.

Prior to commencing ELC and at 24-hour intervals during ELC 1 – 4, three assays of hepatocyte metabolism were performed using intravenous injections of non-radioactive stable isotope tracers (2 mg/kg D_5-phenylalanine; 2 mg/kg ^{15}N-glycine; 1 mg/kg ^{13}C-methacetin). Venous samples were collected in sodium heparin tubes pre- and then at 10-, 20-, 30-, 40-, 50, and 60-minutes post-administration for mass spectrometric analysis. The assays measured hepatocyte conversion of: (i) D_5-

phenylalanine to D_4-tyrosine (cytosolic transamination function); (ii) ^{15}N-glycine to ^{15}N-urea (mitochondrial oxidative function); (iii) ^{13}C-methacetin to blood $^{13}CO_2$ (microsomal cytochrome P450 (CYP1A2) function) (Metabolic Solutions, Nashua, NH, USA).

At the same time-points during ELC 1 – 4, a modified HepQuant SHUNT V2.0 test[339] was performed to measure cholate clearance. Intravenous ^{13}C-cholate (20 mg, pre-mixed with albumin) was administered to the decedents. Blood was sampled into SST tubes at 5, 20, and 60 minutes post-administration simultaneously from four sites: (i) blood leaving decedent to *metra*-ELC; (ii) xenograft portal vein inflow; (iii) xenograft inferior vena cava outflow; (iv) blood returning to decedent from *metra*-ELC (*figure 52*). The flows at these locations were simultaneously measured using a BioProt TT flow sensor. Serum ^{13}C-cholate levels were measured by LC-MS/MS. Total clearance of ^{13}C-cholate by the decedent-*metra*-ELC system was calculated by dividing the ^{13}C-cholate dose by the area under the curve of the ^{13}C-cholate concentration *versus* time plot using a tri-exponential fit. Clearance of ^{13}C-cholate by the donor liver and *metra*-ELC system were calculated by the product of their respective inflows and extraction ratios. Clearance of ^{13}C-cholate by the decedent was determined by the difference between total clearance and clearance by the *metra*-ELC system. In this way, clearance of cholate by the decedent's native liver, the xenograft, and the *metra*-ELC system could be specifically quantified. This calculation assumes instantaneous and uniform mixing of ^{13}C-cholate within the system. Hepatic filtration rates were calculated by dividing the respective clearances by the decedents' body weights (HepQuant, Denver, CO, USA).

Serum cell-free haemoglobin, bile pH, and bile glucose were measured on an ABL90 FLEX arterial blood gas analyser. Perfusate and decedent serum insulin concentrations were measured using ELISA (10-1113-01, Mercodia, Uppsala, Sweden (at eGenesis, Cambridge, MA, USA)).

7.3.7 Xenograft histological assessment

For decedents 1 – 3, xenograft biopsies were obtained at procurement, after 24, 48, 72 hours, and at termination of ELC. Biopsies pre- and during perfusion were peripheral wedges; termination biopsies were central wedges. Tissue samples were formalin-fixed and stained with H&E (Applied Pathology

Systems, Shrewsbury, MA, USA). Immunohistochemistry staining included C4d (University of Pennsylvania, PA, USA), CD14 (macrophages, monocytes, Kupffer cells), CD11b (neutrophils, monocytes), CD57 (natural killer cells), CD3 (cytotoxic and helper T cells), CD19 (B/plasma cells), CD41, CD61 (platelets), IgG, and IgM (Applied Pathology Systems, Shrewsbury, MA, USA). Immunohistochemistry stain-positive inflammatory cells were manually counted within the 400x high power field at the portal tract stroma and hepatic lobule 'hot spot'; positive cells within the portal vein, central veins, or necrotic areas were not counted.

7.3.8 Statistical analysis

Outcomes are reported individually for each study. Where appropriate, parametric data are summarised using mean (range). There is no comparator group. The correlation between xenograft weight and mean platelet count was determined using Pearson's product-moment correlation (two-sided).

7.4 Results

7.4.1 Isolated normothermic perfusion phase

The porcine livers maintained homogeneous parenchymal perfusion and demonstrated metabolic activity during the isolated normothermic perfusion phase, evidenced by lactate clearance, maintenance of perfusate pH, glucose consumption, and urea production (*table 19*).
The perfusate insulin concentration prior to commencing ELC was 1,644 mU/L, 1515 mU/L, and 104 mU/L for ELC 2, 3, and 5, respectively. The mean decedent serum insulin concentration was 567 mU/L (116 – 1123 mU/L, *figure 47*).

ELC	Cold ischaemic time (mins)	NMP duration (mins)	End NMP perfusate lactate (mmol/L)	End NMP perfusate pH	End NMP perfusate potassium (mmol/L)
1	78	437	<0.3	7.34	6.3
2	88	485	0.97	7.19	5.3
3	95	496	2.55	7.29	4.9
4	102	401	<0.3	7.35	4.7
5	312	167	3.4	7.36	4.8

Table 19: The porcine livers demonstrated metabolic activity during the isolated normothermic perfusion phase with evidence of lactate clearance and maintenance of perfusate pH.
The end of isolated normothermic perfusion samples for ELC 1 – 3 were taken shortly following administration of an additional unit of packed red blood cells to the circuit to enable priming of the bypass circuit. This resulted in a transient elevation of perfusate potassium, lactate, and fall in pH. The xenograft used for ELC 5 was preserved by static cold storage during transport back to the study centre, hence the prolonged cold ischaemic time and shortened normothermic perfusion duration. mins: minutes; NMP: normothermic machine perfusion; ELC: extra-corporeal liver cross-circulation.

7.4.2 Feasibility of xeno-ELC (decedent native liver intact, ELC 1 – 4)

7.4.2.1 ELC duration

Decedents 1, 2, and 3 underwent ELC until the pre-defined target of 72 hours. For decedent 2, ELC was continued for an additional 12 hours to test a platelet mitigation therapy (see *7.4.2.4*). After discontinuation of ELC, decedents 2 and 3 were observed for an additional 38 and 26 hours, respectively. ELC 4 was terminated after 36 hours due to low hepatic artery flows, possibly related to a very small-calibre cannula (*figure 52*).

7.4.2.2 Clinical status

The decedents remained haemodynamically stable during ELC except decedent 3, who required increasing vasopressor support during the final day. This was thought to be due to chest sepsis with pre-existing cardiomyopathy. Decedents 2 and 3 developed progressive renal dysfunction prior to commencing ELC: decedent 2 commenced continuous renal replacement therapy (CRRT) prior to

initiation of ELC, while decedent 3 started CRRT after 24 hours of ELC. All decedents demonstrated a transient, mild transaminase and bilirubin elevation upon ELC commencement. Lactate, pH, and creatinine remained stable (*figure 54)*.

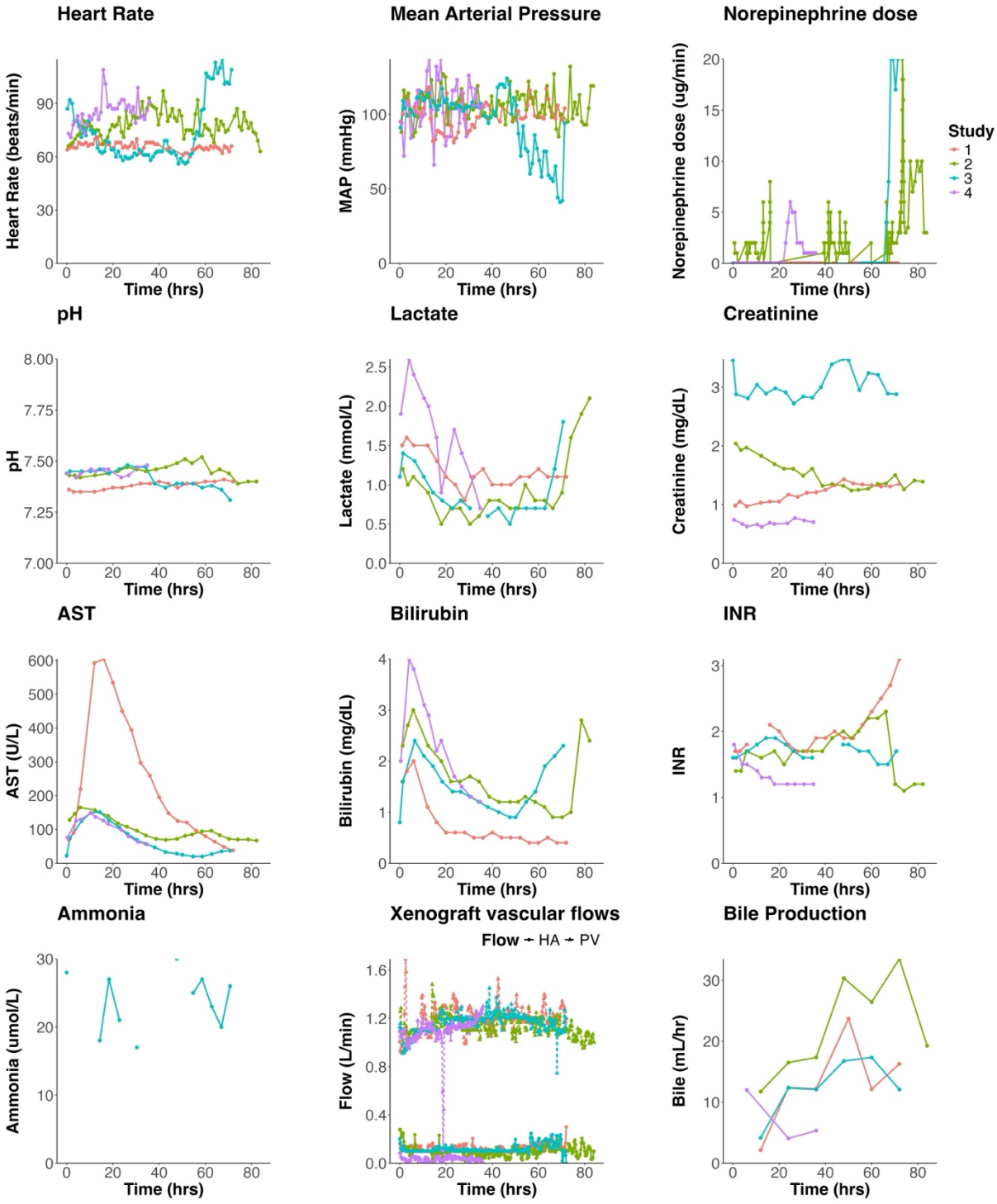


Figure 54: decedents 1 – 4 clinical parameters, blood results, xenograft vessel flows, and bile production during ELC 1 – 4 (with decedent native livers intact).
ELC: extra-corporeal liver cross-circulation. AST: aspartate transaminase. INR: international normalised ratio. HA: hepatic artery. PV: portal vein. Individual studies presented.

7.4.2.3 Xenograft function

The liver xenografts maintained homogeneous parenchymal perfusion during ELC (*figure 53*). In ELC 1 – 3, the xenografts maintained physiological vascular flows (mean hepatic artery flow: 120 (100-130) mL/minute; mean portal vein flow: 1160 (1130 – 1190) mL/minute) and consistent bile production (mean: 19.4 (12.7 – 23.1) mL/hour). In ELC 4, the xenograft demonstrated comparable portal vein flows (mean: 1100 mL/minute), with reduced hepatic artery flows (mean: 30 mL/minute) and bile production (mean: 6 mL/hour) (*figure 54*). Xenograft bile chemistry was measured for ELC 2 – 4: bile pH remained > 7.8 and bile glucose remained ≤ 0.2 mmol/L (*table 20*).

Metabolic testing demonstrated that overall metabolism of D_5-phenylalanine, ^{15}N-glycine, ^{13}C-methacetin, and clearance of cholate were increased during xeno-ELC compared to the decedent pre-ELC baseline (*figures 55, 56*).

ELC	Final bile pH	Final bile glucose (mmol/L)
2	> 8.00	0.00
3	8.30	0.06
4	7.89	0.00

Table 20: final bile chemistry for ELC 2, 3, and 4 after 84, 72, and 36 hours of ELC, respectively.
ELC: extra-corporeal liver cross-circulation.

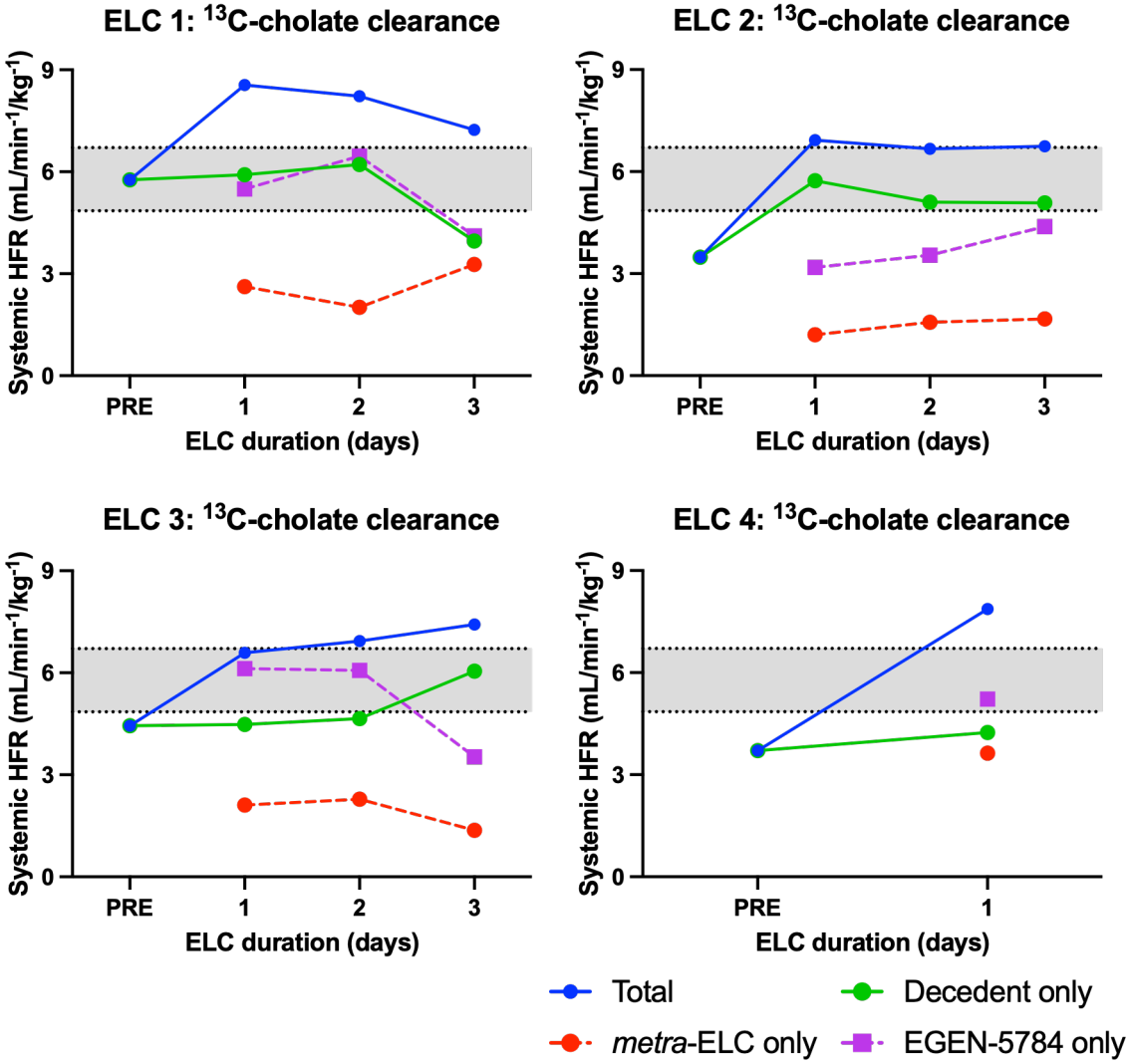


Figure 55: ^{13}C-cholate clearance prior to commencing ELC ('pre') and after 1, 2, and 3 days of ELC during ELC 1 – 4 (decedents' native livers intact).
Blood sampling pre- and post-*metra*-ELC system and xenograft (EGEN-5784) enables quantification of ^{13}C-cholate clearance by the decedent ('decedent only'), xenograft (EGEN-5784 only), and *metra*-ELC system (*metra*-ELC only). Shaded region: healthy control range. ELC: extra-corporeal liver cross-circulation. HFR: hepatic filtration rate.

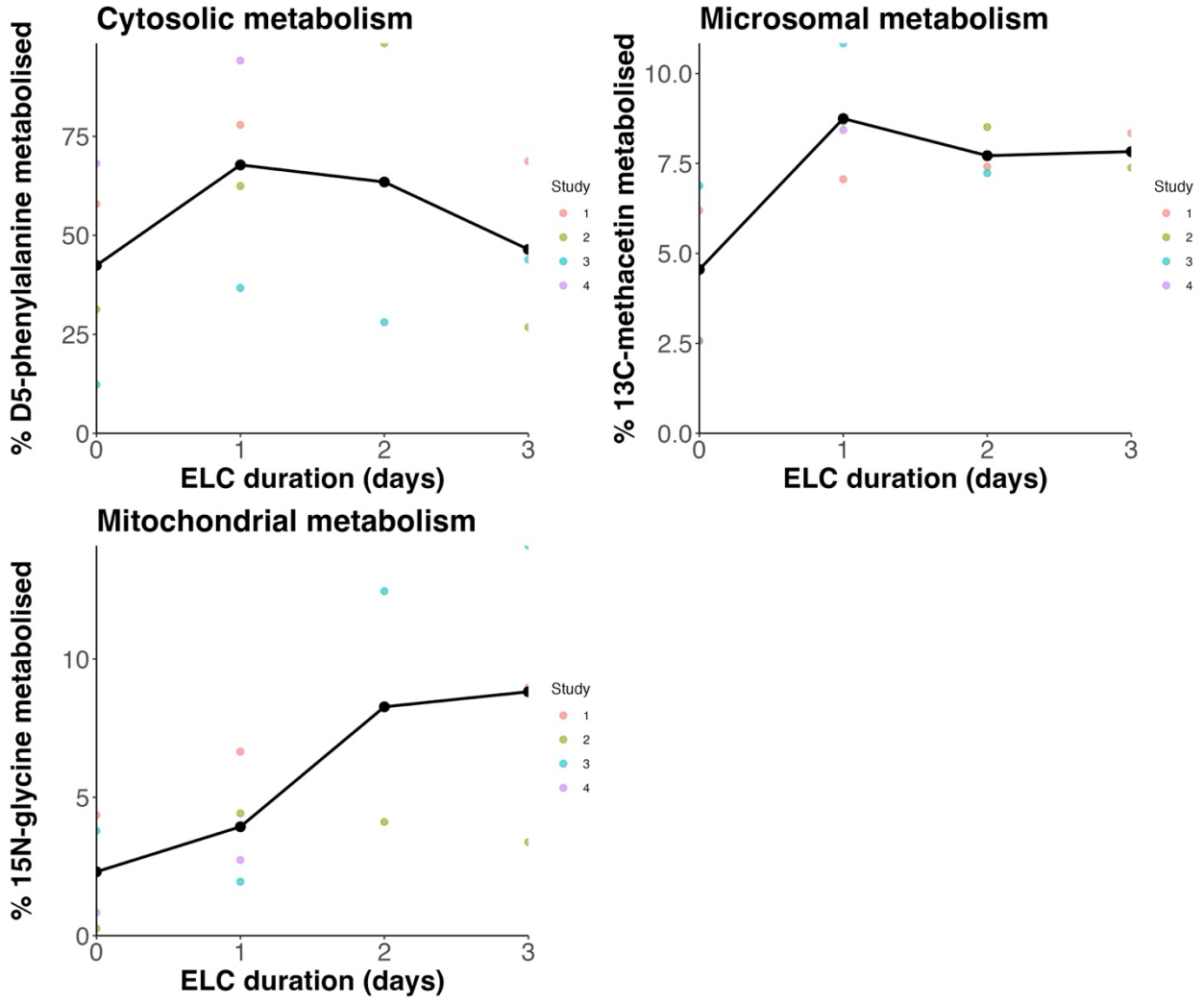


Figure 56: cumulative percentage of D_5-phenylalanine converted to D_4-tyrosine, ^{13}C-methacetin converted to blood $^{13}CO_2$, and ^{15}N-glycine converted to ^{15}N-urea during ELC 1 – 4 (decedents' native livers intact) within 60 minutes of administration (measures of hepatocellular cytosolic, microsomal, and mitochondrial metabolism, respectively). Day 0 refers to the decedents' baseline native liver metabolism prior to commencing ELC. Mean at each time point displayed as solid black dots, with individual ELC studies (1 – 4) coloured as per the legend. ELC: extra-corporeal liver cross-circulation.

7.4.2.4 Haematological impact

During ELC 1, the decedent's INR gradually increased to 3.1. During ELC 2, 10 mg intravenous vitamin K bolus at 67 hours of ELC corrected the INR from 2.3 to 1.2. During ELC 3 and 4, daily 10 mg intravenous vitamin K maintained stable INR levels (*figure 54*). Fibrinogen levels remained within the normal range. D-Dimer levels were substantially elevated in all decedents pre-ELC. The levels decreased during ELC but remained above normal (*figure 57*).

Blood transfusions were required to maintain haemoglobin levels during ELC 2 – 4 (*figure 58*), without evidence of clinical bleeding. Serum cell-free haemoglobin levels are presented in *figure 59*. Peripheral blood smear examination revealed no schistocytes during ELC 1 – 3, but decedent 4 demonstrated schistocytes prior to commencing ELC. Heparin infusion largely maintained the decedents' ACT within the 160 – 200 seconds target range. The mean hourly heparin dose was 2,659 IU and the mean ACT was 194 seconds (*figure 60*).

Thrombocytopenia developed within one hour of starting ELC in all decedents. The mean platelet count during ELC was 24 x 10^9/L (range of study means: 10 – 33 x 10^9/L) with only transient response to platelet transfusion (*figure 61*). Mean platelet count correlated negatively with xenograft weight ($r = -0.82$, $p = 0.09$, *figure 62*). Peripheral blood smear examination excluded pseudo-thrombocytopenia and heparin-induced thrombocytopenia screens were negative. CD41 staining revealed platelet sequestration in the sinusoids of the xenograft after 24 hours of ELC (*figure 63*). During ELC 2 and 3, intravenous 50 g immunoglobulin and 1080 mg pegcetacoplan (C3 inhibitor) were administered respectively, followed by platelet transfusion, without sustained improvement in platelet counts. Prior to ELC 4, the decedent and *metra* circuit were both treated with 22 mg caplacizumab (von Willebrand factor inhibitor). Pre-existing thrombocytopenia in decedent 4 limited interpretation of the impact of caplacizumab. The response to platelet transfusion appeared to improve after discontinuation of ELC 2, to a lesser extent ELC 3, and whilst ELC 5 was paused for native hepatectomy (*figure 61*).

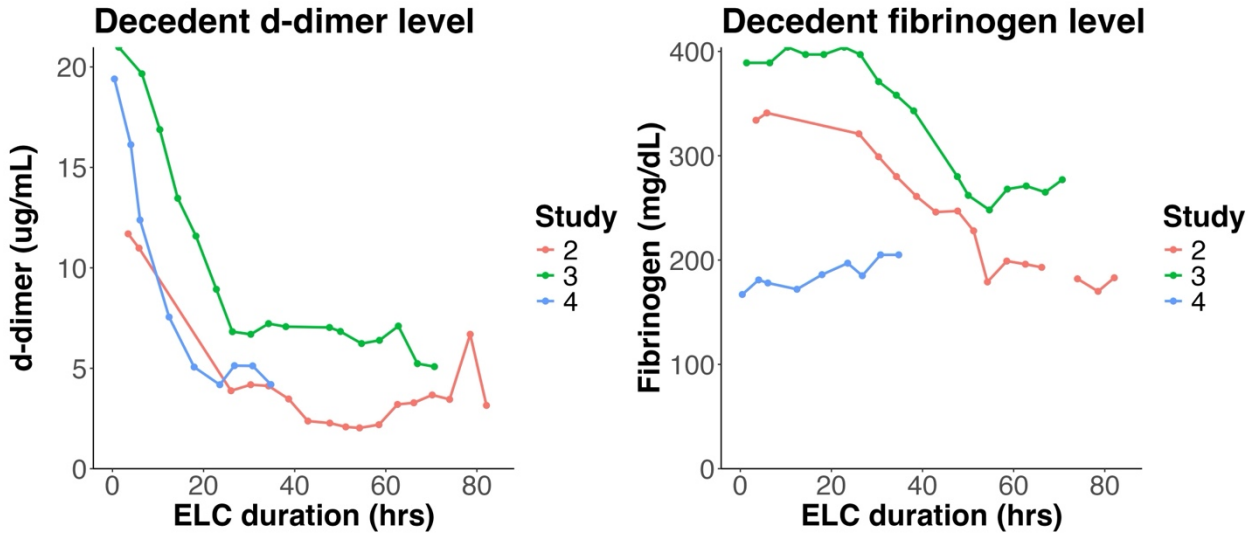


Figure 57: decedent d-dimer and fibrinogen levels during feasibility studies, ELC 2 – 4 (values not available for ELC 1).
ELC: extra-corporeal liver cross-circulation. Individual studies presented.

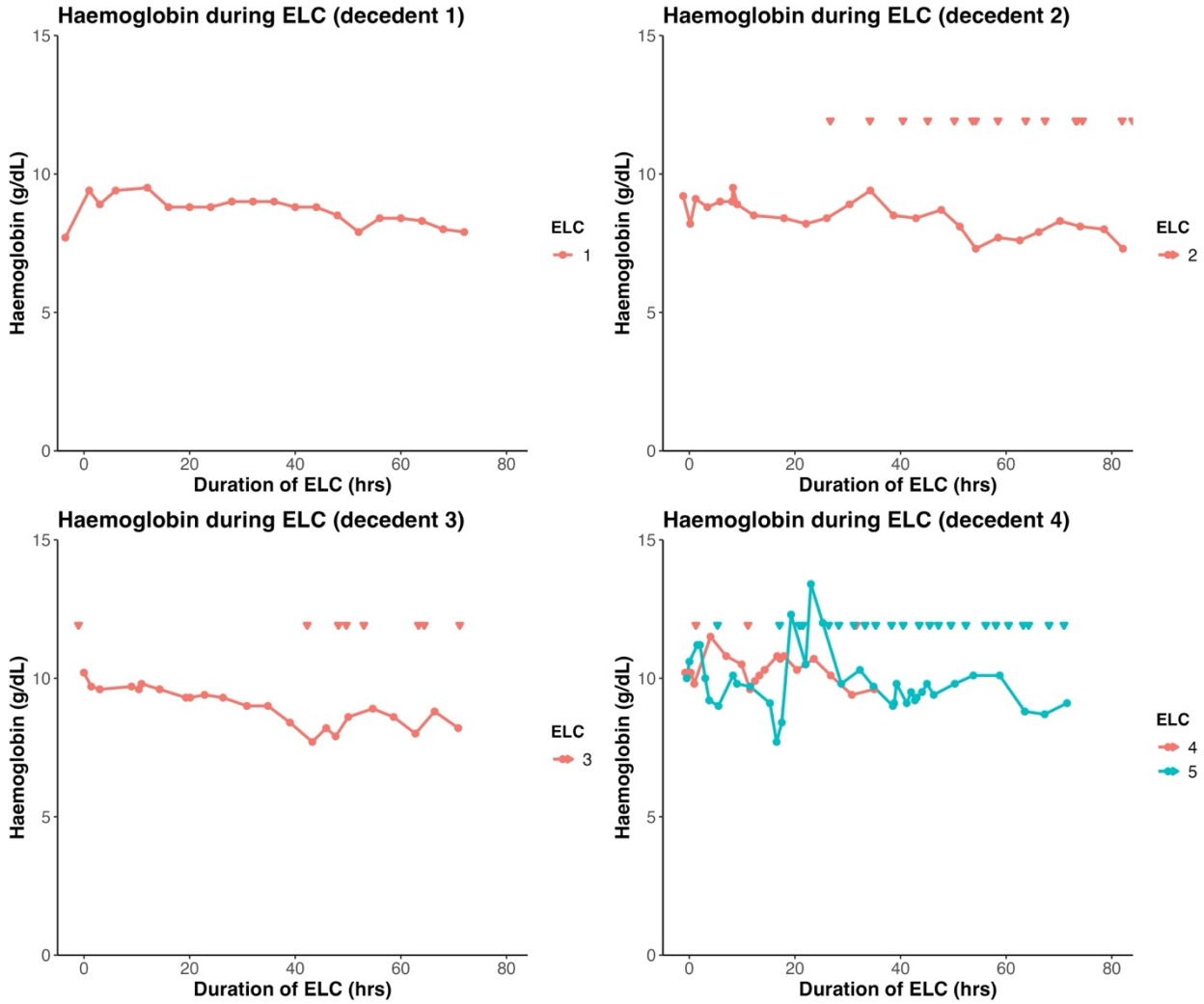


Figure 58: haemoglobin levels of decedents 1 – 4 during ELC 1 - 5.
Small arrows indicate red blood cell transfusions. For ELC 5, data are presented according to time from start of ELC (i.e. including the period when ELC was paused for hepatectomy). ELC: extra-corporeal liver cross-circulation.

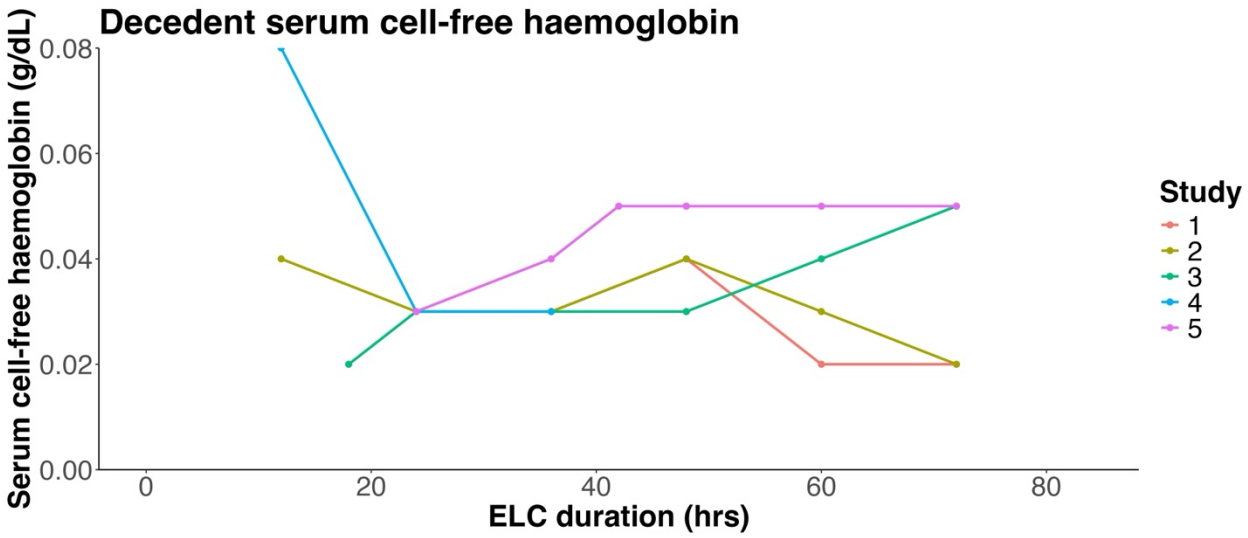
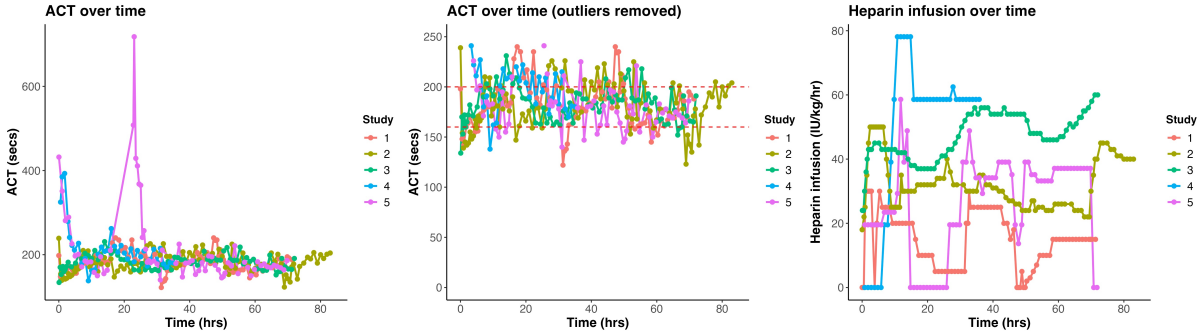


Figure 59: decedent serum cell-free haemoglobin during ELC.
ELC: extra-corporeal liver cross-circulation. Individual studies presented.



ELC	Mean ACT (secs)	Mean hourly heparin dose (IU/hr)	Mean hourly heparin dose (IU/kg/hr)
1	183	1419	16.9
2	184	3647	32.7
3	184	4185	49.1
4	207	2644	51.6
5	211	1399	27.3
Mean	**194**	**2659**	**35.5**

Figure 60: decedent activated clotting time (ACT) and hourly heparin infusion dose during extra-corporeal liver cross-circulation (ELC).
Individual studies presented.

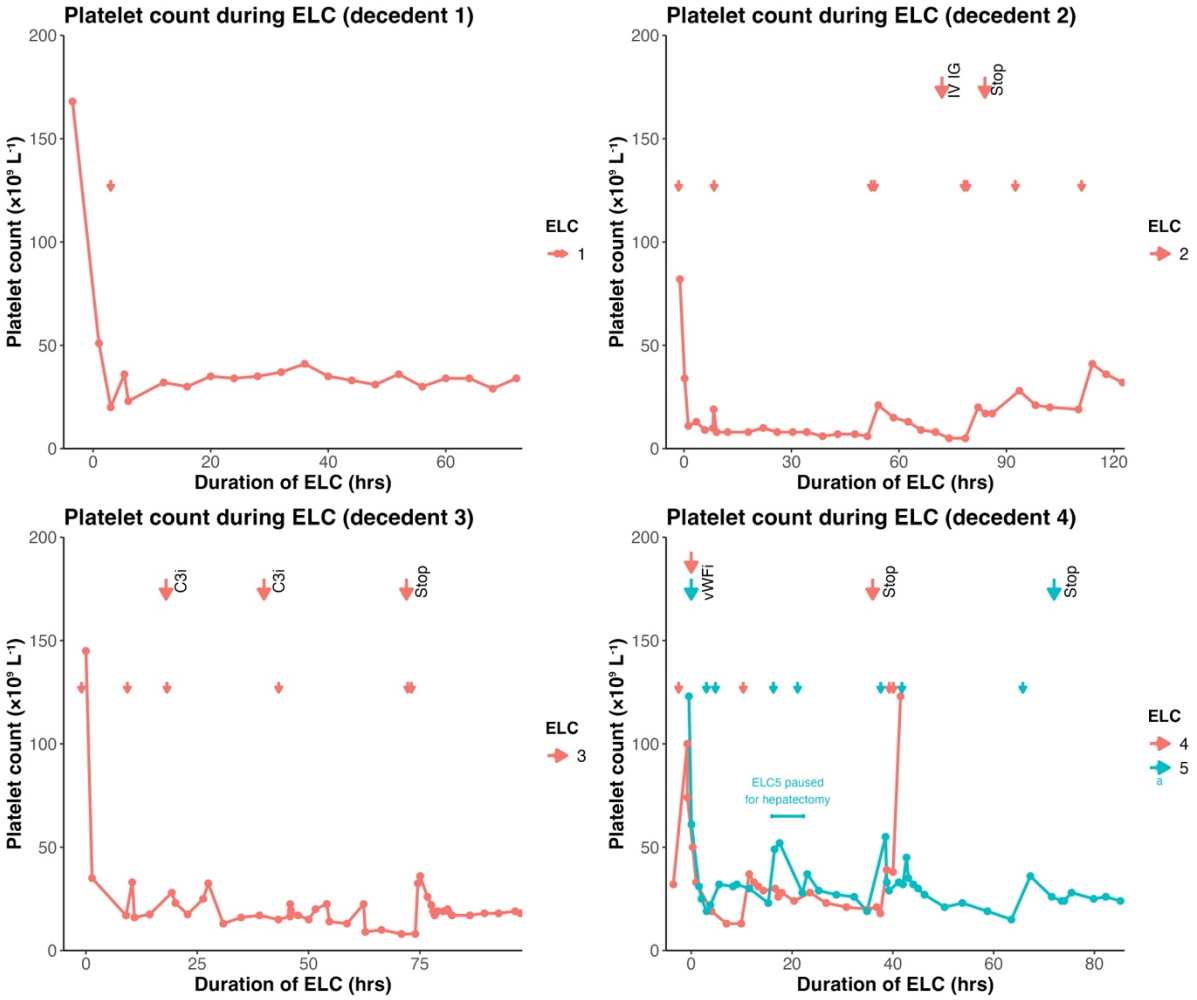


Figure 61: decedent platelet counts during ELC and post-disconnection.
Small arrows mark the timepoints of platelet transfusion. Large arrows mark the timepoints of administration of potential platelet mitigation therapies and when ELC was stopped. For ELC 5, data are presented according to time from start of ELC (i.e. including the period when ELC was paused for hepatectomy). IV IG: intravenous immunoglobulin. C3i: pegcetacoplan (C3 inhibitor). vWFi: caplacizumab (anti-von Willebrand factor). ELC: extra-corporeal liver cross-circulation. Stop: timepoint at which ELC was stopped (decedents 2 – 4).

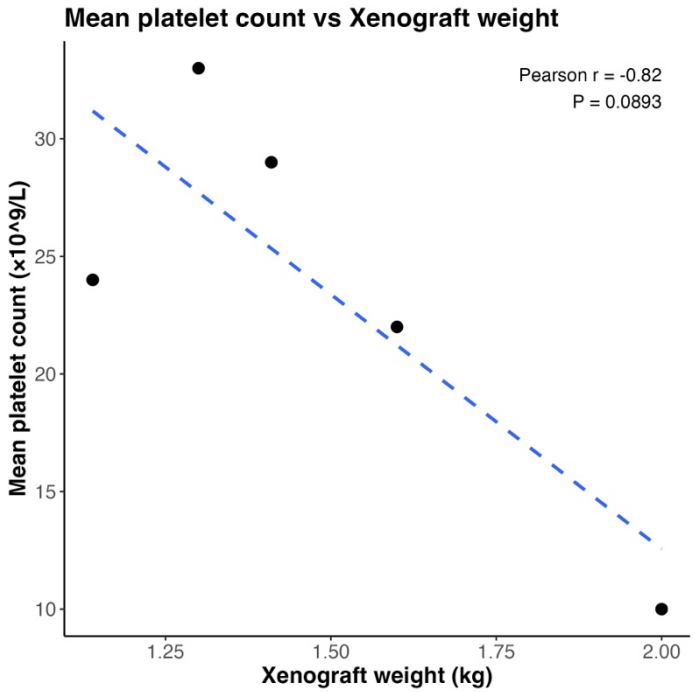


Figure 62: correlation between mean platelet count during extra-corporeal liver cross-circulation and xenograft weight.

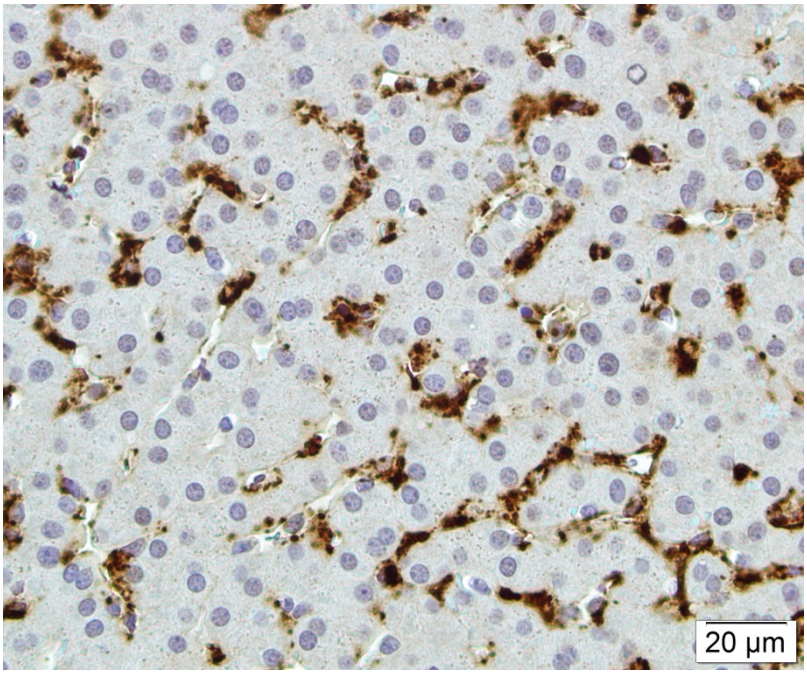


Figure 63: CD41 staining within hepatic sinusoids after 24 hours of extra-corporeal liver cross-circulation.

7.4.2.5 Adventitious agent testing

After completion of ELC, the decedents tested negative for fungal cultures, hepatitis E virus, porcine lymphotropic herpes virus, porcine cytomegalovirus, and porcine circovirus 3. Blood cultures were negative for all decedents. Decedent 3 grew Stenotrophomonas maltophilia and Staphylococcus aureus from sputum culture.

7.4.3 Functional hepatic replacement by xeno-ELC (anhepatic decedent, ELC 5)

Decedent 4 commenced ELC with a second liver ('ELC 5') 5.25 hours after ELC 4 was complete. After 16.1 hours of ELC 5 with the native liver intact, the *metra*-ELC system was temporarily disconnected from the decedent for platelet transfusion followed by hepatectomy. Hepatectomy was completed 20.8 hours after commencing ELC 5. During the period of temporary disconnection from the decedent (a total of 6.2 hours), the xenograft was perfused on the isolated *metra* circuit. 1.25 hours post-hepatectomy, the decedent's lactate rose to 6.8 mmol/L, base excess was -7.3 mmol/L, bilirubin 2.9 mg/dL, and ammonia 67 μmol/L. ELC was resumed 1.5 hours post-hepatectomy (*figure 64*). During the subsequent 48 hours, ELC maintained the decedent in a haemodynamically stable state, with minimal vasopressor support (48-hour post-hepatectomy heart rate 90 beats per minute; mean arterial pressure 86 mmHg; noradrenaline dose 2 μg/minute (0.04 μg/kg/minute)). Decedent lactate, pH, INR, and ammonia were maintained (48-hour post-hepatectomy lactate: 2.3 mmol/L; pH: 7.35; INR: 1.1; ammonia: 50 μmol/L). Creatinine increased mildly from pre-hepatectomy values with maintained urine output (48-hour post-hepatectomy creatinine: 0.97 mg/dL). The xenograft maintained homogeneous parenchymal perfusion, portal vein, and hepatic artery flows, although bile production decreased in the final 12 hours, associated with a rising bilirubin level (*figure 64*). One unit of fresh frozen plasma was given intra-operatively and one unit was administered during ELC, early in the post-hepatectomy phase. Multiple packed red cell transfusions were administered due to surgical site bleeding post-hepatectomy (*figure 58*).

ELC was discontinued electively after 48 hours of cross-circulation post-hepatectomy. The decedent immediately developed rapidly-escalating noradrenaline requirement, acidosis, and rising lactate. Ammonia increased to 89 μmol/L 2.5 hours post-disconnection and the decedent became anuric within 6 hours. 13.5 hours post-disconnection the INR exceeded 10, pH was 7.17, lactate 16 mmol/L, and noradrenaline dose 28 μg/minute (0.55 μg/kg/minute). Circulation ceased 17 hours after discontinuation of ELC.

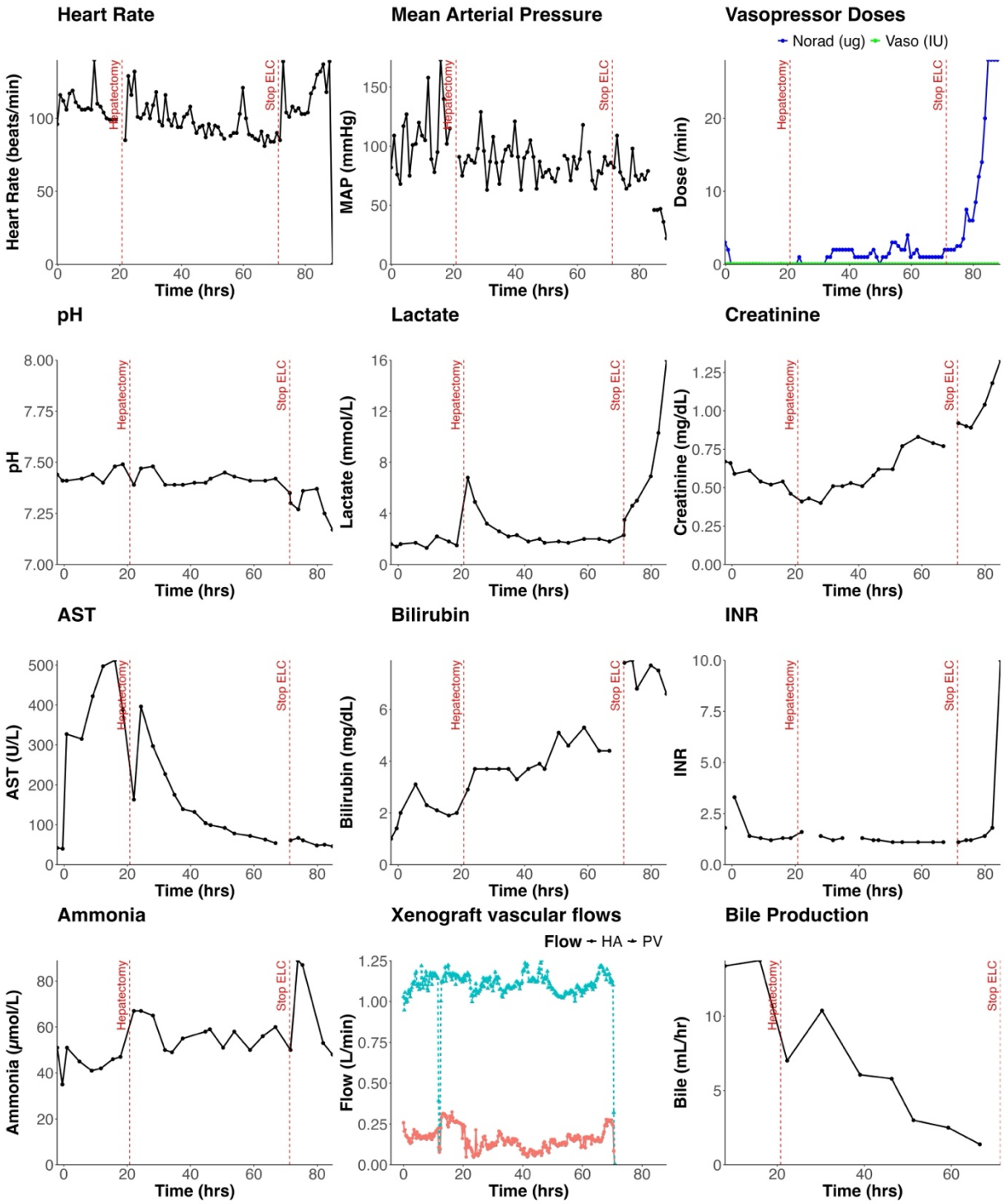


Figure 64: decedent 4 clinical parameters, blood results, xenograft vessel flows, and bile production during ELC 5.
ELC was paused between 16.1 and 22.3 hours. The decedent underwent hepatectomy 20.8 hours into ELC 5. ELC was resumed 1.5 hours later and continued for a further 48 hours, before elective discontinuation. Data are presented according to time from start of ELC (i.e. including the period when ELC was paused for hepatectomy). ELC: extra-corporeal liver cross-circulation. Norad: noradrenaline. Vaso: vasopressin. AST: aspartate transaminase. INR: international normalised ratio. HA: hepatic artery. PV: portal vein.

7.4.4 Xenograft histology

Xenograft biopsies demonstrated preservation of the hepatic parenchyma after 72 hours of ELC. There were variable focal areas of hepatocyte necrosis (spotty foci to localized areas within a lobule) and patchy minimal-to-mild portal tract inflammation with focal portal venule endothelialitis. Bile ducts were largely preserved, with focal bile duct injury and minimal inflammatory infiltrates. Red blood cell extravasation occurred in portal and septal areas, beginning at 24 hours, and expanding to periportal regions and beneath the central veins by 72 hours (*figure 65*).

CD14, CD11b, and CD3 staining gradually intensified during serial biopsies, suggesting that the predominant infiltrating cells were macrophages/monocytes, neutrophils, and T-cells (*figure 65*). CD3-postive T-cells were primarily localised in the portal regions, whereas CD14- and CD11b-positive cells were more prominent within the hepatic lobules. Absence of CD19 staining suggests no B cell presence, and absent or minimal CD57 staining suggests limited NK cell infiltration (*table 21*).

Pre-ELC xenograft biopsies were negative for IgG, IgM, and C4d. IgG staining remained negative, whilst IgM staining markedly increased during ELC, localised to endothelial cells (*figure 65*). After 72 hours of ELC, weak, variable, and focal endothelial C4d staining was noted in some portal venules and arterioles.

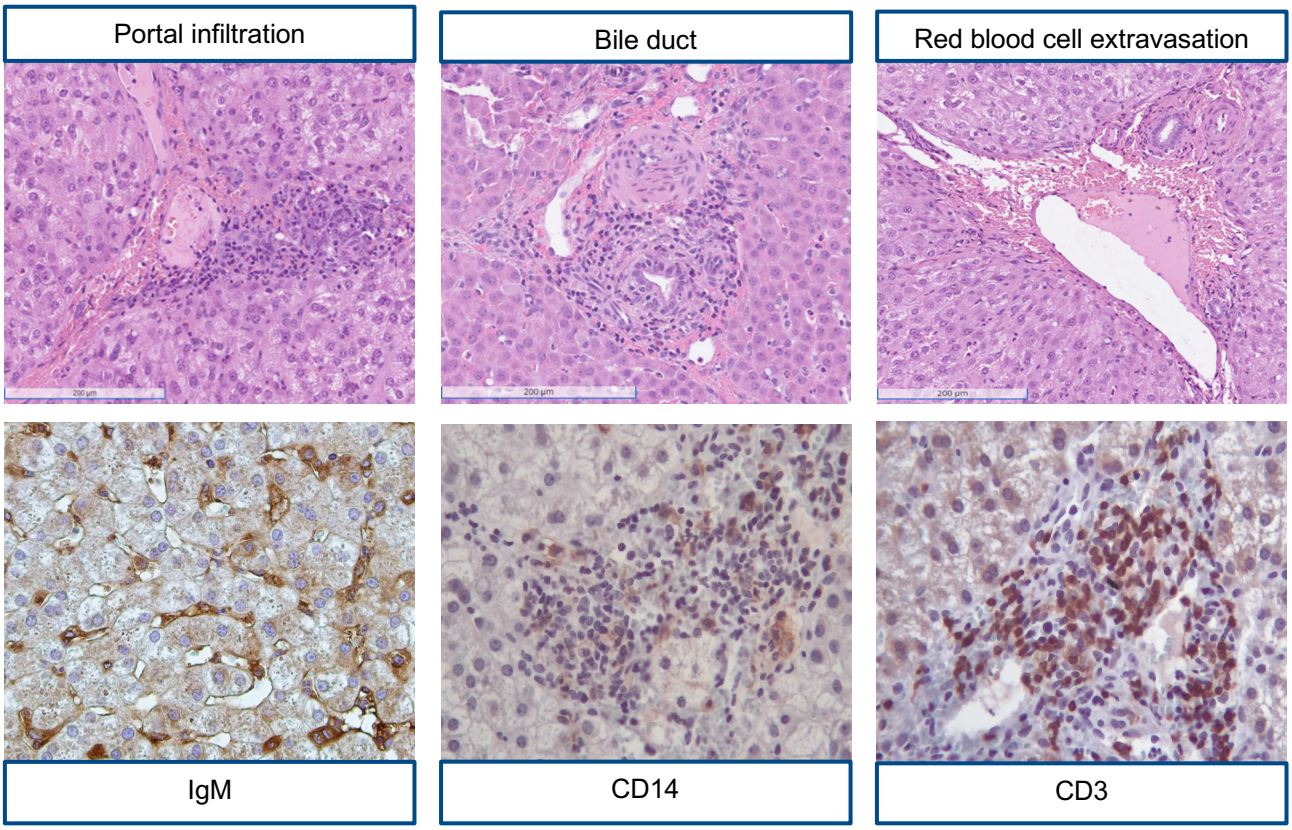


Figure 65: 72-hour xenograft histology and immunohistochemistry demonstrating: portal tract inflammation; inflammatory infiltrate of bile duct; red blood cell extravasation within portal tract; endothelial IgM staining; portal infiltration with CD14-positive and CD3-postive cells.

Timepoint	CD14		CD11b		CD57		CD3		CD19	
	Portal	Lobule	Portal	Lobule	Portal	Lobule	Portal	Lobule	Portal	Lobule
Pre-reperfusion	0 (0-0)	0 (0-0)	18 (15-23)	11 (5-17)	1 (0-3)	0 (0-0)	10 (3-18)	3 (0-7)	0 (0-0)	0 (0-0)
24 hours	7 (0-17)	11 (0-23)	28 (8-45)	52 (31-72)	1 (0-3)	2 (0-6)	55 (19-102)	7 (3-14)	0 (0-0)	0 (0-0)
48 hours	10 (3-22)	33 (8-80)	21 (6-50)	31 (6-70)	0 (0-0)	2 (0-7)	42 (30-64)	2 (1-3)	0 (0-0)	0 (0-0)
End	13 (6-21)	58 (13-136)	52 (10-145)	101 (34-200)	2 (0-6)	3 (0-12)	113 (14-222)	6 (1-20)	0 (0-0)	0 (0-0)

Table 21: xenograft biopsy immune cell immunohistochemistry scoring within the portal tract stroma and hepatic lobule pre-reperfusion (i.e. prior to isolated normothermic perfusion phase), after 24, 48 hours of ELC, and at the end of ELC (ELC 1, 3: 72 hours; ELC 2: 84 hours) for decedents 1 – 3.
Stain-positive cells were manually counted within the 400x high power field at the portal tract stroma and hepatic lobule 'hot spot'.
Mean (range). ELC: extra-corporeal liver cross-circulation.

7.5 Discussion

This study has evaluated the feasibility of 72-hour ELC using transgenic porcine livers with human brain-dead research donors. The impact on the recipients' physiology, xenograft function, the immunological response to the xenograft, and risk of zoonotic transmission has been explored. The potential for ELC using a xenograft to replace hepatic function in an anhepatic recipient for 48 hours has been assessed.

Feasibility was demonstrated by performing ELC with the recipients' native livers intact. During ELC 2 and 3, this was undertaken whilst the decedents were simultaneously supported with continuous renal replacement therapy – an important demonstration since ALF and ACLF are frequently complicated by renal failure[1,43,73].

The reduced pre-connection insulin infusion rate (3.33 IU/hour for decedents 1 – 3, 1.33 IU/hour for decedent 4) resulted in more physiological pre-connection perfusate insulin concentrations than the standard *metra* dosing regimen (see *6.5*, *figure 47*). The decedents' glucose concentrations remained stable throughout commencement of ELC. The shorter isolated normothermic perfusion phase for ELC 5 (2.8 hours *vs.* ELC 1 – 4: 7.6 (6.7 – 8.3) hours) may explain the lower-than-expected perfusate insulin concentration in this case; a pre-connection perfusate sample is not available for ELC 1 or 4. Based on these results, the pre-connection insulin infusion rate in the final ELC protocol will be 1.33 IU/hour. The recipient glucose concentration will be closely monitored over the first 2 hours of connection (see *8.8, 8.9*).

A transient transaminase and bilirubin rise occurred upon commencing ELC, attributed to mild ischaemia-reperfusion injury of the graft at retrieval. The xenografts maintained adequate perfusion parameters, bile production, and bile chemistry (indicating intact cholangiocyte function[97]). During the final six hours of ELC 3, the xenograft hepatic artery flow declined, thought to be a result of vasoconstriction associated with an increasing vasopressor dose administered to the decedent. This was resolved by direct infusion of epoprostenol into the hepatic artery inflow line (rather than the portal reservoir) to increase the effective dose delivered to the arterial tree of the donor liver. The fourth liver also experienced reduced hepatic artery flow, but due to a small donor hepatic artery that

necessitated the use of a reduced calibre cannula (DLP 3mm, Medtronic, Dublin, Ireland). This could again be partially offset by direct infusion of epoprostenol into the hepatic artery line. In this case, the decision was made to replace the liver in the interest of obtaining maximum data from the study. Direct hepatic artery infusion reduces the time for the initial 'first-pass' epoprostenol to reach the recipient from 1.15 to 0.58 minutes. There is a concomitant increase in the overall dose delivered to the recipient from 61 to 70 ng/min (0.87 to 1.00 ng/kg/min for a 70 kg recipient) (*figure 43*). This is half the starting epoprostenol dose for patients with pulmonary arterial hypertension (see *5.5*). In both ELC 2 and 4, when infusion of epoprostenol was moved from the portal reservoir to hepatic artery inflow line, no change in the haemodynamic status of the recipient was observed. This is consistent with the low predicted effective dose and the finding in the porcine ALF model that there was no difference in plasma levels of 6-keto-prostacyclin $F_{1\alpha}$ (longer-lived metabolite of epoprostenol) between intervention and control pigs (see *5.4.4*).

Metabolic testing demonstrated that hepatocyte cytosolic, mitochondrial, microsomal metabolism, and cholate clearance were augmented from the decedent baseline levels during ELC 1 – 4. The only exception was the mean value for day-3 metabolism of D_5-phenylananine. This is partly related to reduced metabolism by the second liver and partly because the baseline mean was elevated by decedent 4, which did not contribute to the day-2 and -3 means (ELC 4 lasted only 36 hours). Total cholate clearance during ELC (i.e. the decedent native livers in combination with the extra-corporeal xenografts) exceeded the range for healthy control patients undergoing the HepQuant assay (5.4 ± 1.0 mL/min/kg, *n = 50*, *personal communication*). The level of xenograft hepatocyte metabolic activity and cholate clearance measured during ELC 4 was comparable to ELC 1 – 3, despite the reduced xenograft hepatic artery flow in the former.

The hepatic parenchyma was largely preserved after 72 hours of ELC. There was limited infiltration by macrophages/monocytes, neutrophils, and T-cells; IgM was deposited without major C4d fixation or tissue damage. Previous attempts at xeno-ELC using plasma-only exchange with wild-type pig livers reported marked centrilobular necrosis with mild deposition of IgM, IgG, and complement[102]. ELC using whole blood exchange with h*CD55*.h*CD59* transgenic pig livers reported similar immune

cell infiltration (predominantly CD3-positive T-cells and neutrophils) to that observed in our study with deposition of IgG, C3 ± IgM, although the ELC durations were limited to 6.5 – 10 hours (*n* = *2*)[103]. The mild complement response in these studies has been attributed to the relative complement deficiency associated with liver failure[111]. Following 10-day heterotopic implantation of a triple glycan knock-out, h*CD46*.h*CD55*.h*THBD* transgenic pig liver into a human decedent, there was moderate IgM and IgG deposition with minimal tissue complement activation; immune cell infiltration of the xenograft was not reported. This occurred under an immunosuppression regimen including T- and B-cell depletion, and complement C5 and TNF-α blockade[205]. In our experiment, the combination of triple glycan knock-out with insertion of human immune, complement, and coagulation transgenes was sufficient to limit the xeno-immune response during 72-hour ELC using steroid-only immunosuppression (although pegcetacoplan was administered to decedent 3, the purpose was to assess its effect on xenograft-associated thrombocytopenia rather than for immunosuppression). The ability to use only mild immunosuppression will be beneficial when treating patients with ALF and ACLF, who are at increased risk of infection[1,43]. Modern perfusion technology may have contributed to better preservation of the xenograft parenchyma in our study than previous ELC reports[102].

ELC using a transgenic xenograft maintained an anhepatic recipient with stable haemodynamic status, renal function, lactate, pH, base excess, INR, and ammonia until elective termination of ELC after 48 hours. The reason for the reduced bile production by the xenograft in the final 12 hours of the post-hepatectomy ELC phase (after 62.6 hours of perfusion in total) is uncertain, but may have been due to malabsorption of bile acids administered enterally to the decedent or the longer cold ischaemic time with this xenograft (ELC 5: cold ischaemic time 5.2 hours *vs*. ELC 1 – 4: 1.5 (1.3 – 1.6) hours). After ELC discontinuation, the decedent rapidly deteriorated with manifestations of ALF, highlighting the support provided by the xenograft.

Based on the xenograft perfusion parameters, histological appearance, and functional data over the five ELC cases, ELC using xenografts would be expected to function for longer than the pre-defined 72 hours examined in this study.

The fourth decedent case demonstrated several logistical findings. Retrieval of the second liver occurred whilst ELC with the first liver was ongoing. The second liver was transported back to the study site under static cold storage ('back-to-base' strategy), as opposed to the other livers that underwent normothermic perfusion during transport ('device-to-donor' strategy). Porcine grafts tolerate cold ischaemia less well than human grafts[340], hence the preference for a 'device-to-donor' strategy. Except for reduced bile production, the second liver demonstrated adequate metabolic function despite the longer cold ischaemic time; although further investigation is required, the 'back-to-base' approach may be feasible in the future. This could reduce the logistical complexity of the process.

On arrival of the second liver at the study centre, ELC of the first liver was discontinued. The perfusion circuit was replaced, re-primed, and the isolated normothermic phase then performed per protocol. ELC was successfully resumed with the second liver after a break of 5.25 hours, confirming that it is possible to replace a xenograft with a short delay if necessary.

ELC 5 was paused whilst decedent 4 underwent hepatectomy. The 'to-patient' and 'to-*metra*' bypass lines were disconnected from the venous cannulae, which were flushed with heparinised saline to prevent clotting. The bypass lines were connected via a 1/4-1/4" straight connector to form a complete loop. The 'to-*metra*' tubing was removed from its roller pump and the ELC exchange system continued at the lowest rate (50 mL/minute): perfusate was slowly circulated from the *metra* circuit, through the bypass loop and back to the *metra* circuit by the 'to-patient' pump. The perfusion of the donor liver otherwise continued unaffected, according to the same protocol as the isolated normothermic perfusion phase (with more frequent ACT monitoring due to the perfusate being whole blood) until ready to resume ELC after 6.2 hours. This confirms that the design of the *metra*-ELC system enables temporary pausing of ELC, during which the donor liver can be successfully maintained by normothermic perfusion. When used clinically, this will permit the recipient to be moved from the ICU (e.g. for a scan) or undergo a procedure (see *below*) and resume ELC once complete.

In ELC 1 – 3, the perfusate volume used to prime the bypass lines prior to starting ELC was replaced with packed red cells. Whilst using fresh blood for this purpose in the porcine ALF study (*chapter 5*)

did not significantly alter the perfusate biochemistry, stored red cells increased perfusate potassium concentration, lactate, and reduced the pH (*table 19*). This necessitated a further period of *ex situ* haemofiltration to re-correct the perfusate potassium concentration before starting ELC. To prevent this, in ELC 4 and 5, the perfusate volume required for priming was replaced with 5% human albumin solution. The perfusate volume required to prime the bypass line is 236 mL. If the perfusate haemoglobin concentration is ≥ 82 g/L prior to priming the bypass line (and assuming a *metra* circuit perfusate volume of 1.5 L), administration of 250 mL 5% human albumin solution during priming will result in a perfusate haemoglobin concentration ≥ 70 g/L at the time of connection to the recipient (i.e. ≥ the threshold for blood transfusion for most ICU patients[341]). This approach will be used for the planned phase-1 clinical trials (*chapters 8, 9*).

The main complication, and one seen in all these cases, was rapid, severe thrombocytopenia that developed within one hour of commencing ELC. There was no clinical evidence of bleeding (except surgical site bleeding post-hepatectomy in ELC 5), although post-ELC exploratory laparotomy to exclude intra-abdominal bleeding was only performed for ELC 3 and the protocol did not include a post-ELC CT head scan to exclude intra-cranial haemorrhage. Thrombocytopenia has been reported following xenotransplantation of porcine livers into non-human primates, with various mechanisms proposed (see *6.5*)[307,316-318,320-322]. There was evidence of platelet sequestration in the xenograft in this study; the extent of platelet sequestration may be under-estimated as a result of the delay between time of platelet clearance (< 1 hour of ELC) and first biopsy (after 24 hours of ELC). Neither blocking Fc receptors nor complement inhibition significantly affected the degree of thrombocytopenia or response to platelet transfusion. Interpretation of the effect of blocking vWF with caplacizumab is limited by the pre-existing thrombocytopenia of decedent 4. Accepting the small sample size, there appeared to be a negative correlation between xenograft weight and mean platelet count. The single outlier, with a lower-than-anticipated platelet count, was the first liver of the fourth decedent, which had pre-existing thrombocytopenia. Most of the hypothesised mechanisms for xenograft-associated thrombocytopenia might be expected to correlate with liver weight (e.g. vWF load, Kupffer

cell/sinusoidal endothelial mass for phagocytosis, endothelial surface area for antibody-mediated platelet tethering).

Rather than a progressive decline over time, a floor in the platelet count was reached early during each ELC session (approximately 10 – 30 x 10^9/L). In other studies, this has been a self-limiting phenomenon: the platelet count recovered 7 days after heterotopic auxiliary liver xenotransplant in a human decedent[205], and 5 – 11 days after orthotopic xenotransplant in a non-human primate study[203]. Removal of the xenograft in the non-human primate model has been associated with rapid recovery of the platelet count[201]. Similarly, the response to platelet transfusion appeared to improve shortly after ELC discontinuation or pausing, although extrapolation must be cautious due to the heterogeneity and subtlety of the observed effect. If confirmed, it suggests that platelet clearance either occurs within the xenograft or is driven by a secreted factor with a short half-life. For example, this could be consistent with platelets adhering to endothelium-bound vWF within the xenograft, but not xenograft-derived circulating vWF driving platelet aggregation within the decedent (the half-life of vWF in plasma is about 16 hours, albeit with substantial variation[342] and this may differ in the xeno setting). An improved response to platelet transfusion shortly after ELC disconnection may prove to be an important safety feature: ELC may be temporarily paused (e.g. to facilitate intervention such as ascitic drain placement) or permanently discontinued (e.g. in the event of bleeding complication), followed by reversal of heparin anticoagulation using protamine and platelet transfusion to increase the platelet count, if indicated.

Mitigation of the risk associated with thrombocytopenia during xeno-ELC should also include careful patient selection to exclude those at greatest risk of haemorrhagic complication, minimisation of heparin dose, and strict stopping criteria. If the observed negative correlation between platelet count and xenograft weight is confirmed in a larger sample size, exclusion of xenografts above a specific weight may mitigate risk (for example, by reducing the likelihood of inducing platelet counts < 10 x 10^9/L). Ultimately, a combination of pharmacological approaches, or a plasma exchange membrane filter separating the circulations of the recipient and xenograft, may be required to reduce the severity of thrombocytopenia. This is further explored in *9.3.11*.

There was a notable requirement for red cell transfusion during ELC 2 and 3, despite the absence of clinical evidence of bleeding. This may be accounted for by the significant blood volume that was sampled from the decedents for experimental analysis: 600 mL on day 1, followed by 400 mL per day thereafter (i.e. 1.4 L over 72 hours); a further 500 mL was sampled at 72 hours in ELC 2. Blood volume was additionally sampled throughout ELC as necessary for the clinical management of the decedents. However, potential explanations might also include haemolysis and xenograft-associated erythrophagocytosis.

In ELC 2 and 3, the serum cell-free haemoglobin level remained ≤ 0.05 g/dL and schistocytes were absent from peripheral blood smears. The Extra-corporeal Life Support Organisation (ELSO) defines moderate haemolysis during ECMO as plasma cell-free haemoglobin 0.05 – 0.1 g/dL and severe haemolysis as plasma cell-free haemoglobin > 0.1 g/dL (sustained for two consecutive days)[343]. Although it is likely that a degree of haemolysis occurred, the severity appears to have been mild.

As discussed in *6.5*, previous studies have suggested that CMAH knock-out pig livers do not exhibit the profound erythrophagocytosis of human red cells that is associated with wild-type pig livers[306,308,311]. This is supported, and extended to the 3KO.7TG±RI livers, by the observation that the perfusate haematocrit remained relatively well-preserved during the xenoperfusions reported in *chapter 6*. It is reassuring that red cell transfusion was not required during ELC 1. However, definitive exclusion of erythrophagocytosis is challenging. It was not reported on pathologist review of the H&E-stained 72-hour xenograft biopsies[311], but this has been shown to be an unreliable measure in haemophagocytic lymphohistiocytosis[344]. If concern arises as experience accrues, erythrophagocytosis may be further investigated in the decedent model by a combination of Perl's staining, CD68 immunohistochemistry, and electron microscopy of xenograft biopsies[311] ± labelled red cell studies[345].

The decedent model provides a pathway between evaluating ELC in the pre-clinical large animal and *ex situ* isolated xenoperfusion models (*chapters 5, 7*), and clinical translation. As discussed in *7.1*, the pathophysiology of brain-death complicates this model. Brain-death is associated with activation of the coagulation cascade by tissue factor release[346]. All decedents had elevated d-dimers pre-ELC and decedent 4 had probable pre-existing disseminated intravascular coagulation with an International Society of Thrombosis and Haemostasis (ISTH) score of 5[347] and evidence of schistocytes on

peripheral blood smear examination prior to starting ELC. Chest sepsis in decedent 3 resulted in haemodynamic instability, and the requirement for CRRT in decedents 2 and 3 limits interpretation of the effect of xeno-ELC on decedent pH, lactate, and creatinine levels in these cases.

As with the results presented in *chapter 5*, the small sample size of this study is a limitation. In particular, the efficacy of ELC in supporting a recipient with liver failure was tested in a single case with a single model of liver failure (i.e. hepatectomy). The heterogeneity of clinical liver failure is not represented in this sample. Extrapolation of the results to the clinic must be guarded and made with these important caveats.

This study provides evidence that ELC using livers from genetically-modified pigs is feasible and can provide near-physiological levels of liver function in human recipients, suggesting that this approach may provide effective temporary hepatic support. Investigation of the mechanism of liver xenograft-associated thrombocytopenia, and potential pharmacological mitigation, remains a priority for future experimental work. Future studies will evaluate xeno-ELC with caplacizumab pre-treatment in a decedent with stable platelet counts within the normal range.

7.6 Declaration

I designed and ran the study in conjunction with the University of Pennsylvania team included under *Acknowledgements*. I performed the liver perfusions, ELC, and sample collection (with support from the Transplant Research Fellows and OrganOx clinical specialists included under *Acknowledgements*); data analysis; and interpretation.

Recipient pig surgery was performed by Professor Peter Friend, the Transplant Research Fellows, and the University of Pennsylvania team. Recipient pig anaesthesia was performed by the veterinary staff at Accuro Farms. Decedent medical management was performed by the staff at the Gift of Life Donor Care Centre. Laboratory, histological, and immunohistochemical analyses were performed in the USA, as specified in the Methods section.

8 Extra-corporeal liver cross-circulation using declined human livers in the treatment of acute-on-chronic liver failure: a safety and feasibility study (ELC-1 study design)

8.1 Research question

Is 72-hour ELC, using deceased donor human livers declined for transplantation, safe and feasible in the treatment of ACLF?

8.2 Aims

The primary aims are to test the safety and feasibility of the *metra*-ELC system using human donor livers in the treatment of ACLF (IDEAL-D Stage 1[348]).

The secondary aims are to generate preliminary effect and endpoint data, through comparison with matched historical controls receiving standard-of-care, to inform the design of an efficacy trial.

8.3 Study overview

This is a sequential, non-randomised, open-label controlled study involving 2 centres: King's College Hospital and Royal Free Hospital. The intervention group will comprise 20 ACLF patients. These will be compared with 40 matched historical controls treated at the study centres in the past 5 years. Enrolment to both groups will be restricted to patients not listed for transplant.

Once a participant has consented to the study, the Organ Donation and Transplantation (ODT) Hub (responsible for co-ordination of organ offers in the UK) will be informed and will identify potential donor livers for ELC. Such livers will be those that have been offered for transplantation but declined by all UK centres.

If a donor liver is accepted for the trial, it will be retrieved by the National Organ Retrieval Service (NORS), cold-stored, and transported to the study centre. On arrival, the donor liver will undergo initial isolated normothermic perfusion on a modified *metra* set to assess its suitability for ELC by

attainment of perfusion viability criteria. Once viability has been confirmed, *ex situ* haemofiltration of the perfusion will commence to make the perfusate electrolyte composition suitable for patient connection.

The participant will be in a study centre liver intensive care unit (ICU). A dual-lumen cannula will be placed in a central vein. The participant will receive the pre-ELC medication followed by connection to the *metra*-ELC system. The exchange rate will be increased incrementally to 400 mL/minute.

The study will consist of 2 stages:

(i) Stage 1: 5 patients, maximum of 24 hours ELC.

(ii) Stage 2: 15 patients, maximum of 72 hours ELC (if evidence at 24 hours of both clinical benefit and donor liver viability).

Planned safety reviews will occur after 28-day follow-up of participants 4 (which will inform progression to stage 2) and 9. The primary outcome will be 28-day overall survival. The total follow-up period per participant will be 90 days. The planned study length is 36 months: 9 months set-up; 18 months recruitment; 3 months follow-up; 6 months analysis/reporting (*figures 66, 67*).

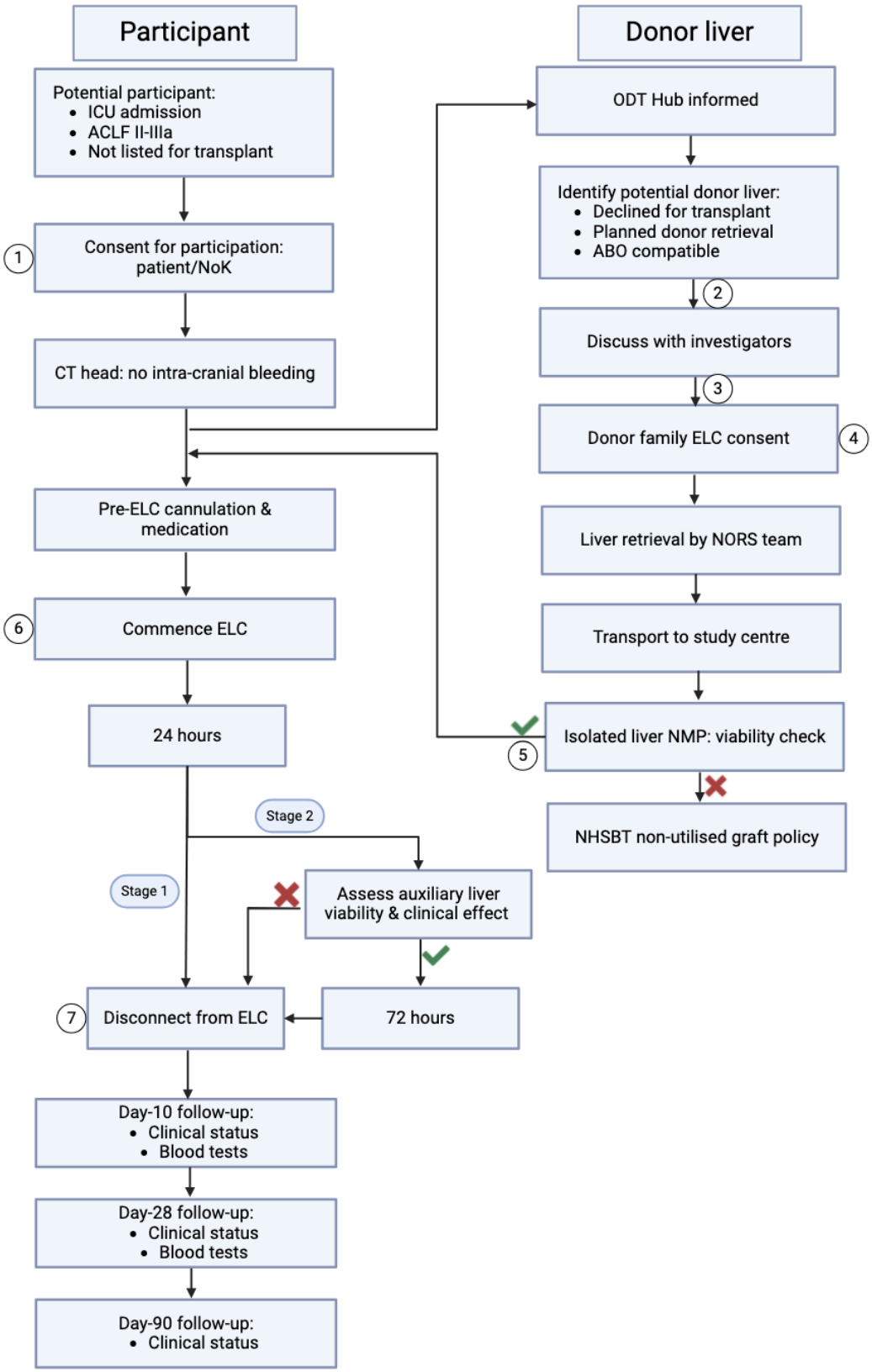


Figure 66: ELC-1 participant flow diagram.
Numbers correspond to feasibility outcomes (*table 22*): 1. Participant consent rate. 2. Liver offer rate. 3. Liver acceptance rate. 4. Donation consent rate. 5. Liver recruitment rate. 6. Participant enrolment rate. 7. ELC treatment completion rate. ICU: intensive care unit. ACLF: acute-on-chronic liver failure. NoK: next-of-kin. CT: computed tomography. ODT: Organ Donation and Transplantation. ELC: extra-corporeal liver cross-circulation. NORS: National Organ Retrieval Service. NMP: normothermic machine perfusion. NHSBT: NHS Blood and Transplant.

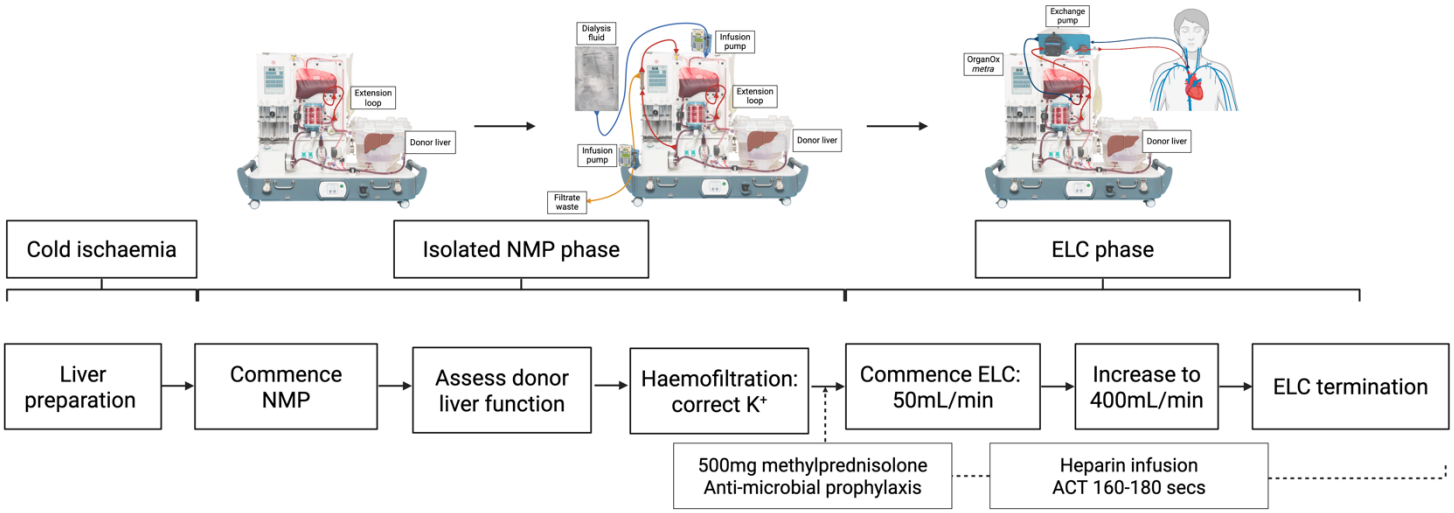


Figure 67: ELC-1 *metra*-ELC protocol.
The donor liver commences the isolated normothermic perfusion phase without *ex situ* haemofiltration. Donor liver function is assessed by attainment of normothermic viability criteria, starting at 2 hours of perfusion (up to 6 hours of perfusion if required). Once viability criteria have been attained, *ex situ* haemofiltration is commenced to bring the perfusate potassium concentration within the physiological range prior to commencing ELC. NMP: normothermic machine perfusion. K^+: potassium. ELC: extra-corporeal liver cross-circulation. ACT: activated clotting time.

8.4 Participant eligibility criteria

The participant inclusion criteria are:

(i) Age ≥ 18 years

(ii) Admitted to study centre liver ICU

(iii) Diagnosis (radiological and/or histological) of advanced chronic liver disease.

(iv) Acute decompensation event (any of hepatic encephalopathy, jaundice, and/or ascites) within 6 weeks of screening.

(v) ACLF grade II - IIIa defined per the EASL-CLIF definition[1]

(vi) Serum bilirubin > 205 μmol/L and/or West Haven hepatic encephalopathy grade 3 – 4[1].

(vii) Computed tomography (CT) head scan (within 72 hours of screening): no evidence of intra-cranial bleeding or lesion at high risk of intra-cranial bleeding

(viii) Anatomically-suitable central vein(s)

(ix) Participant informed consent, or (if participant unable to consent) written informed consent provided on behalf of participant by next-of-kin or legal representative

The participant exclusion criteria are:

(i) Listed for liver transplantation

(ii) > 7 days from diagnosis of ACLF

(iii) Mean arterial pressure < 65 mmHg refractory to administration of fluid or vasopressors/inotropes (e.g. noradrenaline dose > 0.5 μg/kg/minute, or multiple vasopressor/inotropes [excluding terlipressin if used for variceal haemorrhage and/or hepato-renal syndrome])

(iv) PaO_2/FiO_2 ratio < 200mmHg

(v) Active bleeding (transfusion > 2 units packed red cells) within 72 hours of screening

(vi) Surgery (major abdominal/thoracic/intra-cranial) within 72 hours of screening

(vii) Untreated medium-to-large oeseophageal grade 3 varices or stigmata of recent hemorrhage identified on upper gastro-intestinal endoscopy, or variceal bleed within 3 months of screening and did not receive adequate treatment with sclerotherapy or ligation

(viii) Platelets < 40 x 10^9/L or > 50% reduction within 24 hours of screening

(ix) Disseminated intravascular coagulation (International Society for Thrombosis and Haemostasis (ISTH) score ≥ 5[347])

(x) Heparin hypersensitivity or heparin-induced thrombocytopenia

(xi) Sepsis refractory to antibiotics at 72 hours (defined as recurrent positive blood cultures and/or failure to achieve appropriate clinical response) or fungaemia

(xii) Intra-abdominal sepsis (excluding spontaneous bacterial peritonitis)

(xiii) Ischaemic hepatitis

(xiv) Irreversible brain injury

(xv) Uncontrolled seizures

(xvi) Pregnancy or lactating

(xvii) Creutzfeldt-Jakob disease

(xviii) Received investigational drug or device within 30 days, or scheduled to receive another investigational drug or device in the course of the study

(xix) Active malignancy, unless adequately treated or in complete remission for five years or more

(xx) Not considered appropriate for full active treatment including organ support or with Do Not Attempt Cardio-Pulmonary Resuscitation (DNACPR) order.

(xxi) Significant extra-hepatic co-morbidity (e.g. coronary artery disease, cerebrovascular disease, pulmonary disease, chronic renal failure, serious psychiatric disease) precluding safe participation and completion of the study, determined by the site Principal Investigator (PI)

Participant eligibility criteria will be confirmed during screening and re-confirmed at the time of commencing ELC.

8.5 Participant recruitment and consent

Patients admitted to the liver ICU with acutely decompensated liver disease, and/or their next-of-kin (if lacking capacity), will be informed of the study and provided with a participant information leaflet. Patients meeting the study eligibility criteria will be approached to obtain informed consent. The informed consent discussion will be undertaken by an appropriately qualified consultant liver intensivist at the study site. The discussion will incorporate explanation of the aims, methods, potential benefits, and hazards of the study, as well as the facts that patients are under no obligation to enter the trial and that they can withdraw at any time without needing to give a reason. The patient/next-of-kin will be able to ask questions regarding the study prior to giving consent. Adequate time will be given for the patient to consider whether to provide consent; the time at which the consent documents have been given to the patient will be recorded. Once the informed consent form has been signed, one copy will be given to the patient, one copy will be placed in the medical notes, and the original will be retained at the study site. The participant will be offered the option to nominate a representative at the point of consent who will act under their presumed wishes with respect to ongoing study involvement if they lose the capacity to consent during the study; the nominee must agree to act in this capacity and this will be documented in the site medical notes. Once

appropriate consent has been provided, this will remain valid until study completion, withdrawal, or exclusion.

Consent of patients lacking capacity due to hepatic encephalopathy will be based on an advance directive or wishes previously expressed by the patient, in conjunction with next-of-kin and/or legal representative discussion: written informed consent will be obtained from the participant's next-of-kin or legal representative in accordance with local ethical and legal requirements. If a participant regains capacity, their consent will be sought for continuing involvement.

Screening assessments will be performed once informed consent has been obtained; no clinical trial procedures will be conducted prior to obtaining informed consent.

A participant is considered enrolled upon commencing ELC.

8.6 Donor liver eligibility criteria

The donor liver inclusion criteria are:

(i) Deceased donor liver offered for transplantation and declined by all UK centres

(ii) Planned organ retrieval by UK NORS team for another organ(s)

(iii) Written informed donor next-of-kin consent for liver donation for ELC

(iv) ABO blood-type compatible with participant

(v) Fulfilment of normothermic perfusion viability criteria (see *8.8*)

The donor liver exclusion criteria are:

(i) Anticipated cold ischaemic time > 10 hours

(ii) Donor malignancy precluding transplantation

(iii) Donor infection precluding transplantation

(iv) Anatomical variant precluding normothermic perfusion

(v) Retrieval injury precluding normothermic perfusion

(vi) Severe hepatic fibrosis or cirrhosis (by radiological, histological, or visual (i.e. at retrieval) assessment).

8.7 Donor liver recruitment

Once a participant has been consented and negative CT head scan confirmed, the ODT Hub will be informed. All deceased donor liver offers will be screened for the donor liver eligibility criteria; this will include donor livers declined prior to retrieval, after retrieval at the donor centre, or after retrieval at the implanting centre. The donor liver offer will be discussed with the study investigators. If the donor liver is accepted, the Specialist Nurse in Organ Donation will approach the donor next-of-kin to provide study information and seek written informed consent for liver donation for ELC. The donor liver will be transported to the study site under static cold storage using NHSBT/IMT transport.

8.8 Donor liver isolated normothermic perfusion phase

On arrival at the study site, the donor liver will undergo back-table preparation according to local standard liver transplant process in an operating theatre.

The OrganOx *metra* will be modified by insertion of the pre-portal reservoir extension tubing loop to enable connection to the ELC bypass lines. The *ex situ* haemofiltration circuit will be attached to the circuit but remain disconnected from the perfusion until viability has been confirmed.

The *metra* will be primed with 3 – 4 units of packed red cells and 500 mL 5% human albumin solution (target perfusate haemoglobin concentration > 9 g/L). The following bolus medications will be administered to the *metra*: (i) 5,000 IU heparin; (ii) 10 mL 10% calcium gluconate; (iii) anti-microbial prophylaxis (per local infectious disease team recommendation); (iv) 8.4% sodium bicarbonate to target perfusate pH 7.25 – 7.35. The following infusions will be commenced: (i) epoprostenol (8.33 μg/hour); (ii) actrapid insulin (1.33 IU/hour). The standard *metra* bile salt and heparin infusions will be omitted. Parenteral nutrition infusion (Nutriflex Special, B Braun) (4 mL/hour) will be commenced once perfusate glucose concentration is less than 10 mmol/L. Perfusate lactate, glucose, pH, sodium, potassium, ionised calcium, haematocrit, haemoglobin concentration,

and ACT will be monitored at least hourly during the isolated normothermic perfusion phase. If perfusate ACT is less than 200 seconds, 1,000 IU heparin will be administered to the perfusate.
After 2 hours of isolated normothermic perfusion, the following perfusate viability criteria will be assessed:

(i) alanine transaminase < 6000 IU/L

(ii) lactate < 2.8 mmol/L

(iii) pH > 7.2 without continuing bicarbonate supplementation.

(iv) glucose rate of fall ≥ 1 mmol/L/hour

(v) portal vein flow ≥ 500 mL/minute.

If these criteria have not been met after 2 hours, assessment will be repeated hourly up to 6 hours of normothermic perfusion. If the donor liver fails the viability criteria it will be disposed of according to NHSBT and local trust guidelines for donor livers declined prior to implantation.
If the criteria are met, *ex situ* haemofiltration of the perfusate will commence at a rate of 999 mL/hour until the perfusate potassium concentration is 3.5 – 5.2 mmol/L and the perfusate haemoglobin concentration is confirmed ≥ 82 g/L.

8.9 Extra-corporeal liver cross-circulation

A dual-lumen cannula capable of supporting flows > 500 mL/minute will be placed in an appropriate central vein of the participant. An arterial line and central venous catheter will be placed for invasive arterial pressure monitoring and intravenous medication administration respectively, if not already present.
The extension tubing loop will be connected to the ELC bypass system, and the ELC bypass lines primed using the ELC exchange pump. 250 mL 5% human albumin solution will be administered to the circuit to replace the perfusate volume used to prime the bypass lines; the portal reservoir will be confirmed to be 50 – 90% full post-priming of the bypass lines. The portal reservoir height ± approximately 100 mL will be marked. The perfusate potassium will be re-confirmed to be 3.5 – 5.2

mmol/L. The *ex situ* haemofiltration circuit will be disconnected from the *metra*. The *metra* insulin infusion will be terminated.

The participant will receive intravenous 500 mg methylprednisolone, heparin bolus (to target ACT 200 seconds), and anti-microbial prophylaxis (per local infectious disease team recommendation). The ELC exchange pump will be paused and the bypass sash divided to connect to the dual-lumen cannula (using heparinised saline to exclude all air during connection). The ELC exchange rate will be commenced at 50 mL/minute, and increased incrementally from 50 mL/minute to 400 mL/minute in 50 mL/minute increments every 10 minutes, confirming haemodynamic stability at each increment before progressing. Recipient glucose level will be closely monitored over the first two hours of ELC. Anticoagulation by heparin infusion will be titrated to an ACT 160 – 180 seconds. After the initial methylprednisolone dose, patients will receive daily intravenous 500 mg doses throughout ELC. Bile salts excreted by the donor liver will be replaced using enteral ursodeoxycholic acid (4 mg/kg three times per day).

ELC may be paused during treatment up to a maximum of 3 hours (stage 1) or 6 hours (stage 2). When paused, the donor liver will continue to be perfused *ex situ* on the isolated metra circuit.

8.10 Stage 2 24-hour continuation assessment

In stage 2, the decision to continue ELC beyond 24 hours will be dependent upon evidence at 24 hours of both:

(i) clinical benefit: improvement in CLIF-C OF score[349], individual organ score, or ammonia compared to baseline.

(ii) donor liver viability: homogenous parenchymal perfusion and portal vein flow ≥ 500 mL/minute.

8.11 ELC discontinuation criteria

ELC will be discontinued before completion of the target duration in the event of the following:

(i) Mean arterial pressure < 60 mmHg refractory to administration of fluid or vasopressors/inotropes (e.g. noradrenaline dose > 1 μg/kg/min)

(ii) Progressive respiratory failure (PaO_2/FiO_2 ratio < 150 mmHg)

(iii) Uncontrolled bleeding (> 2 units packed red blood cell transfusion over 24 hours)

(iv) Intra-cranial bleeding

(v) Platelet count < 40 x 10^9/L despite 4 pools of platelets

(vi) Disseminated intravascular coagulation, per ISTH criteria[347]

(vii) Thrombo-embolism, diagnosed by imaging and requiring treatment.

(viii) Infection specifically ascribed to ELC

(ix) Uncontrolled sepsis

(x) Uncontrolled seizures

(xi) Excessive haemolysis (serum cell-free haemoglobin > 0.1 g/dL)

(xii) Donor liver portal vein flow < 500 mL/minute for > 2 hours

(xiii) Withdrawal of participant consent

(xiv) ELC is unsafe to continue in the opinion of the PI

8.12 Safety monitoring and study stopping criteria

A Data Safety Monitoring Committee (DSMC) review will occur after 28-day follow-up of patient 4 to assess for treatment-related safety concerns. Progression to stage 2 will require recommendation by the DSMC based on this and authorisation by the Trial Steering Committee (TSC). A second safety review will occur after 28-day follow-up of participant 9, with additional reviews triggered by serious adverse events. The DSMC will evaluate adverse event, device deficiency, and ELC discontinuation data during reviews[350].

Serious adverse event reporting to the Medicines and Healthcare products Regulatory Agency (MHRA) and Research Ethics Committee (REC) by the Sponsor will be in accordance with MEDDEV 2.7/3 and ISO 14155 (2020). The study may be halted on safety grounds by the TSC, following notification by the Chief Investigator/DSMC of treatment-related safety concerns.

Causality will be assessed by medically-qualified site PIs, based on clinical judgement, device- and disease-related expected events in the Investigator's Brochure and Clinical Investigation Pack, and the presence of confounding factors (e.g. natural history of the disease, concomitant treatment/interventions, concurrent illness/risk factors). Expectedness will be confirmed by a Nominated Person, based on Reference Safety Information in the Investigator's Brochure.

8.13 Control data

Historical control data will be obtained from prospectively-maintained study centre databases. 40 historical controls will be matched 2:1 to intervention patients. A higher ratio would increase statistical power but at the expense of the quality of matching (given the size of the control database, which comprises 600 ACLF cases treated at the study centre ICUs over the last 5 years)[351].

The historical controls will be matched to the intervention group using the following criteria:

- treated at the same study centre within the past 5 years
- not listed for transplant at 'time zero'
- hierarchically matched to intervention group by: (i) CLIF-C OF score[349] at 'time zero'; (ii) age (within 5 years); (iii) leucocyte count at 'time zero' (iv) aetiology.

'Time zero' for the intervention group will be time of ELC commencement. For the controls, 'time zero' will be matched to the intervention group by number of days in ICU.

The matching criteria are predictive of prognosis in ACLF. CLIF-C OF (composite of: bilirubin; creatinine; INR; hepatic encephalopathy grade; mean arterial pressure; respiratory function (PaO_2/FiO_2)) predicts 28-day mortality[349]; age, leucocyte count, and aetiology are independent predictors[1,349].

Matching will be pre-programmed and overseen by the Clinical Trials Unit (CTU) statistical team to avoid selection bias. If more than 2 matches are available, the most recent pair will be chosen. The data will be analysed using a conditional logistic regression model to remove selection bias introduced through matching.

8.14 Outcomes

See *table 22*[352].

Outcome	Objective	Measure	Timepoints
Primary	Safety*	• Overall survival	• 28 days
Secondary	Safety[350]	• Incidence of adverse events, serious adverse events • Incidence of treatment-related adverse events, serious adverse events • Incidence of adverse device effects, serious adverse device effects, unexpected serious adverse device effects • Incidence of device deficiencies	• 90 days
	Feasibility	• Participant consent rate • Participant blood group • Liver offer rate[1] • Donor liver blood group • Liver acceptance rate[2] • Donation consent rate • Liver recruitment rate[3] • Participant enrolment rate[4] • Time from consent to ELC commencement • ELC completion rate[5] • Reason for ELC discontinuation • Data completeness per outcome	• Event
	Biochemical and organ impact*	• CLIF-C OF score** • Bilirubin** • Creatinine** • Requirement for renal replacement therapy • West Haven grade** • International normalised ratio** • Mean arterial pressure** • Vasoactive inotropic score** ▪ PaO_2/FiO_2** ▪ Requirement for mechanical ventilation	▪ Pre-connection ▪ Daily during ELC ▪ Day-1 post-disconnection ▪ Day-10 ▪ Day-28
	Clinical impact*	• Transplant-free survival • Overall survival • Length of ICU stay • Length of hospital stay • ACLF recurrence rate	• 90 days
Exploratory	Biochemical and organ impact	• Conjugated, unconjugated bilirubin**; bilirubin/albumin ratio** • Ammonia**, bi-spectral index** • Lactate**, pH**, base excess** • Alanine transaminase**, aspartate transaminase** • APTR, thromboelastogram • Fibrinogen**, factor V**, VII** • Human mercaptalbumin**, human non-mercaptalbumin-1/-2** • Indocyanine green clearance**	• Pre-connection • Daily during ELC • Day-1 post-disconnection • Day-10
	Immunological and inflammatory impact	• Blood cultures • Pro-calcitonin**; cytokines**; damage-associated molecular patterns**; endotoxin**. • White cell count**; monocyte HLA-DR expression**; lymphocyte subset counts/function. • Donor-specific antibodies; calculated reaction frequency (pre-connection and day-28)	
	Extra-corporeal liver during isolated normothermic perfusion	• Macroscopic appearance • Hepatic artery, portal vein flows • Bile production, bile pH, bile glucose • Perfusate alanine transaminase, lactate, pH, glucose, potassium, sodium, ionised calcium, haematocrit, haemoglobin • Perfusate cytokines, damage-associated molecular patterns, endotoxin, blood cultures, insulin, serum cell-free haemoglobin (pre-reperfusion and pre-ELC)	• Pre-reperfusion • Hourly during perfusion • Pre-ELC

	Extra-corporeal liver during ELC	• Macroscopic appearance • Hepatic artery, portal vein flows • Bile production, bile pH, bile production • Lactate, ammonia, and bilirubin at inflow versus outflow of *metra*-ELC • Histology (H&E, PAS, CD68, CK7); immunohistochemistry (C5b-9, C4d, C3, IgG, IgM, CD14, CD11b, CD3, CD19, CD57) (pre-NMP and end of ELC)	• Daily during ELC
	Haematological complications	• Platelet count** • Serum cell-free haemoglobin**; haptoglobin**; blood-film microscopy; lactate dehydrogenase**	• Pre-connection • 8-hourly during ELC • Day-1 post-disconnection

Table 22: ELC-1 clinical trial primary, secondary, and exploratory outcomes.
A participant is considered enrolled in the study at the point of connection to ELC. For the intervention group, baseline is defined as the start of ELC. Baseline for the control group will be matched with the intervention group for number of days in ICU.
*To be compared between intervention and control groups. **Serial data available for intervention group.
[1]no. livers offered per consented participant. [2]no. livers accepted per consented participant. [3]no. livers passing viability testing per consented participant. [4]no. participants connected to ELC per consented participant [5]no. participants that complete the intended ELC duration per enrolled participant.
ELC: extra-corporeal liver cross-circulation. ICU: intensive care unit. ACLF: acute-on-chronic liver failure. APTR: activated partial thromboplastin time ratio.

8.15 Schedule of activities

See *tables 23 – 25.*

	Screening	Pre-isolated NMP	During isolated NMP (hourly)	End isolated NMP/pre-ELC	Notes
Participant					
Informed consent	X			X	
Participant eligibility criteria	X			X	
Demographics	X				
Weight, height	X				
Blood group	X				
Medical history	X				
Liver disease history	X				
Physical examination	X				
Prior/concomitant medications	X			X	
ACLF grade	X			X	CLIF-C OF grade
Days since ACLF diagnosis	X			X	
Serum pregnancy test	X				For participants of child-bearing potential
CT head	X				
Liver function tests	X			X	ALT, AST, bilirubin (conj., unconj.), albumin.
Urea and electrolytes	X			X	Cr, urea, eGFR, Na, K, calcium, phosphorus
Full blood count	X			X	
Coagulation	X			X	INR, APTT, PT
Vital signs	X			X	MAP, HR, SaO_2, FiO_2, RR, temperature
Vasopressors, inotropes	X			X	Vasoactive inotropic score
Hepatic encephalopathy grade	X			X	West Haven grade
Renal replacement therapy	X			X	
Urine output	X			X	Urine output over previous 24 hours
Mechanical ventilation	X			X	
CLIF-C OF score	X			X	
Ammonia				X	
Bi-spectral index				X	
Thromboelastogram				X	
Fibrinogen, FV, FVII				X	
Pro-calcitonin				X	
Blood culture				X	
Donor-specific antibodies				X	
Calculated reaction frequency				X	
Exploratory SST serum				X	HMA, HNA-1/-2, storage
Exploratory EDTA plasma				X	Cytokines, DAMPs, endotoxin, storage
Exploratory citrate plasma				X	Storage
Exploratory EDTA				X	Monocyte HLA-DR expression, lymphocyte subset counts/function
ICG clearance				X	
Serum cell-free haemoglobin				X	
Haptoglobin				X	
Lactate dehydrogenase				X	
Blood-film microscopy				X	
ACT				X	
Arterial blood gas	X			X	pH, pO_2, pCO_2, HCO_3^-, base excess, lactate, sO_2
Donor liver offered	X				Donor liver offered by ODT Hub to investigators
Donor liver accepted	X				Donor liver offer accepted by investigators
Donor liver recruited				X	Donor liver passes perfusion viability check
ELC commenced				X	Participant connected to ELC
Survival				X	
AEs, SAEs				X	
Drug administration					
IV 500 mg methylprednisolone				X	
IV heparin				X	Target ACT 200 seconds
Anti-microbial prophylaxis				X	According to local microbiology recommendation
Donor liver					
Donor next-of-kin informed consent	X				
Donor liver eligibility criteria	X			X	
Donor medical history	X				
Donor liver disease history	X				
Donor blood group	X				

Donor weight, height	X				
Donor liver function tests	X				
Donor liver biopsy		X			Peripheral wedge: formalin, RNA-later, snap frozen
Cold ischaemic time		X			
Macroscopic appearance		X	X	X	Colour, perfusion homogeneity, mottling, bleeding
Hepatic artery, portal vein flow			X	X	
Bile production rate			X	X	
Bile pH, bile glucose			X	X	
Perfusate biochemistry		X	X	X	ALT, lactate, glucose, pH, K, Na, iCa, haematocrit, hb
Perfusate exploratory EDTA		X		X	Cytokines, DAMPs, endotoxin, storage
Perfusate exploratory SST		X		X	Storage
Perfusate exploratory citrate		X		X	
Perfusate blood culture				X	
Perfusate insulin concentration		X		X	
Serum cell-free haemoglobin		X		X	
Isolated normothermic perfusion duration				X	
Ex situ haemofiltration rate			X	X	
Ex situ haemofiltration duration			X	X	

Table 23: schedule of activities: participant screening, donor liver screening, isolated normothermic perfusion phase, and pre-ELC assessment.
NMP: normothermic machine perfusion. ELC: extra-corporeal liver cross-circulation. ACLF: acute-on-chronic liver failure. CT: computed tomography. SST: serum separator tube. EDTA: ethylenediaminetetraacetic acid. ICG: indocyanine green. ACT: activated clotting time. AEs: adverse events. SAEs: serious adverse events. IV: intravenous. ALT: alanine aminotransaminase. AST: aspartate aminotransaminase. Cr: creatinine. eGFR: estimated glomerular filtration rate. Na: sodium. K: potassium. INR: international normalised ratio. PT: prothrombin time. APTR: activated partial thromboplastin time ratio. MAP: mean arterial pressure. HR: heart rate. RR: respiratory rate. HMA: human mercaptalbumin. HNA: human non-mercaptalbumin. DAMPs: damage-associated molecular patterns. ODT: Organ Donation and Transplantation. iCa: ionised calcium. Hb: haemoglobin.

Time from ELC commencement	24 hours	48 hours	72 hours/end ELC	Notes
Participant				
Physical examination	X	X	X	
Concomitant medications	X	X	X	
ACLF grade	X	X	X	CLIF-C OF grade
Liver function tests	X	X	X	ALT, AST, bilirubin (conj., unconj.), albumin.
Urea and electrolytes	X	X	X	Cr, urea, eGFR, Na, K, calcium, phosphorus
Full blood count	X	X	X	8-hourly
Coagulation	X	X	X	INR, APTT, PT
Vital signs	X	X	X	MAP, HR, SaO_2, FiO_2, RR, temperature
Vasopressors, inotropes	X	X	X	Vasoactive inotropic score
Hepatic encephalopathy grade	X	X	X	West Haven grade
Renal replacement therapy	X	X	X	
Urine output	X	X	X	Urine output over previous 24 hours
Mechanical ventilation	X	X	X	
CLIF-C OF score	X	X	X	
Ammonia	X	X	X	
Bi-spectral index	X	X	X	
Thromboelastogram	X	X	X	
Fibrinogen, FV, FVII	X	X	X	
Pro-calcitonin	X	X	X	
Blood culture	X	X	X	
Exploratory SST serum	X	X	X	HMA, HNA-1/-2, storage
Exploratory EDTA plasma	X	X	X	Cytokines, DAMPs, endotoxin, storage
Exploratory citrate plasma	X	X	X	Storage
Exploratory EDTA	X	X	X	Monocyte HLA-DR expression, lymphocyte subset counts/function
ICG clearance	X	X	X	
Serum cell-free haemoglobin	X	X	X	8-hourly
Haptoglobin	X	X	X	8-hourly
Lactate dehydrogenase	X	X	X	8-hourly
Blood-film microscopy	X	X	X	8-hourly
ACT	X	X	X	Hourly
Arterial blood gas	X	X	X	6-hourly. pH, pO_2, pCO_2, HCO_3^-, BE, lactate, sO_2
ELC duration			X	
Reason for ELC discontinuation			X	
Survival	X	X	X	
AEs, SAEs, ADEs, SADEs, USADEs, DDs	X	X	X	
Drug administration				
IV 500 mg methylprednisolone	X	X	X	
Heparin infusion	X	X	X	Target ACT: 160 – 180 seconds
Anti-microbial prophylaxis	X	X	X	According to trust microbiology recommendation
Enteral ursodeoxycholic acid	X	X	X	4 mg/kg TDS
Donor liver				
Macroscopic appearance	X	X	X	Colour, perfusion homogeneity, mottling, bleeding
Hepatic artery, portal vein flow	X	X	X	
Bile production rate	X	X	X	12-hourly
Bile pH, bile glucose	X	X	X	
metra-ELC inflow *vs* outflow: lactate, ammonia, bilirubin	X	X	X	
Perfusate exploratory EDTA	X	X	X	Stored for mechanistic analysis
Perfusate exploratory SST	X	X	X	
Perfusate exploratory citrate	X	X	X	
Donor liver biopsy			X	Central wedge: formalin, RNA-later, snap frozen

Table 24: schedule of activities: during ELC.
ELC: extra-corporeal liver cross-circulation. ACLF: acute-on-chronic liver failure. CT: computed tomography. SST: serum separator tube. EDTA: ethylenediaminetetraacetic acid. ICG: indocyanine green. ACT: activated clotting time. AEs: adverse events. SAEs: serious adverse events. ADEs: adverse device events. SADEs: serious adverse device events. USADEs: unexpected serious adverse device events. DDs: device deficiencies. IV: intravenous. ALT: alanine aminotransaminase. AST: aspartate aminotransaminase. Cr: creatinine. eGFR: estimated glomerular filtration rate. Na: sodium. K: potassium. INR: international normalised ratio. PT: prothrombin time. APTR: activated partial thromboplastin time ratio. MAP: mean arterial pressure. HR: heart rate. RR: respiratory rate. HMA: human mercaptalbumin. HNA: human non-mercaptalbumin. DAMPs: damage-associated molecular patterns. ODT: Organ Donation and Transplantation. iCa: ionised calcium. Hb: haemoglobin. TDS: three times per day.

Time from ELC commencement	1-day post-disconnection	10 days	28 days	90 days	Notes
Participant					
Concomitant medications	X	X	X	X	
ACLF grade	X	X	X		CLIF-C OF grade
Liver function tests	X	X	X		ALT, AST, bilirubin (conj., unconj.), albumin.
Urea and electrolytes	X	X	X		Cr, urea, eGFR, Na, K, calcium, phosphorus
Full blood count	X	X			
Coagulation	X	X	X		INR, APTT, PT
Vital signs	X	X	X		MAP, HR, SaO_2, FiO_2, RR, temperature
Vasopressors, inotropes	X	X	X		Vasoactive inotropic score
Hepatic encephalopathy grade	X	X	X		West Haven grade
Renal replacement therapy	X	X	X		
Mechanical ventilation	X	X	X		
CLIF-SOFA score	X	X	X		
Ammonia	X	X			
Bi-spectral index	X	X			
Thromboelastogram	X	X			
Fibrinogen, FV, FVII	X	X			
Pro-calcitonin	X	X			
Blood culture	X	X			
Exploratory SST serum	X	X			HMA, HNA-1/-2, storage
Exploratory EDTA plasma	X	X			Cytokines, DAMPs, endotoxin, storage
Exploratory citrate plasma	X	X			Storage
Exploratory EDTA	X	X			Monocyte HLA-DR expression, lymphocyte subset counts/function
Donor-specific antibodies			X		
Calculated reaction frequency			X		
ICG clearance	X	X			
Serum cell-free haemoglobin	X	X			
Haptoglobin	X	X			
Lactate dehydrogenase	X	X			
Blood-film microscopy	X	X			
Arterial blood gas (if in ICU; SaO_2 only if not)	X	X			pH, pO_2, pCO_2, HCO_3^-, BE, lactate, sO_2
Survival	X	X	X	X	
AEs, SAEs, ADEs, SADEs, USADEs, DDs	X	X	X	X	
Data completeness per outcome				X	
Transplant-free survival				X	
Length of ICU stay				X	
Length of hospital stay				X	
ACLF recurrence				X	

Table 25: schedule of activities: post-ELC follow-up.
ELC: extra-corporeal liver cross-circulation. ACLF: acute-on-chronic liver failure. CT: computed tomography. SST: serum separator tube. EDTA: ethylenediaminetetraacetic acid. ICG: indocyanine green. ACT: activated clotting time. AEs: adverse events. SAEs: serious adverse events. ADEs: adverse device events. SADEs: serious adverse device events. USADEs: unexpected serious adverse device events. DDs: device deficiencies. IV: intravenous. ALT: alanine aminotransaminase. AST: aspartate aminotransaminase. Cr: creatinine. eGFR: estimated glomerular filtration rate. Na: sodium. K: potassium. INR: international normalised ratio. PT: prothrombin time. APTR: activated partial thromboplastin time ratio. MAP: mean arterial pressure. HR: heart rate. RR: respiratory rate. HMA: human mercaptalbumin. HNA: human non-mercaptalbumin. DAMPs: damage-associated molecular patterns. ODT: Organ Donation and Transplantation. iCa: ionised calcium. Hb: haemoglobin.

8.16 Data recording

Data will be recorded in real-time in the subject source documents and entered into an electronic Case Report Form within a reasonable timeframe. The CTUs (Centre for Healthcare Randomised Trials (CHaRT), University of Aberdeen and Surgical Intervention Trials Unit (SITU), University of Oxford) will manage the study database. At the end of the study, the database will be locked and analysed.

8.17 Outcome reporting and statistical analysis

Descriptive statistics will be provided for all primary, secondary, and exploratory outcomes. Categorical data will be summarised by the mean count and percentage for each variable category. Continuous data will be summarised by mean and standard deviation (parametric) or median and range (non-parametric).

The primary aim of the study is to demonstrate that ELC can be performed in the clinical environment without significant safety issues. Safety and feasibility outcomes will be descriptive in the context of published ACLF[1,75,353,354] and comparable device[11-14,16,84,85,355-357] data. The primary safety outcome, 28-day overall survival, will be presented as proportion alive at 28 days, to reflect the existing literature. Treatment-related adverse events, serious adverse events, adverse device effects, serious adverse device effects, unexpected serious adverse device effects, and device deficiencies will be summarised by treatment causality and affected organ system. The number of patients who discontinue ELC or the study, and the reason for doing so, will be summarised.

The secondary aim is to evaluate markers of clinical and biochemical effect as preliminary evidence of efficacy and to guide endpoint selection in a subsequent RCT. For this purpose, fields marked with a single asterix (*table 22*) will be compared between intervention and control groups as follows:

(i) survival data: time-to-death analysis. The method of statistical analysis will be determined by a test of proportional hazard: proportional hazards with balanced co-variates: log-rank; proportional hazards with unbalanced co-variates: Cox; non-proportional hazards: restricted mean survival times[358].

(ii) serial data: two-way repeated measures ANOVA.

(iii) single time-point data: parametric: two-tailed Student's t-test; non-parametric: Mann-Whitney U test.

For fields with a double asterix (*table 22*), serial data (pre- and during ELC) in the intervention group will be analysed by repeated measures ANOVA (parametric) or Friedman test (non-parametric) to determine the impact of ELC.

The dose effect of ELC duration on survival, clinical, and biochemical outcomes will be analysed using a method appropriate to the statistical distribution of ELC duration and outcome variable.

A CTU statistician will oversee statistical analysis. All participants who commence ELC will be included in the analysis. Statistical analysis will be performed when the study is complete; the results will be communicated to the DSMC.

8.18 Sample size determination

The sample size of 20 intervention cases reflects first-in-human study design guidance[359-361] and previous first-in-human liver support studies[11,14,85,355-357]. It has been selected to allow rigorous assessment of the main safety and feasibility outcomes, balanced against participant exposure, study duration, and cost. As reporting of the safety and feasibility outcomes will be primarily descriptive and prediction of the effect size of ELC in patients with ACLF is not possible from the pre-clinical studies, a formal power calculation is not planned.

8.19 Donor liver and participant recruitment timeline

The NHSBT Research Operational Feasibility Group (ROFG) has approved in principle access to declined deceased donor livers for the purpose of this trial. The timely availability of suitable declined donor livers and the feasibility of commencing ELC within an appropriate timeframe are important considerations of the study. 570 livers from deceased donors undergoing retrieval surgery by a NORS team are declined annually in the UK, 48% (i.e. 270 donor livers) of which may be expected to have

adequate hepatic function for ELC (see *1.10*). This corresponds to one offer with predicted adequate hepatic function every 32.5 hours.

The donor liver will be ABO compatible with the participant (i.e. O recipients can only receive O-type donor livers, whilst AB recipients can receive any donor liver). Since this study makes use of livers that cannot otherwise be used clinically and more than one ELC treatment cannot occur simultaneously, equity of access does not require that recipients are ABO matched to the donor livers in the phase-1 trial. Given the prevalence of each blood type amongst deceased donor livers declined for transplant in the UK (2001 – 2023 NHSBT registry data, see *1.10, table 4*), the median expected waiting time for a compatible liver with predicted adequate function ranges from 22.5 hours for AB participants to 50.0 hours for O participants. The expected time is less than 24 hours for the 28th percentile of participants of all blood-types (*table 26*). Assuming that the distribution of blood groups in the study centre ACLF population reflects the UK population as a whole[362], and calculating the weighted probabilities accordingly, 36% of consented patients would be expected to receive an offer of a functional liver within 24 hours. This group would comprise disproportionately more patients of blood-type A than O due to the shorter waiting time of the former: blood-group data will be collected and analysed as a component of the feasibility assessment.

The study site liver ICUs treat more than 180 patients with ACLF per 18-month period. Enrolment of 11% of patients will complete study recruitment within 18 months. This corresponds to approximately one-third of patients expected to receive a functional liver offer within 24 hours. The study limitations on the time from initial screening to ELC commencement are that the participant must still be within 7 days of ACLF diagnosis and meet the eligibility criteria at the point of connection.

Blood type	Population prevalence (%)	Declined liver prevalence (%)	Probability: liver being compatible (by recipient blood group)	Median: time to functional liver offer (hrs)	28th percentile: time to functional liver offer (hrs)	Probability: functional liver offer ≤ 24 hours
O	50	45	0.45	50.0	23.7	0.28
A	36	41	0.86	26.2	12.4	0.47
B	11	10	0.55	40.9	19.4	0.33
AB	3	4	1.00	22.5	10.7	0.52

Table 26: estimated time to a functional liver offer for participants according blood-type.
Approximately 270 livers predicted to have adequate function for ELC are declined annually in the UK (λ = 0.0308 functional liver offers per hour). Declined liver blood type prevalence data were derived from the NHSBT registry dataset for UK deceased donors where at least one solid organ was retrieved between January 1, 2001 and October 31, 2023 (*table 4*). The expected arrival rate of a compatible, functional liver, $\lambda_c = p.\lambda\ (hr^{-1})$, where p = probability of a liver being compatible for each recipient blood type. Modelling as a Poisson process, the expected median time to arrival of a compatible, functional liver $= \frac{ln\,2}{\lambda_c}\ (hr)$. The equivalent for the 28th percentile $= -\frac{ln\,0.72}{\lambda_c}\ (hr)$. The probability of receiving a liver within 24 hours, P(T ≤ 24), $= 1 - e^{-\lambda_c \times 24}$. The sum of the probabilities of receiving a liver within 24 hours weighted by the population prevalence of each blood-type provides the proportion of consented patients expected to receive a functional liver offer within 24 hours (i.e. 36%).

8.20 Study sites and staffing

The King's and Royal Free Liver units have extensive experience in ACLF research and novel extra-corporeal devices. The King's liver failure programme is one of the most active in Europe. It incorporates the only UK ECMO service for liver failure patients; this has been used as a bridge to transplantation[363]. ECMO shares technical similarities with ELC.

Building upon the local liver ICU, transplant, and ECMO infrastructure, the study has been costed to facilitate 24/7 participant recruitment and delivery of ELC, including:

(i) investigators with appropriate clinical skills (hepatology, liver transplant surgery, ECMO) at both sites.

(ii) training of study centre perfusionists/ECMO specialist nurses, hepatologists, and ICU nurses in the use of the *metra*-ELC system.

(iii) clinician time for screening of ICU admissions and informed consent of participants (by study centre liver intensivists)

(iv) hepatologist input during ELC: 3 hours for ELC commencement/initial monitoring and a further 6 hours for safety monitoring per ELC session

(v) liver transplant surgeon input: 3 hours per ELC session for liver back-table preparation and manipulation during perfusion, if required.

(vi) cardio-pulmonary perfusionist or ECMO specialist nurse: continuous *metra*-ELC device monitoring and management throughout the 24 – 72 hours of ELC (in shifts). This is in addition to the standard-of-care one-to-one ICU nursing for the participant's clinical management.

(vii) dedicated clinical research fellow: co-ordination of participant screening; consenting; donor liver acceptance, isolated perfusion and pre-ELC assessment; ELC delivery; data/sample collection and analysis under the supervision of experienced investigators.

(viii) OrganOx clinical specialists will provide on-site support in device use and trouble-shooting.

8.21 Patient and public involvement

The study design has been informed by two workshops with patient and public involvement (PPI) representatives. The workshops were followed by email communication and virtual meetings to provide more detailed feedback and review specific elements of the study. The first group (6 people with lived experience of chronic liver disease and liver failure) was recruited through the King's liver PPI group. The second group (9 people who had either received a liver transplant or provided next-of-kin consent for deceased organ donation) was recruited in collaboration with the UK ODT Research Network (ODTRN) and Donor Family Network. The two groups represent the key stakeholders who will be involved in the study: patients with liver failure who will undergo ELC and donor families who will consent for next-of-kin liver donation for ELC.

The representatives were supportive of the proposed treatment ('amazing', 'transformational'). The participant journey was reviewed through recruitment, intervention, and follow-up. The representatives were confident that this would be acceptable to patients and proportionate to the severity of the condition. For patients who may go on to lose capacity prior to recruitment, the importance of informing patients of the trial prior to developing encephalopathy (if possible) was identified.

Donor family representatives supported the use of organs that could not be transplanted. They provided feedback on the information that should be provided to donor families, including the eventual outcome of the donor liver and access to the overall study results. This informed the planned donor next-of-kin consent process and discussions with ODT.

The proposed study outcomes were reviewed to ensure that these reflect what is important to representatives. From this discussion, assessment of the donor consent process was included as a secondary outcome.

Three PPI representatives (representing patients with liver failure and the next-of-kin of organ donors) will be actively involved throughout the study. Their roles will include being members of the TSC and Trial Management Group (TMG), advising on the recruitment and consent process, co-developing participant-facing documents, reviewing screening logs, public communication, and results dissemination.

A PPI activity log will be maintained and reviewed annually in collaboration with the PPI representatives to audit this approach. Impact will be evaluated using the GRIPP2 guidelines.

8.22 Ethical and regulatory approval

The set-up period includes approvals from the Sponsor, Trust Research and Development, and parallel submission to the REC/Health Research Authority (HRA) and MHRA.

The study will adhere to the UK Framework for Health and Social Care Research. Specific ethical issues include:

(i) Enrolment of participants lacking capacity. The planned consent process (see *8.5*) is based on established guidance, liver support trial precedence[14], and PPI feedback.

(ii) Enrolment of participants with substance misuse history. Unlike transplantation, ELC is not complicated by considerations of graft utilisation or recidivism. These patients may benefit significantly from ELC due to reduced access to transplantation. It allows time for recovery, or a more detailed assessment for transplant suitability.

(iii) Disconnection of a participant benefitting from ELC. The maximum duration at each stage reflects pre-clinical testing and enables appropriate dose escalation. The 72-hour limit is the maximum duration over which the system has been evaluated in replicate in the decedent model, and therefore considered the upper limit for safe deployment in a first-in-human trial. However, it must be acknowledged that, if ELC is effective, withdrawal of therapy at 72 hours may be associated with substantial, temporally-linked deterioration that may be fatal. The possibility of deterioration post-disconnection must be explicitly discussed with the patient and/or next-of-kin during the informed consent discussion, and there must be clear communication throughout the process. There must also be recognition that deterioration post-disconnection risks moral injury to the investigators. A professional ethicist should be involved in the trial design and management. This will be combined with robust trial oversight. In cases where the potential for moral injury to investigators is recognised, structured debrief and peer support should be offered.

metra-ELC is a Class III medical device, which uses the *metra* outside its UKCA/CE mark. The study will be registered with the MHRA as a device trial, and has been designed in accordance with MHRA guidance[361].

8.23 Potential risks and mitigation

Potential risks associated with the use of the *metra*-ELC system in this phase-1 trial are outlined, with the corresponding mitigation strategies, in *table 27*.

Participant risk will be minimised through rigorous selection criteria, confirmation of graft suitability for ELC during isolated normothermic perfusion, and a phased approach to extending ELC duration. Participants will be managed and closely monitored in specialist liver ICUs, with one-to-one nursing and safety supervision provided by a liver intensivist. A professional experienced with extra-corporeal circuits (either perfusionist or ECMO specialist nurse) and trained in the use of *metra*-ELC system by OrganOx will undertake continuous device and ELC monitoring. A clinical research fellow trained in liver perfusion and ELC will co-ordinate research activities and delivery of the intervention. ELC will be safely discontinued immediately if concern arises.

The pre-clinical data, in combination with robust participant, donor liver selection, and safety monitoring, support a low risk of complications.

Risk	**Mitigation**
Haemodynamic instability	• Exchange rate will start low (50 mL/minute) and be incrementally increased, whilst confirming haemodynamic stability at each stage. • Exchange pump flow balancing software, combined with manual offset when required by professional continuously monitoring device. • The device will alarm if flow imbalance > 50 mL/minute detected and stop if flow imbalance > 100 mL/minute detected. The occlusive nature of the exchange pumps means that blood exchange will stop when the pumps pause. • Participant selection criteria exclude patients with mean arterial pressure < 65 mmHg or requiring noradrenaline > 0.5 μg/kg/minute. • Continuous participant monitoring with one-to-one ICU nursing and invasive arterial pressure monitoring.
Haemorrhage	• Heparin dose minimised: 5,000 IU initial perfusate bolus; recipient initial dose to target ACT 200 seconds; heparin infusion with target ACT 160 – 180 seconds thereafter. • Hourly (at least) ACT measurement during ELC. • One-to-one ICU nursing for early detection of clinical evidence of bleeding. • CT head pre-ELC excludes pre-existing intra-cranial haemorrhage or lesion at high-risk of bleeding. • Exclusion criteria include active bleeding or surgery within 72 hours; platelets < 40 x 10^9/L; disseminated intravascular coagulation. • ELC discontinuation criteria include uncontrolled (> 2 units packed red cell transfusion over 24 hours) and intra-cranial bleeding.
Infection	• Pre-ELC anti-microbial prophylaxis according to local policy. • Sterile technique throughout liver retrieval, transport, back-table preparation, perfusion, and ELC. • *metra*-ELC system is a closed circuit: lid of bowl containing liver only opened when absolutely necessary (e.g. to inspect liver if concern regarding flows) and using sterile technique. • Daily blood cultures may detect infection early if it does occur. • ELC discontinuation criteria include uncontrolled sepsis.
Air embolism	• Arterial filter on bypass line from *metra* to patient is filled during priming to reduce likelihood of bubble passing beyond the filter towards the patient. • Bubble detector on exchange line (after bubble filter) detects bubble, immediately switching off pump and alarming. The exchange pump is occlusive so there will be no blood flow once paused. Monitoring professional will then apply tubing clamp to vascular line and expel air bubble via bubble filter bypass line to return to the portal reservoir. There is no risk once in portal reservoir – air can be expelled via three-way tap at top of the reservoir if necessary.
Thrombo-embolism	• Heparin infusion with target ACT 160 – 180 seconds reduces likelihood of thrombosis. • Arterial filter on the bypass line to patient prevents passage of thrombo-embolic material to patient. • Portal reservoir filter prevents passage of thrombo-embolic material to donor liver. • ELC discontinuation criteria include participant thromboembolism (diagnosed on imaging and requiring treatment).
Vascular line kinking	• Professional monitoring ELC system will detect increased pressure displayed by pressure sensor.

	• If the pressure exceeds 300 mmHg, the exchange pumps will stop, preventing further exchange of blood.
Thrombocytopenia	• Exclusion criteria include platelets < 40 x 10^9/L or > 50 % reduction in platelet count within 24 hours prior to ELC. • 8-hourly monitoring of platelet count during ELC. • ELC discontinuation criteria include platelet < 40 x 10^9/L despite 4 pools of platelets. • Biocompatibility testing being undertaken to ensure compatibility of *metra* and ELC perfusion circuit with blood.
Haemolysis	• 8-hourly monitoring of serum cell-free haemoglobin, haptoglobin, blood film microscopy, and LDH during ELC. • ELC discontinuation criteria include serum cell-free haemoglobin > 0.1 g/dL.
Transfusion complication upon commencing ELC	• Packed red cells for perfusate will be provided by the study site according to local transfusion policy and standards. • Packed red cells for perfusate will be ABO-compatible with patient and donor liver. • *Ex situ* haemofiltration of perfusate prior to commencing ELC will ensure electrolyte composition of perfusate is physiological at connection. • Participant will be closely monitored at the start of ELC, including for potential transfusion reaction.
Inadequate donor liver function	• The donor liver function will be assessed during *ex situ* normothermic perfusion prior to commencing ELC. The perfusion viability criteria reflect those used to guide suitability for transplant. • During ELC, the donor liver will be monitored: vascular flows; bile production; clearance of lactate, ammonia, and bilirubin. The impact on the participants' blood results will also be monitored (but must be interpreted in the context of the underlying natural history of their illness). • ELC discontinuation criteria include donor liver portal vein flow < 500 mL/minute for > 2 hours. The donor liver may also be disconnected if there are any other investigator concerns regarding function.
Material-related toxaemia (e.g. leachables, particulate, degradation products, packaging incompatibility)	• The *metra* and ELC bypass circuit and system are undergoing biocompatibility testing to ensure that all appropriate regulations are met. • Arterial filter on ELC to patient bypass line protects against passage of particulate matter to recipient.
ELC exchange pump malfunction	• The ELC exchange pump is undergoing electrical, software, and stress testing to ensure all appropriate regulations are met.
Misuse of *metra*-ELC system	• The *metra*-ELC system will be continuously monitored and operated by a local clinical professional experienced in the use of extra-corporeal circuits (e.g. perfusionist, ECMO specialist nurse) • Staff managing the *metra*-ELC system will undergo rigorous training in the use of the device by OrganOx. • An OrganOx clinical specialist and/or engineer will be available for advice and guidance during every ELC session.

Table 27: potential risks associated with use of the *metra*-ELC system with mitigation strategies.
ELC: extra-corporeal liver cross-circulation. ICU: intensive care unit. ACT: activated clotting time. LDH: lactate dehydrogenase. ECMO: extra-corporeal membrane oxygenation.

8.24 Funding

This trial has been awarded a National Institute for Health and Care Research i4i Product Development Award grant (NIHR208501), which is due to commence in October 2025.

8.25 Discussion

This study will assess the safety and feasibility of the *metra*-ELC system in the treatment of patients with grade II – IIIa ACLF who are not listed for transplant.

Given that this will be the first-in-human use of a novel system, recruitment is limited to those not listed for transplant to ensure that study involvement does not negatively impact a patient's access to transplant (e.g. by an adverse event necessitating de-listing). Additionally, although only relevant to a small proportion of patients (~1%[20]), ACLF tier waitlist prioritisation results in transplantation within a median of 3 days: given that the median expected time to a suitable compatible donor liver offer for ELC is 1 – 2 days, this could result in enrolled participants undergoing only brief ELC prior to receiving a transplant (as occurred in the trial of the MARS device in the treatment of transplant-listed ALF patients[80]). Although being listed for transplant at enrolment is an exclusion criterion, this does not mean that patients who 'recompensate' as a result of ELC cannot be considered for subsequent transplantation[364,365].

Patients with ACLF II – IIIa will, as a result of the level of organ support required, most often be managed in an ICU. ICU admission is a stipulation of the trial to ensure appropriate monitoring of the ELC system. The high mortality of this cohort (in the absence of transplant) means that they potentially have the most to gain from ELC. Inclusion will be limited to patients within 7 days of ACLF diagnosis to maximise the potential benefit (since deaths are skewed to the first week) and ensure a more homogeneous cohort. The requirement for elevated bilirubin or hepatic encephalopathy at enrolment is to select participants with ACLF driven primarily by hepatic insufficiency, rather than renal failure[1].

The primary safety outcome is 28-day overall survival. Expected survival in this cohort is well described in the literature: 23% of patients with ACLF II – III who did not receive transplants survived 28 days in a large multi-centre observational study[75]. Substantial short-term mortality in both the intervention and control group in this study is therefore anticipated. Adverse event, adverse device effect, and device deficiency data will be interpreted in the context of this, other liver support device trials (e.g. DIALIVE[14]), and the observed benefit of ELC in this study. Adverse event data are not recorded for the historical control group so will not be compared between groups.

An important risk of ELC is haemorrhage due to the use of a heparin infusion to prevent device clotting. The heparin dose will be tightly controlled using the lowest feasible ACT target based on the pre-clinical studies; the regimen is comparable to that used in ECMO. The eligibility criteria will exclude participants at increased risk of haemorrhage and ELC will be discontinued if there is evidence of uncontrolled or intra-cranial bleeding. Although thrombocytopenia is prominent during xeno-ELC (*chapter 7*), there was no significant difference between the platelet counts of the control and intervention groups when allo-ELC was used in the porcine ALF model (*chapter 5*). Instead, a dilution effect due to the initial mixing of the recipient's circulation with the platelet-free perfusate combined with a gradual decline in platelet count (a well-recognised complication of extra-corporeal circuits[229,280]) on a background of pre-existing mild-moderate thrombocytopenia (a complication of chronic liver disease[1]) may be expected in this study. The platelet count will be monitored 8-hourly and ELC will be discontinued if it cannot be maintained above 40 x 10^9/L with platelet transfusions or disseminated intravascular coagulation occurs. Hourly ACT and daily INR, APTR, and thromboelastography will monitor overall coagulation status. The bleeding risk associated with ELC is further explored in *9.2.1*.

Feasibility at each stage of the *metra*-ELC protocol will be interrogated, including the acceptability of ELC to patients and their next-of-kin; the logistics of obtaining donor livers for ELC; timely availability of donor livers with sufficient function for ELC; the predictive capacity of the proposed donor liver perfusion criteria for function during ELC; and feasibility of delivering ELC in a clinical environment. This evaluation will occur in liver transplant centres with dedicated liver ICUs; extrapolation to other settings will require careful consideration.

Assessment of efficacy will focus on prognostically-significant parameters (e.g. bilirubin, INR, hepatic encephalopathy grade, vasopressor requirement), which will be compared between the treatment groups and measured longitudinally in individual participants. The EASL ACLF guidelines recommend using the CLIF-C OF score (composite of individual organ failure scores – see *8.13*) in the context of the CLIF-C ACLF score (which also incorporates age and leucocyte count) for longitudinal monitoring of disease severity[349,366]; the CLIF-C OF score alone has been selected as a secondary outcome in this trial because connection to ELC may impact the white cell count independently of the severity of the patient's ACLF. Being a phase-1 first-in-human trial, this study has not been powered to definitively assess efficacy. Instead, it has been designed to provide information on the potential effect size and appropriate primary endpoint in a future RCT.

The historical controls will be drawn from prospectively-maintained study centre databases that contain the necessary data. Whilst concurrent controls could enable more detailed comparison between groups (e.g. cytokine levels) and potentially better standardisation of patient management, this would limit the number of patients available for recruitment to the intervention arm. This may become relevant because the rate at which ELC will be commenced for consented patients (i.e. the enrolment rate) is not yet known (it will be an important outcome of the study). The use of concurrent controls would also reduce the quality of matching on prognostic variables: there will be 180 prospective control cases over 18 months *versus* 600 retrospective controls over the previous 5 years. This will facilitate matching separately to CLIF-C OF score, age, and leukocyte count rather than combining these variables in the single CLIF-C ACLF score[349] (which would group heterogenous patients in the same score). The intensive care management of ACLF has not changed substantially at the study centres over the last 5 years. For these reasons, the decision was taken to use historical controls. Longitudinal assessment of biochemical and clinical parameters prior, during, and after ELC allows each participant to act as their own control in determining the impact of ELC, maximising the efficacy data obtained from the trial.

The exploratory investigations will provide further insight into the mechanism of action of ELC, by evaluating the extent of functional hepatic replacement by the extra-corporeal liver (e.g. ammonia detoxification, coagulation protein synthesis, excretory function) and whether ELC impacts the

molecular basis for the systemic inflammatory response of ACLF. The extra-corporeal liver will be characterised in detail, including perfusion viability, metabolic and excretory function, maintenance of microscopic parenchymal architecture, and immunological reaction in the context of steroid-only immunosuppression. This will determine the functional capacity of the extra-corporeal livers after 72 hours of ELC. Generation of donor-specific antibodies by the recipient as a result of ELC will be assessed to understand the impact it may have on access to future liver transplantation. This will inform expansion of the indication for ELC to a 'bridge-to-transplantation' in a future trial. Demonstration of safety and feasibility in this phase-1 trial would support a subsequent multi-centre efficacy RCT. Depending on the findings of the phase-1 trial, the duration of ELC may be extended beyond 72 hours and the indication expanded to include both non-transplant and transplant candidates. The number of participants in each arm would be informed by evidence of efficacy in the phase-1 trial. To support recruitment and assess feasibility across more units, the efficacy trial would aim to run at all UK liver transplant centres. This would incorporate a full health economic analysis, including measurement of the cost of ELC, quality of life (e.g. EQ-5D-5[367], by proxy at baseline if necessary), and follow-up to 5 years to evaluate hospital and ICU stay, access to transplantation, ACLF recurrence rate, and long-term survival. Acceptability of ELC to both clinicians and recipients will be evaluated.

9 Discussion and conclusions

This thesis has developed and evaluated a system to provide temporary hepatic support by ELC using allografts and xenografts.

9.1 Key findings

i. Three *ex situ* renal replacement techniques (haemofiltration using haemoconcentrator; haemodiafiltration using standard dialysis filter; haemodialysis using high cut-off filter) can be used during normothermic liver perfusion to improve perfusate electrolyte balance, osmolality, and remove metabolic waste. High cut-off haemodialysis removes a range of cytokines from the perfusate; haemofiltration removes interleukin-6. Haemofiltration using a haemoconcentrator is the optimal technique to correct perfusate electrolyte composition prior to ELC due to greater fluid volume stability.

ii. In a porcine model of ALF, ELC using donor allografts ('allo-ELC') effectively replaced lost native hepatic metabolic, synthetic, excretory, and detoxification function. ELC maintained stable biochemical parameters, modulated plasma proteomic and metabolomic changes associated with ALF, and reduced plasma levels of damage-associated molecular patterns and interleukin-6. This translated into improved clinical hallmarks of ALF. Significant thrombocytopenia or haemolysis were not observed.

iii. Viable *ex situ* isolated xenoperfusion of transgenic porcine livers (triple glycan knock-out, seven human transgene insertion, porcine endogenous retrovirus inactivation) with pooled human whole blood can be maintained for 48 hours. Significant erythrophagocytosis of human red cells by the pig livers was not observed. The pig livers demonstrated evidence of CYP450 activity equivalent to human CYP3A4 and CYP2C19.

iv. Short duration *ex situ* isolated xenoperfusion of transgenic porcine livers may be used as model to investigate liver xenograft-associated thrombocytopenia. In a pilot study using this model, control and pegcetacoplan (C3 inhibitor) pre-treated xenoperfusions demonstrated rapid, severe

thrombocytopenia, which could be delayed by pre-treatment with caplacizumab (vWF inhibitor). Beginning within 15 minutes, *ex situ* isolated xenoperfusion is associated with widespread sinusoidal endothelial vWF expression and platelet aggregation in hepatic sinusoids.

v. In the decedent model with the native liver intact, ELC using transgenic porcine livers ('xeno-ELC') is feasible for 72 hours with steroid-only immunosuppression. The xenografts maintained adequate perfusion parameters, produced bile, and supplemented native hepatocellular function. Xenograft biopsies demonstrated largely preserved hepatic parenchyma, with mild portal tract inflammation and IgM deposition. All cases developed severe thrombocytopenia within one hour of starting ELC. There was only transient response to platelet transfusion, which was not improved by intravenous immunoglobulin or pegcetacoplan. Caplacizumab was trialled, but the impact could not be assessed due to pre-existing thrombocytopenia in this decedent.

vi. In the decedent model with the native liver removed, ELC using a transgenic porcine liver provided effective temporary hepatic support for 48 hours, with maintenance of stable haemodynamics and normal pH, lactate, ammonia, and INR until elective discontinuation.

vii. The *metra*-ELC protocol has been developed iteratively. There are separate protocols for livers with low *versus* high index of suspicion regarding severity of ischaemia-reperfusion injury (IRI). The protocols differ as to when donor liver function is assessed during the isolated normothermic perfusion phase: either before starting *ex situ* haemofiltration (high IRI risk) or at the end of the isolated perfusion phase (low IRI risk) (*figure 68*).

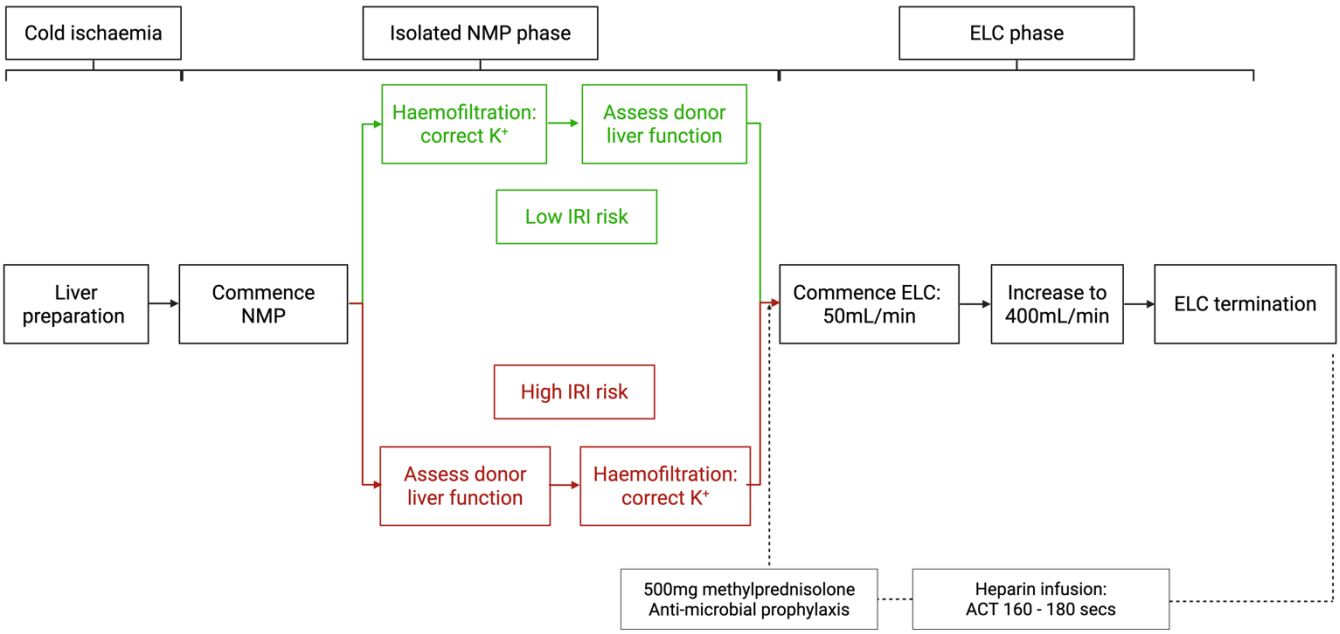


Figure 68: *metra*-ELC protocol for donor livers at low and high risk of ischaemia-reperfusion injury.
NMP: normothermic machine perfusion. ELC: extra-corporeal liver cross-circulation. IRI: ischaemia-reperfusion injury. K^+: potassium. ACT: activated clotting time.

9.2 Phase-1 clinical trials of allo-ELC and xeno-ELC: considerations

These findings provide the basis for two phase-1 clinical trials of ELC, one to evaluate allo-ELC (*chapter 8*) and the other xeno-ELC. The study design for the xeno-ELC trial reflects that of the allo-ELC trial: both will assess the safety and feasibility of ELC in the treatment of 20 patients with ACLF grade II – IIIa who are not listed for transplant at the time of enrolment. Whilst the allo-ELC trial will commence with 24-hour ELC and extend to 72-hour ELC after 5 participants, the xeno-ELC trial will start with 72-hour ELC (with a safety check after 24 hours) and increase to 120 hours after 6 participants.

9.2.1 Bleeding risk

9.2.1.1 Thrombocytopenia

Baseline platelet counts in ACLF II and III are 98 ± 67 and 77 ± 56 x 10^9/L, respectively[1]. In the porcine ALF model, in which the perfusate for the isolated phase was leuco-depleted blood, allo-ELC did not lower platelet counts relative to controls (*chapter 5*). In the phase-1 trial of allo-ELC, a decline in platelet count comparable to ECMO might be expected due to initial dilution of the recipient's circulation with platelet-free perfusate, the known effect of an extra-corporeal circuit, and pre-existing thrombocytopenia associated with chronic liver disease. Xeno-ELC will be associated with more severe thrombocytopenia. To mitigate this risk, additional exclusion criteria for the xeno-ELC trial include systolic blood pressure > 180 mmHg, previous history of cerebrovascular accident/intra-cranial haemorrhage, and dual-antiplatelet therapy. If an invasive procedure (e.g. ascitic drain) is required during either study, ELC will be paused, heparin reversed ± platelet transfusion, if indicated.

Labelled platelet studies suggest that a minimum platelet count of 7 – 8 x 10^9/L is necessary to maintain vascular homeostasis and endothelial integrity[368]. The threshold for prophylactic platelet transfusion in bone marrow failure is 10 x 10^9/L[369-372]; increasing this threshold to 20 – 30 x 10^9/L does not reduce risk of clinically-significant bleeding[373-376]. In the xeno-ELC trial, ELC will be discontinued if the platelet count falls below 10 x 10^9/L; this will also prompt trial pausing for review by the DSMC before resuming recruitment.

The most important bleeding risk is intra-cranial haemorrhage. The PLADO study randomised patients with thrombocytopenia in the context of haematological malignancy to low, medium, or high-dose platelet transfusion at a threshold of 10 x 10^9/L; platelet counts and bleeding events were recorded daily. The overall daily bleeding risk was increased at platelet counts below 5 x 10^9/L but remained similar at counts between 6 and 80 x 10^9/L[377]. The daily risk of central nervous system haemorrhage (including retinal haemorrhage, but excluding lumbar puncture-related bleeding) at each platelet count threshold is shown in *table 28*. The extrapolated risk attributed to thrombocytopenia over 3 days (i.e. the starting duration of xeno-ELC) would be less than 1% for platelet counts 10 – 30 x 10^9/L.

Platelet count (x 10^9/L)	Daily CNS bleed probability (%)	CNS bleed probability over 72 hours (%)
≤ 10	0.06	0.18
11 – 20	0.14	0.43
21 – 30	0.14	0.42
> 30	0.21	0.64

Table 28: daily probability of central nervous system (CNS) bleed from PLADO study.
CNS bleed includes retinal haemorrhage without visual impairment but excludes lumbar puncture-related bleed[377-379].

9.2.1.2 Anticoagulation

During ELC bleeding risk is compounded by simultaneous heparin anticoagulation. Estimation of the risk associated with the latter may be guided by ECMO data. The intra-cranial haemorrhage rate during ECMO using heparin anticoagulation is reported as 7.5%, 7.8%, and 21% in three studies[380-382]; the corresponding daily rates are 0.8%, 1.4% and 2.65%, and 72-hour rates are 2.3%, 4.1% and 7.9%. The higher rate reported in one study may be due to incidental imaging diagnoses in the absence of neurological symptoms[380]. The average nadir platelet counts in the three studies were 44 – 49 x 10^9/L[380-382]. ACT values were reported for two studies (mean: 458 seconds[381] and target: 180 – 220 seconds[380], respectively). One study found an association of intra-cranial haemorrhage rate with platelet count[380], whilst the other two did not[381,382].

In a study of ECMO in 29 patients undergoing liver transplant (i.e. similar to the patient population for ELC), one patient died due to pulmonary haemorrhage; there were no cases of intra-cranial haemorrhage. The median number of days on ECMO was 6.0 and the median platelet count nadir was 43 x 10^9/L; ACT values were not reported but 5/29 patients did not receive anticoagulation due to concern regarding bleeding[363].

9.2.1.3 Coagulopathy

With regard to xeno-ELC, previous non-human primate studies have been complicated by coagulopathy, which could be managed with continuous infusion of prothrombin complex concentrate[203]. In *chapter 7*, daily administration of intravenous 10 mg vitamin K during xeno-ELC maintained stable INR levels for 72 hours with the native liver intact (decedent 3) and for 48 hours post-native hepatectomy (decedent

4). The latter was achieved with one unit of FFP administered during the hepatectomy operation and one unit shortly after resuming ELC. Significant coagulopathy was therefore not observed within our 48 – 72 hours study. The difference compared to previous non-human primate studies could be explained by: (i) use of multiple gene edited xenografts (3KO.7TG.RI *versus* α-Gal knock-out alone in the non-human primate studies); (ii) use of human rather than non-human primate recipients; (iii) shorter duration of the study. INR similarly remained stable during 10-day heterotopic auxiliary implantation of a 3KO.3TG transgenic porcine liver into a human decedent[205].

In the xeno-ELC trial, the exclusion criteria include INR > 3.0, there will be regular safety monitoring of coagulation profile (prothrombin time, activated partial thromboplastin time, fibrinogen, d-dimer, thromboelastography, factor VII), and the ELC discontinuation/study pausing criteria include INR > 6.0. The protocol will include daily intravenous 10 mg vitamin K as in the decedent series.

9.2.1.4 Cirrhosis

Cirrhosis itself is a risk factor for intra-cranial haemorrhage[383]. It is not known whether this risk is additional to the background risk associated with the thrombocytopenia and coagulopathy of liver failure: in a case series of spontaneous intra-cranial haemorrhage in patients with cirrhosis, all cases had low platelets and prolonged INR[384].

9.2.1.5 Composite risk

The baseline risk of death due to intra-cranial haemorrhage in decompensated cirrhosis (including ACLF) is 0.15% over 28 days[1], corresponding to 0.016% during 72 hours. The impact of transient thrombocytopenia, heparin anticoagulation ± prolonged INR on this risk is not known. The closest parallel is ECMO, in which critically-unwell thrombocytopenic patients are anticoagulated and connected to an extra-corporeal circuit. The platelet count during allo-ELC might be expected to be similar to the described ECMO studies, resulting in a comparable bleeding risk (< 8%[363,380-382] risk of intra-cranial haemorrhage during 72-hour allo-ELC). The platelet count in xeno-ELC will be lower,

although extrapolation from studies of thrombocytopenia in haematological malignancy suggests that maintaining the counts above 10 x 10^9/L may not be associated with significant additional elevation of risk[369-377]. This risk should be interpreted in the context of the natural history of severe ACLF without transplant: 28-day survival with standard-of-care management is 23%[75]. It is reassuring that no cases of intra-cranial haemorrhage occurred in the study that evaluated ECMO peri-liver transplant[363].

In previous trials of artificial, bio-artificial liver support systems, and plasma exchange in ALF and ACLF, one case of intra-cranial haemorrhage in 600 patients (0.17%) is reported, although most studies do not explicitly report the intra-cranial haemorrhage rate (*table 29*). The single case was a subdural haematoma in a patient receiving DIALIVE therapy[14]. These therapies differ from ELC in mostly treating patients in discrete sessions rather than continuously and with (generally) more mild anticoagulation regimens. In clinical reports of ELC since 1990, there were no documented cases of bleeding, although complications were not systematically reported (*table 3*). Where the information is available, these studies used anticoagulation regimens comparable to the *metra*-ELC protocol. In a review of all published reports of ELC since 1964, bleeding was the most frequent complication (33%) and 9% of patients died due to gastrointestinal haemorrhage; the intra-cranial haemorrhage rate was not reported[29].

Therapy	Number treated (PP)	Pathology	Number of sessions	Session length (hrs)	Total duration (hrs)	Blood exchange (mL/min)	Anticoagulation	Baseline platelets ($x10^9$/L)	Baseline INR	Bleeding (%)	ICH (%)
MARS[11]	71	ACLF	6.5	6.5	42.3	100 – 250	Local policy	130	1.7	17.9 *v.* 9.6*, *p 0.15*	NR
Prometheus[12]	72	ACLF	8.1	5.7	46.2	200	RCA ± heparin (1,000 IU ± 400 IU/hr; ACT 120-150)	93	2.0	43 *v.* 49, *p 0.79*	NR
SPAD[81]	32	ALF, ACLF, PNF	2.0	7.25	14.5	100 - 150	RCA or heparin (ACT 140-200)	70	1.6	NR	NR
DIALIVE[14]	17	ACLF	3.0	8.0	24	200	Heparin 2 mL/hr (1,000 IU/hr[250])	237	1.9	5.9 *v.* 6.7	5.9 *v.* 0
ELAD[16]	96	SAH	≤ 120 hrs	Continuous	≤ 120	≤ 200	Local haemofiltration policy	NR	2.0	7.4 *vs.* 5.6**	N/A
HepatAssist[84]	80	ALF, PNF	2.9	6	17.4	100[85]	NR	NR	NR	NR	NR
HV PLEX[86]	92	ALF	2.4	6	14.4	~ 50	Local policy: heparin, epoprostenol or nil	96	5.5	No statistical difference	NR
SV PLEX[87]	20	ALF	2.15	3.9	8.4	~ 40	RCA	155	3.8	NR	NR
SV PLEX[88]	120	ALF	3	NR	NR	NR	Local policy	82	5.6	NR	NR

Table 29: liver support device clinical trials: bleeding risk.
Intervention *versus* control where applicable. Average values quoted (mean or median depending on which provided by the study). Total duration calculated by multiplying average session duration by average number of sessions. *'significant bleeding'; **'gastrointestinal haemorrhage'. PP: per protocol. INR: international normalised ratio. ICH: intra-cranial haemorrhage. HV PLEX: High-volume plasma exchange. SV PLEX: Standard-volume plasma exchange. ALF: acute liver failure. ACLF: acute-on-chronic liver failure. PNF: primary non-function. SAH: Severe alcoholic hepatitis. NR: not reported. RCA: regional citrate anticoagulation.

9.2.2 Xeno-ELC-specific considerations

9.2.2.1 Zoonotic transmission

The informed consent process will include detailed discussion of the risk of zoonotic transmission (including for close contacts). The porcine donor will be screened for an extensive panel of potential zoonoses including porcine circovirus 1 – 3, porcine lymphotropic herpes virus 1 and 2, porcine cytomegalovirus, and hepatitis E virus. The donor liver will be retrieved under sterile conditions in a dedicated surgical suite at a designated pathogen-free animal facility. Participants will be actively screened for potential porcine pathogens pre-ELC and up to one year from ELC. Archived samples will be stored for up to 10 years for future testing if needed. Informed consent will include the requirement to inform current and future close contacts of the potential risk of zoonosis; agreement to not become pregnant/impregnate partner within 2 years of xeno-ELC; agreement to not donate blood/body fluids/body parts for use in humans within 2 years of xeno-ELC. Close contacts of the participants and healthcare providers will be educated and monitored for potential zoonoses using a passive approach, for which informed consent will be obtained. Pregnant healthcare staff will not be involved in the care of the participant.

9.2.2.2 Xeno-immune response

As with the pre-clinical experiments of allo- and xeno-ELC, the only immunosuppression will be intravenous methylprednisolone. Minimisation of immunosuppression is beneficial in patients with ACLF, who are at increased risk of infection-related morbidity and mortality[1,2,68]. Xenograft immunohistochemical assessment will include immunoglobulin, complement deposition, and immune cell infiltration; recipient cytokine levels will be measured. These assessments reflect those in the allo-ELC trial, which will facilitate differentiation of the allo- from the xeno-immune response. The xeno-ELC study will additionally incorporate assays of xenograft spatial transcriptomics, peripheral immune cell phenotype, circulating complement and immunoglobulin levels to provide further characterisation. An important aim will be to determine whether steroid-only immunosuppression is sufficient in the clinical scenario and up to 120 hours. To understand whether xeno-ELC impacts future access to transplantation, the study will monitor donor-specific antibodies, calculated reaction frequency and, if the participant does go on to receive a transplant, outcome of allotransplantation up to one-year post-ELC.

During xeno-ELC the recipient may generate antibodies against proteins synthesised by the graft. This has not been studied in non-human primate xenotransplant experiments, but has been observed in patients receiving recombinant porcine factor VIII in the treatment of acquired haemophilia A, an autoimmune condition characterised by anti-human factor VIII autoantibodies: 5/18 (28%) patients treated with recombinant porcine factor VIII developed *de novo* neutralising anti-porcine FVIII antibodies after 8 – 85 days[385-387]. The extent to which this will occur during the timeframe of ELC in patients without a history of anti-hFVIII autoimmunity is not known and should be measured during the phase-1 trial.

9.2.2.3 Functional compatibility

The efficacy of xeno-ELC will be determined by the extent to which the xenograft provides life-supporting hepatic function compatible with human physiology. Previous non-human primate and decedent studies have limited this consideration to standard biochemical tests and clotting factor synthesis[203,205]. In the study of allo-ELC in porcine ALF (*chapter 5*), a combined plasma metabolomic, proteomic, tissue proteomic, and bile metabolomic approach enabled more detailed evaluation of the impact of ELC on the pathways deranged in ALF than is possible with standard biochemical tests. As a mechanistic follow-up study of the phase-1 allo- and xeno-ELC trials, this same approach (with the addition of plasma lipidomics and tissue metabolomics) will analyse exploratory samples of: participant plasma pre-, during, and post-ELC; donor liver tissue pre- and post-ELC; and donor liver bile pre- and during ELC. Accepting the limitation of a sample size of 20 in each study, this will define: (i) a plasma multi-omic signature of ACLF (through comparison with healthy control plasma samples); (ii) the impact of allo-ELC on this signature; (iii) the impact of xeno-ELC on this signature; (iv) the differential impact of xeno- *versus* allo-ELC. These findings will be interpreted in the context of changes in the tissue/bile multi-ome of allografts and xenografts during ELC.

9.3 Refinement of *metra*-ELC

The *metra*-ELC system will be refined based on the experience of the phase-1 allo- and xeno-ELC trials. Potential areas for refinement are discussed.

9.3.1 Perfusate pre-conditioning during isolated normothermic perfusion phase

During normothermic liver perfusion, cytokines and DAMPs are released into the perfusate, which correlate with the severity of IRI[224-226,388,389]. The systemic inflammatory response of ALF and ACLF is also associated with increased circulating levels of cytokines and DAMPs[14,87,236,271,390-395]. At the start of ELC, when the perfusate of the donor liver is connected to the circulation of the recipient, a potential cytokine/DAMP load may be introduced from the perfusate that could worsen the recipient's systemic inflammatory response.

Predicting the clinical relevance of this is challenging due to considerable inter-study variation in the reported levels of specific cytokines/DAMPs (*table 30*), relating to differences in the patient populations, donor livers, and assays. Although this means that direct comparison between the circulating plasma levels in ALF/ACLF and perfusate concentrations is not feasible, these studies suggest that the perfusate levels may be a similar order of magnitude to those in patient plasma. Upon mixing the perfusate and recipient circulations, the perfusate is diluted by a factor of 4.33. Nonetheless, sufficiently high concentrations of cytokines/DAMPs in the perfusate may be clinically relevant.

In the studies of ELC described in *chapters 5* and *7*, a worsening of the systemic inflammatory response was not observed; in *chapter 5*, allo-ELC was associated with lower circulating levels of interleukin-6, cell-free DNA, and extra-cellular histones. However, the ischaemic injury of the donor livers in both studies was relatively mild. Whilst this will be the case for clinical xeno-ELC, the use of declined deceased donor livers for clinical allo-ELC introduces potential for greater injury.

For the phase-1 allo-ELC trial, this risk will be mitigated by careful graft selection and the requirement to meet perfusion viability criteria comparable to those for transplantation. *Ex situ* haemofiltration during the isolated normothermic perfusion phase will remove perfusate interleukin-6, as shown in *chapter 3*. The exchange rate will start low (50 mL/minute) to minimise initial exposure of the recipient to the perfusate: the initial perfusate volume will be infused into the recipient over approximately 20 minutes. The exchange rate will be incrementally increased with close clinical monitoring to facilitate early detection of a worsening systemic inflammatory response.

Detailed analysis of the perfusate levels of cytokines/DAMPs at the end of the isolated normothermic perfusion phase, as well as plasma levels in the recipient before, during, and after ELC, will be included in both phase-1 trials. This will determine whether there may be benefit in greater clearance of these proteins from the perfusate of declined allografts prior to connection. This could be achieved by perfusate high cut-off haemodialysis (see *chapter 3*), perfusate plasma exchange at the end of isolated normothermic perfusion, or incorporation of a filter to specifically adsorb cytokines (e.g. Cytosorb[393] (CytoSorbents Corporation, Princeton, NJ, USA)), DAMPs (e.g. NucleoCapture (Santersus AG, Zürich, Switzerland) to adsorb nuclear DAMPs[224]), or a combination of cytokines, DAMPs (e.g. HMGB1), and

PAMPs (e.g. endotoxin) (e.g. the oXiris filter (Baxter, Deerfield, IL, USA)[14,396]) during isolated perfusion.

Cytokine/DAMP	ALF (pg/mL)	ACLF (pg/mL)	NMP perfusate (pg/mL)
Il-6	54 – 458[87,394,395]	31 – 385[14,393,395] *	277 – 706[225,388,389]
TNF-α	11 – 45[87,394]	8[14]	21 – 169[225,389]
HMGB1	121 x 10^3 [87]	4 – 83 x 10^3 [236,390]	119 x 10^3 [226] **

Table 30: reported average levels of illustrative cytokines/DAMPs (interleukin-6, TNF-α, HMGB1) in ALF, ACLF, and perfusate during normothermic liver preservation.
Inter-study variation is more substantial for cell-free DNA, histones, and nucleosomes; these have not been included in this table. *Additional outlier study reported average interleukin-6 concentration in ACLF as 77 ng/mL[391]. ** Porcine liver normothermic perfusion study (NB: HMGB1 perfusate concentration units variably reported as pg/mL and ng/mL in this publication). ALF: acute liver failure. ACLF: acute-on-chronic liver failure. NMP: normothermic machine perfusion. DAMP: damage-associated molecular pattern.

9.3.2 Optimisation of ELC exchange rate

The MARS, Prometheus, and DIALIVE artificial liver support systems exchange blood between recipient and device at rates of 100 – 250 mL/minute. The effectiveness of a given exchange rate depends upon the clearance efficiencies of their respective albumin dialysis, adsorption, and exchange systems, which are not publicly available[11,12,14]. In plasma exchange, plasma is exchanged at a rate of 1.5 L/hour, corresponding to 25 mL/minute (equivalent to 42 mL/minute blood)[86,87].

Most reports of ELC from the 1990s do not explicitly state the exchange rate between recipient and device. Two of the studies that use a direct exchange system between recipient and graft (i.e. without a capacitance reservoir or plasma separator) report graft perfusion rates of 500 – 700 mL/min/kg graft[109] and 800 mL/minute[103]. For the former, although graft weight is not provided, the porcine donors were 11 – 14 kg. Assuming a liver weight 2% of body weight, the grafts weighed about 0.25 kg and flows were approximately 150 mL/minute[397]. At these rates, Chari et al. found consistent improvement in bilirubin, ammonia, and hepatic encephalopathy[109]; Levy et al. reported decreased levels of bilirubin but inconsistent impact on ammonia and INR[103]. However, based on the short duration of these perfusions (0.5 – 10 hours), graft viability likely had a greater impact on biochemical effect than the exchange rate.

In the pre-clinical *metra*-ELC studies, an exchange rate of 400 mL/minute was sufficient to provide effective temporary hepatic support in pigs with complete inflow hepatic devascularisation (*n = 6, chapter 5*) and an anhepatic decedent (*n = 1, chapter 7*). In both contexts this exchange rate maintained stable ammonia, lactate, pH, INR, haemodynamics, and, in the porcine study, bilirubin and bi-spectral index. In the decedent study, rising bilirubin was secondary to lack of bile production by the xenograft in the final 12 hours; increasing the exchange rate in the absence of bile production would not clear more bilirubin. These data suggest that an exchange rate of 400 mL/minute is sufficient to replace absent native hepatic function, at least for 24 – 48 hours.

This can be explored theoretically by considering the clearance capacity of the *metra*-ELC system compared to a healthy native liver. An exchange rate of 400 mL/minute is 27% of physiological hepatic blood flow (1500 mL/minute[398]). However, the donor liver is perfused at close-to physiological flows (*chapter 5* mean: 1300 mL/minute) and the design of the *metra*-ELC system means that blood from the patient can pass through the donor liver multiple times. Assuming an exchange rate 400 mL/minute and donor liver vascular flows equivalent to the *chapter 5* mean values (portal vein flow: 1000 mL/minute; hepatic artery flow: 300 mL/min), the fraction of blood circulating on the *metra* that returns to the patient on each pass is 30.8% ($\frac{400}{1300}$), whilst 69.2% ($\frac{900}{1300}$) completes a further pass. The fraction of blood that passes through the liver *n* times, f_n, is $0.308 \times 0.692^{n-1}$. Computing the geometric series over the f_n weights, if *H* is the extraction ratio of the donor liver for a substance of interest, the effective extraction ratio of the *metra*-ELC system (*M*) at an exchange rate of 400 mL/minute, is given by:

$M = 1 - \frac{0.308 \times (1-H)}{1-0.692 \times (1-H)}$ (*figure 69*). The device clearance then equals $M \times 400\ (\mathrm{mL/min})$. Assuming the extraction ratio of the donor liver, *H*, is equivalent to a healthy native liver, physiological hepatic clearance would equal $H \times 1500\ (\mathrm{mL/min})$. The fraction of physiological hepatic clearance provided by the *metra*-ELC system at an exchange rate of 400 mL/minute therefore equals $0.27 \times \frac{M}{H}$.

Table 31 shows the clearance of substances with different extraction ratios by a healthy liver and the *metra*-ELC system at exchange rates of 400, 500, and 800 mL/minute. *Table 32* displays the clearance by the *metra*-ELC system as a percentage of the clearance by a healthy liver. For a given substance, the extraction ratio of the *metra*-ELC system is higher at lower exchange rates because more blood passes

through the liver multiple times. This effect is more pronounced for substances with lower first-pass extraction. However, increasing the exchange rate more than offsets the reduction in extraction ratio. The *metra*-ELC system therefore provides greater overall clearance of substances at higher exchange rates.

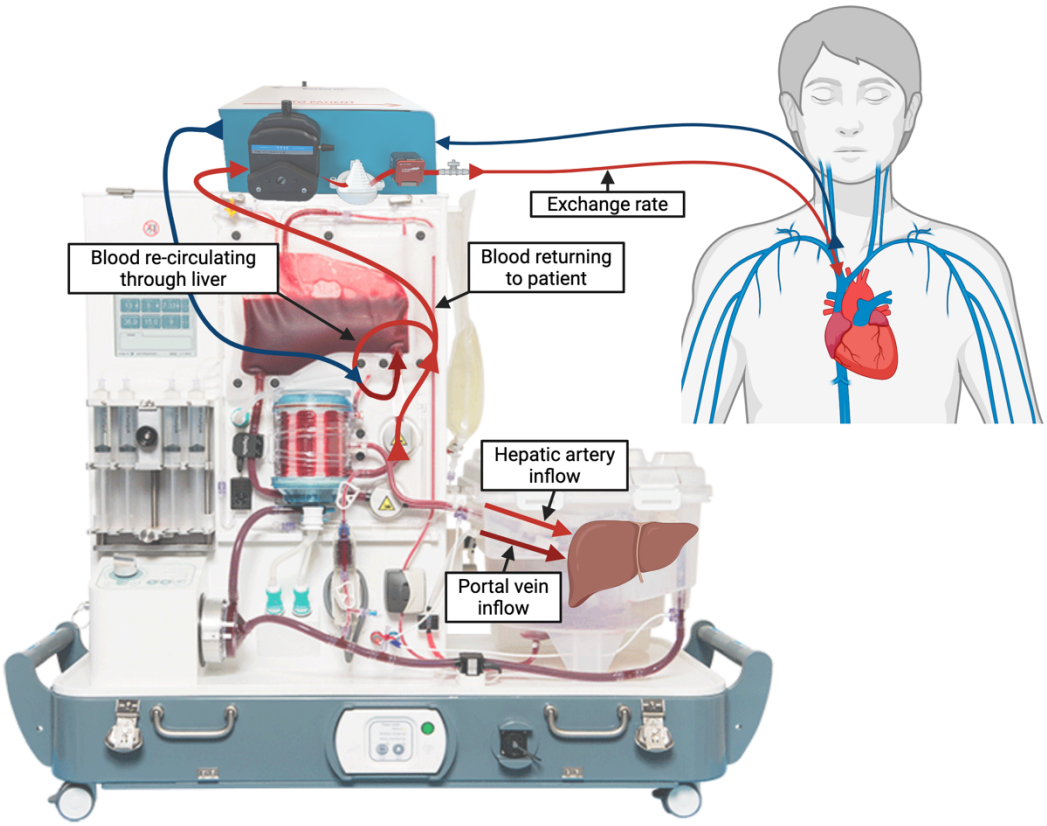


Figure 69: fractional blood exchange between device and recipient.

With each pass through the liver, the fraction of blood that returns to the patient equals $\frac{exchange\ rate}{hepatic\ artery\ flow+portal\ vein\ flow}$. The fraction of blood that recirculates through the liver equals $1-\frac{exchange\ rate}{hepatic\ artery\ flow+portal\ vein\ flow}$. Using the mean values from the allo-ELC experiment described in *chapter 5* and an exchange rate of 400 mL/minute, the fraction returning to the recipient equals $\frac{400}{300+1000}=0.308$ and the fraction re-circulating equals 0.692. The fraction re-circulating exactly twice equals $0.692\times0.308=0.213$. The fraction re-circulating n times, f_n, equals $0.308\times0.692^{n-1}$.

On each occasion that blood passes through the donor liver, the fraction of a substance with donor liver extraction ratio H that remains equals $1-H$. After n passes, the fraction remaining equals $(1-H)^n$. The fraction that has been cleared by the donor liver after n passes therefore equals $1-(1-H)^n$.

To determine the overall extraction ratio of the *metra*-ELC system for this substance, the fraction of blood that passes through the liver n times and the fraction of the substance cleared after n passes must be combined over infinite passes. This is the sum of the series between $n=1$ and $n=$ infinity of:

$0.308\times0.692^{n-1}\times(1-(1-H)^n)$, which computes as:

metra-ELC system extraction ratio (at exchange rate 400 mL/minute), $M=1-\frac{0.308\times(1-H)}{1-0.692\times(1-H)}$.

Healthy liver		*metra*-ELC at exchange rate 400 mL/min		*metra*-ELC at exchange rate 500 mL/min		*metra*-ELC at exchange rate 800 mL/min	
Extraction ratio (*H*)	**Clearance (mL/min)**	**Extraction ratio (*M*)**	**Clearance (mL/min)**	**Extraction ratio (*M*)**	**Clearance (mL/min)**	**Extraction ratio (*M*)**	**Clearance (mL/min)**
1.0	1500	1.0	400	1.0	500	1.0	800
0.9 (ammonia[399])	1350	0.97	390	0.96	480	0.94	750
0.7 (cholate[400])	1050	0.88	350	0.86	430	0.79	630
0.5 (lactate[401])	750	0.77	310	0.72	360	0.62	495
0.3 (midazolam[402])	450	0.58	230	0.53	260	0.41	330
0.05 (bilirubin[403])	75	0.15	58	0.12	60	0.08	63

Table 31: estimated clearance of substances with different extraction ratios by a healthy liver and the *metra*-ELC system at exchange rates of 400, 500, and 800 mL/min.
This calculation assumes: (i) donor liver has physiological hepatic function at all exchange rates; (ii) portal vein flow: 1000 mL/min, hepatic artery flow: 300 mL/min; (iii) physiological hepatic total blood flow: 1500 mL/min.

Healthy liver	*metra*-ELC system: % of normal hepatic clearance			
Extraction ratio (*H*)	**400 mL/min**	**500 mL/min**	**800 mL/min**	**1000 mL/min**
1.0	27	33	53	67
0.9 (ammonia[399])	29	35	55	68
0.7 (cholate[400])	34	41	60	72
0.5 (lactate[401])	41	48	66	75
0.3 (midazolam[402])	52	59	73	80
0.05 (bilirubin[403])	78	80	84	85

Table 32: estimated percentage of healthy hepatic clearance of substances of different hepatic extraction ratios provided by the *metra*-ELC system at exchange rates of 400 mL/min, 500 mL/min, 800 mL/min, and 1000 mL/min.
This calculation assumes: (i) donor liver has physiological hepatic function at all exchange rates; (ii) portal vein flow: 1000 mL/min, hepatic artery flow: 300 mL/min; (iii) physiological hepatic total blood flow: 1500 mL/min.

For substances with high extraction ratios in a healthy liver (e.g. ammonia), the *metra*-ELC system provides 29%, 35%, and 55% of normal hepatic function at 400, 500, and 800 mL/minute, respectively. For substances with low extraction ratios (e.g. midazolam), these values are 52%, 59%, and 73%, respectively. This assumes that the donor liver function and total flow remains constant at higher exchange rates in the presence of greater deoxygenation of the portal vein (see *2.4*). The upper limit of the exchange rate is the portal vein flow because the pre-portal reservoir line supplies the bypass line: the portal vein flow was about 1000 mL/min with perfused pig livers in the porcine ALF and decedent models (*chapters 5, 7*).

The extraction ratio of cholate for a human liver is approximately 0.7[400]. Using the described theoretical approach, at an exchange rate of 400 mL/minute, the *metra*-ELC system is predicted to clear cholate at a rate 34% of a healthy liver. The daily HepQuant data for ELC 1 – 4 demonstrate that the *metra*-ELC

system cleared ^{13}C-cholate at a rate 44.8 ± 22.8% of the decedent's native liver; excluding two out of the ten daily results that were anomalously high gives a value of 38.7 ± 5.2% (*chapter 7*). Although a discrepancy exists between the predicted and measured values, given the multiple assumptions inherent in the calculations and potential differences in cholate clearance between human and pig livers, these data provide preliminary support for the proposed approach to estimating effective hepatic function at different exchange rates. Actual clearance will require empirical determination.

Whereas a substantial first-pass effect is observed with hepatic detoxification, metabolic, and excretory function, the time course for hepatic synthetic function is much slower. The shortest half-life of a clotting factor (factor VII) is about 5 hours. This is multiple orders of magnitude greater than the blood circulation time on the *metra* (1.15 mins) or the time for blood to exchange between recipient and *metra* device (18 seconds). The donor liver does not substantially contribute to the clearance of the proteins it produces. For these reasons, essentially all of the proteins produced by the donor liver reach the recipient regardless of exchange rate or the number of passes through the liver: it can be assumed that the *metra*-ELC system delivers 100% of the hepatic synthetic function provided by the donor liver.

The liver exhibits considerable functional reserve. In liver transplantation, a graft-to-native liver volume ratio > 30 – 40% (or graft-to-recipient weight ratio > 0.8%) is recommended to avoid small-for-size syndrome[404-407], although smaller grafts have been used successfully[408]. In the context of hepatic resection with a healthy native liver, a future liver remnant 20 – 30% of the initial liver volume is the lower limit considered safe, with the reported incidence of post-hepatectomy liver failure being generally less than 6%[409-414]. In ALF, > 75% hepatocyte necrosis is associated with increased mortality without a transplant[415]. In the porcine model, resection up to 70 – 80% liver volume can be tolerated, whilst 90% resection is associated with liver failure[416,417]. Based on these studies, the fraction of normal hepatic function to maintain life is approximately 20 – 30%. Significantly, a major component of the pathophysiology of small-for-size syndrome is abnormal portal haemodynamics generated by size discrepancy rather than absolute metabolic insufficiency[407,418]; this is not an issue with the *metra*-ELC system, which maintains physiological haemodynamics of the graft.

The threshold of normal hepatic function required to support different liver activities may vary. Bilirubin and INR are the most sensitive indicators of small-for-size syndrome after liver transplantation and heptacetomy[404,419,420]. This may suggest that bilirubin excretion and synthetic capacity require greater fractions of residual liver function than other activities, or simply reflect the discriminative utility of these tests in the clinical environment. There does appear to be greater reserve capacity for ammonia clearance: resections up to 75% in livers with good background parenchymal function are associated with rapid adaptive increases in ammonia clearance per gram of liver tissue, which prevent a rise in systemic ammonia levels[421]. This concept of function-specific thresholds for residual liver activity has not been systematically investigated and so remains speculative.

At an exchange rate of 400 mL/minute, the predicted clearances by the *metra*-ELC system of ammonia, lactate, and bilirubin are, respectively, 29%, 41%, and 78% of those provided by a healthy liver (*table 32*); synthetic function is approximately 100% independent of exchange rate. The fact that these activities equal (ammonia detoxification) or exceed (lactate metabolism, bilirubin excretion, synthetic function) the lower limit for residual liver volume supports the empirical observation from *chapters 5* and *7* that an exchange rate of 400 mL/min is sufficient to support recipients with absent native hepatic function. Patients with ALF and ACLF will likely have some residual hepatic function. In practice, the optimal exchange rate will need to be determined in early-stage trials based on clinical impact, and may be specific for the individual patient. This analysis will be guided by the effect of ELC on recipient biochemical data (lactate, pH, bilirubin, INR, ammonia); the gradient of lactate, ammonia, and bilirubin across the donor liver; indocyanine green clearance; and perfusion haemodynamic parameters. The aim is to use the minimum exchange rate necessary to provide effective hepatic support. This might exceed 400 mL/minute for specific patients. Ensuring reliable exchange volume stability at rates greater than 400 mL/minute will require further optimisation of the ELC exchange balance system.

9.3.3 Improved ELC exchange flow balancing

The current ELC exchange system balances flows between recipient and *metra* by real-time measurement of blood flow through flow sensors. Due to the significant flows (400 mL/minute), a small

proportional error in flow detection results in clinically meaningful volume shifts that will be poorly tolerated by critically unwell patients with liver failure: a 2.5% flow error at 400 mL/minute results in a 200 mL blood volume shift over 20 minutes. In the planned phase-1 trials, this risk is mitigated by continuous supervision by a professional trained in extra-corporeal devices and the *metra*-ELC system, who will manually offset the exchange pumps if a volume imbalance is noted based on a changing portal reservoir filling height. The degree of filling of the portal reservoir reflects the volume status of the *metra* device.

Whilst there is room for further optimisation of the exchange system flow sensing system, a small error of flow detection is inevitable. Changing the exchange system algorithm to balance flow according to portal reservoir filling may improve volume balance by integrating flow discrepancy over time. In this case, the exchange system algorithm would be integrated with the *metra* software to suspend the portal reservoir volume metric during an IVC collapse episode of the donor liver (IVC collapse results in transient decrease in portal reservoir filling independent of exchange volume balance status, as blood volume is temporarily taken on by the donor liver).

9.3.4 Optimisation of anti-coagulation regimen

The current *metra*-ELC anticoagulation regimen comprises: (i) 5,000 IU heparin bolus administered to the *metra* circuit at the start of the isolated normothermic perfusion phase; (ii) heparin bolus administered to recipient to target ACT 200 seconds prior to commencing ELC; (iii) heparin infusion titrated to ACT 160 – 180 seconds during ELC.

Minimisation of anticoagulation burden will reduce the risk of haemorrhagic complications. The current regimen has been developed iteratively by gradually reducing the dose over the course of the experiments described in this thesis. In this context, clotting of the device would end the experiment prematurely, whereas recipient bleeding could be managed. The preference was for the anticoagulation regimen to ‘fail high’ rather than ‘fail low’, reflected in the fact that the circuit did not clot in any experiment.

This preference is reversed in the clinical context. Whilst the current regimen compares favourably to ECMO dosing[380-382], there may be room for reduction: in the decedent studies, the perfusate ACT remained > 1000 seconds throughout isolated normothermic perfusion following the 5,000 IU heparin bolus and there were periods of several hours during ELC where the ACT was < 160 seconds without the device clotting. Further optimisation of the anticoagulation regimen will be guided by the phase-1 clinical trial outcome data (principally, rates of bleeding and device clotting) combined with coagulation assay data. Due to the complex haemostatic status of patients with ACLF, the latter will combine clinical assays (ACT, INR, activated partial thromboplastin time, thromboelastogram) with a follow-up mechanistic study that undertakes detailed analysis of specific coagulation components using the exploratory plasma samples (thrombin generation assay, clot lysis time, fibrin clot quality, vWF, ADAMTS13, factors II, VII, VIII, X, XIII, fibrinogen, antithrombin, plasminogen, thrombin-antithrombin complexes, plasminogen activator inhibitor type 1)[422,423].

Previous artificial liver support systems have used regional citrate anticoagulation as an alternative to heparin (*table 29*). Regional citrate anticoagulation was developed in the context of dialysis as a technique to minimise exposure of the patient to anticoagulation. Citrate is infused into the outflow line from the patient to chelate calcium and prevent clotting on device. The citrate-calcium complexes are mostly filtered out by the dialysis system. Calcium is replaced by an infusion into the return line to the patient. Any residual citrate is metabolised by the patient's liver. Potential complications include hypocalcaemia and metabolic alkalosis (as bicarbonate is generated during citrate metabolism)[424]. Although regional citrate anticoagulation is more challenging in patients with liver failure, it is possible[425]. The primary obstacle to using regional citrate anticoagulation for ELC is that there is a functioning liver on the device that needs to be anticoagulated. The perfused donor liver will metabolise citrate, increasing the risk of device clotting and rendering the perfusate alkalotic. Conversely, the donor liver will be exposed to a low perfusate ionised calcium concentration that may adversely impact function. Regional citrate anticoagulation is therefore likely not feasible for ELC.

9.3.5 Centrifugal exchange pumps with pinch valve safety shut-off

The current ELC exchange system uses roller pumps. Since the pumps are occlusive, this design ensures no flow if the system is paused, which is a vital safety feature. There is evidence to suggest that at the flows used for ELC, roller pumps are associated with less haemolysis than centrifugal pumps[215-217]. Indeed, excessive haemolysis was not observed in the porcine ALF ELC study (*chapter 5*). However, if excessive haemolysis is observed in the phase-1 trials, an option to consider would be to switch to centrifugal exchange pumps. To prevent blood flow under gravity when the exchange is paused, this would require incorporation of automated occlusive pinch valves (beyond the bubble sensor) that close when the pump is paused.

9.3.6 *metra* circuit refinement

The *metra* is CE-marked for normothermic liver perfusion of human livers up to 24 hours. In the experiments of this thesis, transgenic porcine livers have been perfused up to 48 hours *ex situ* and up to 92 hours with ELC (8 hours isolated normothermic perfusion + 84 hours xeno-ELC for decedent 2). To facilitate 48-hour *ex situ* perfusion, the standard *metra* circuit was modified to incorporate *ex situ* haemofiltration. During ELC, many of the physiological homeostatic mechanisms that are lacking during *ex situ* perfusion are provided by the recipient, supporting prolonged perfusion despite the added challenge of the intact immune system. Nonetheless, several potential modifications to the *metra* circuit may support longer durations of ELC:

i. IVC collapse feedback algorithm refinement. During liver perfusion on the *metra*, the negative pressure generated by the centrifugal pump acting on the IVC outflow can collapse the IVC. This occurs in the event of a temporary reduction in IVC outflow (the centrifugal pump rate is transiently too high relative to the actual flow) or due to the anatomical position of the IVC cannula (the openings of the cannula oppose the endothelium of the IVC rather than the ostia of the hepatic veins, as intended). The standard *metra* algorithm resets the centrifugal pump to a slow rate (750 rpm) before gradually increasing back to a lower set-point than the original that generated the collapse. During an IVC collapse, the liver temporarily takes on more blood volume and there is a transient reduction in flow as the centrifugal pump speed returns towards the set-point. A newly

developed *metra* algorithm adjusts the centrifugal pump in a more responsive manner to negative IVC pressure without a major reduction in pump rate. This results in fewer IVC collapse episodes and less perturbation in flow if an episode occurs (*H. Sadik, B. Kaye, K. Gyoten: unpublished data*).

ii. Physiological portal vein pressure is 5 – 10 mmHg[426]. Portal vein pressure when perfused on the *metra* is determined by the height of the portal reservoir, and frequently exceeds 10 mmHg. The standard *metra* circuit includes a proportional pinch valve on the portal reservoir-to-portal vein line. The standard algorithm uses this as an 'all-or-nothing' valve, closing in response to an empty portal reservoir to prevent air embolism. A novel algorithm incorporates a portal vein pressure sensor and closed loop feedback of the portal pinch valve aperture to maintain physiological portal vein pressures (*H. Sadik, B. Kaye, K. Gyoten: unpublished data*).

This may be particularly relevant to xeno-ELC. In *chapters 6* and *7*, there was histological evidence of red cell extravasation on xenograft biopsies (*figures 48*, *65*). A similar phenomenon has been observed in wild-type porcine liver and kidney perfusions with autologous blood, suggesting that this is neither a xeno- nor liver-specific phenomenon. One explanation may be that porcine endothelium is more vulnerable to excess pressure than the human donor livers for which the *metra* is optimised. In particular, the porcine donors are more juvenile than the human donors with potentially less well-developed vascular supportive connective tissue. Additionally, *in vivo* hepatic arterial resistance varies with portal vein flow: reducing portal vein flow increases hepatic artery flow ('hepatic arterial buffer response[427]'). This is preserved during *ex situ* perfusion[428]. During xeno-ELC, hepatic artery flows at the lower end of the target range were observed, partially related to the smaller hepatic artery of the Yucatan minipig compared to a human and partially related to administration of vasopressors to the decedents. Maintenance of more physiological portal vein pressures during xeno-ELC may improve hepatic artery flow.

iii. *In vivo* the portal vein supplies the liver with partially deoxygenated blood; physiological portal vein oxygen saturation ranges from 85% in the fasting state to lower values post-prandially[426]. In keeping with this, in the *chapter 5* study the pO_2 and pCO_2 of a donor pig portal vein sampled *in vivo* during laparotomy were 5.4 kPa and 7.58 kPa, respectively. The standard *metra* circuit supplies the portal vein with fully oxygenated blood, which has been associated with increased

vasoconstriction during *ex situ* perfusion[212]. Excessive oxygen delivery may exacerbate IRI[429]. The oxygen gradient along the hepatic sinusoid from portal tract to central vein contributes to the zonal differentiation of hepatocyte metabolism that is vital to hepatic function[213]; the significance of this is likely to become more important as perfusion duration increases.

The *metra*-ELC system mixes central venous blood of the recipient with the post-oxygenator blood of the *metra* circuit. The result is partial deoxygenation of the portal supply of the donor liver in proportion to the relative oxygen content and flows of the portal supply and exchange blood. This was confirmed during ELC in the third feasibility case of *chapter 4*. The flows were: exchange rate 500 mL/minute; donor liver hepatic artery inflow 300 mL/minute; donor liver portal inflow 900 mL/minute. The pO_2 values were: recipient pig mixed venous sample: 6.9 kPa (SO_2 78%); donor liver hepatic artery inflow (equivalent to pre-reservoir portal line): 12.0 kPa (SO_2 97%); donor liver portal inflow: 9.1 kPa (SO_2 91%). Although more physiological than the standard *metra* circuit, this still exceeds physiological portal vein oxygen saturation and cannot be regulated other than by changing the exchange rate. A refined circuit design may exchange the recipient's venous blood with the *metra* pre-oxygenator and incorporate a bypass line with a proportional pinch valve that runs from a site between the exchange entry point and oxygenator, to the portal reservoir. The proportional pinch valve aperture would be feedback-controlled based on the oxygen saturation of the line entering the portal vein, for example with a target oxygen saturation 80 – 85%. A manually controlled version of this portal deoxygenation method for the standard *metra* circuit has been developed (*K. Gyoten: unpublished data*).

iv. The *metra* perfuses the hepatic artery with a non-pulsatile pump at a pressure of 65 – 70 mmHg, whereas *in vivo* the hepatic artery blood supply is pulsatile. Several of the groups that have achieved prolonged normothermic liver preservation employ pulsatile hepatic artery perfusion[30,430,431], which has been associated with reduced haemolysis compared to continuous perfusion[30]. Pulsatile arterial perfusion may promote release of endothelium-derived nitric oxide (with vasodilatatory[432], anti-thrombotic[433], anti-inflammatory[434,435] and anti-oxidative[436] effects), reduce leucocyte adherence to the endothelium, reduce erythrocyte stasis, and improve preservation of the microcirculation[437-442]. In the context of cardio-pulmonary bypass, pulsatile

perfusion has been associated with less renal injury[443,444]. Translation of these findings to *ex situ* perfusion of the liver, with its unique dual afferent blood supply, has not yet been systematically investigated.

These refinements of the base *metra* circuit may support prolonged perfusion of the donor liver by reproducing *in vivo* physiology more closely.

9.3.7 Incorporation of renal replacement therapy ± DAMP/PAMP removal during ELC

Many patients with ALF and ACLF have concurrent renal failure necessitating renal replacement therapy (RRT). Whilst decedent cases 2 and 3 demonstrated that it is possible to run ELC simultaneously with RRT, it would be possible to incorporate RRT into the *metra*-ELC system by continuing one of the techniques described in *chapter 3* during ELC. Since this circuit would comprise three volume compartments (recipient, *metra* circuit, dialysate), reliable automated volume balancing would be a pre-requisite.

A central component of the pathophysiology of ALF and ACLF is the systemic inflammatory response driven by DAMPs and PAMPs: both conditions are associated with elevated levels of circulating cell-free DNA, histones, HMGB1, endotoxin, and cytokines[14,52,86,87,236,237,270-272,390-395,445]. Since ELC provides a functioning reticulo-endothelial system, it may contribute to physiological management of these pro-inflammatory molecules. Preliminary support for this concept is provided by the lower circulating levels of cell-free DNA and extra-cellular histones observed in ALF pigs undergoing ELC (*chapter 5*). However, this cause-effect relationship remains hypothetical. Incorporation of additional techniques to the *metra*-ELC system that remove DAMPs/PAMPs during ELC may be beneficial (e.g. adsorption of cytokines (Cytosorb[393]), nuclear DAMPs (NucleoCapture[224]) or combined DAMP-PAMP-cytokines (oXiris[14,396])). These techniques could commence in the isolated normothermic perfusion phase to pre-condition perfusate prior to patient connection (see *9.3.1*) and continue during ELC (*figure 70*).

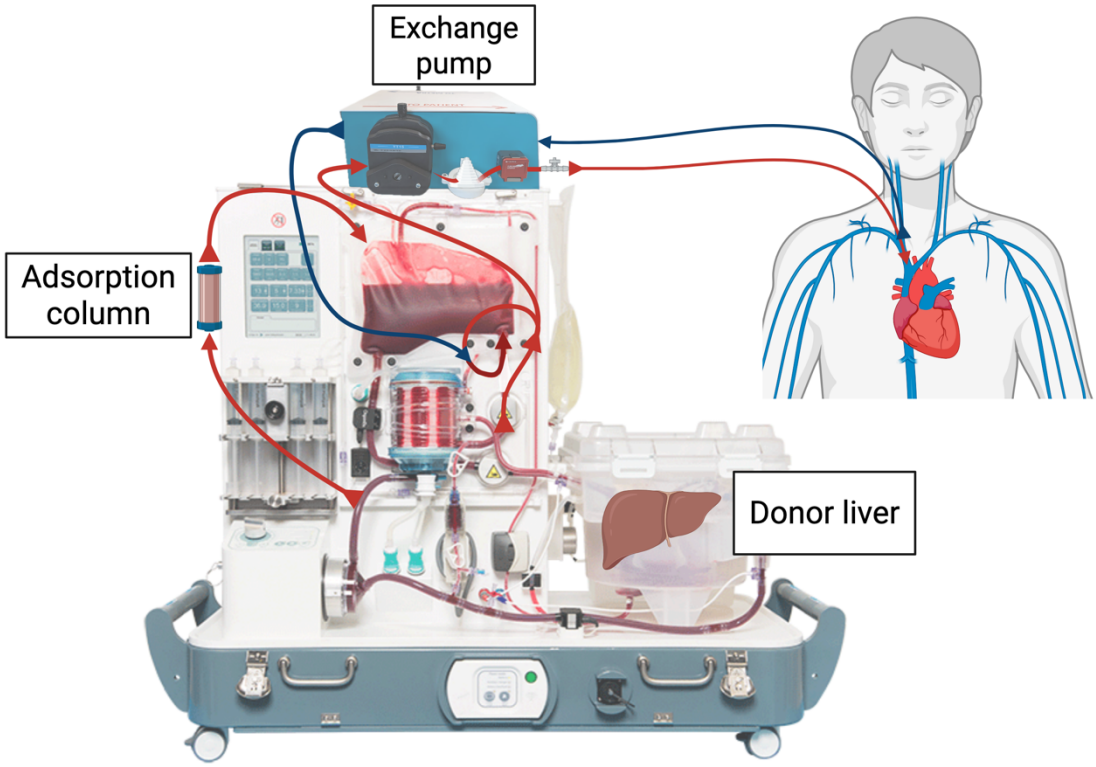


Figure 70: incorporation of adsorption column into *metra*-ELC system to remove damage- and pathogen-associated molecular patterns (DAMPs, PAMPs) during ELC.
Examples include Cytosorb (cytokine adsorption), NucleoCapture (nuclear DAMP adsorption), and oXiris (combined adsorption of cytokines, DAMPs (e.g. HMGB1) and PAMPs (e.g. endotoxin)). An alternative approach would be to incorporate renal replacement therapy (e.g. haemofiltration) during ELC in a similar manner.

9.3.8 ELC-specific declined allograft selection criteria

For allo-ELC, the donor liver viability criteria may be modified to increase the proportion of donor livers that proceed to ELC. The criteria proposed for the phase-1 trial are based on liver transplant criteria (with bile chemistry criteria removed because cholangiocyte function is not essential for the short-term life sustaining function of the donor liver[3,97]). These have been used to maximise safety in the first-in-human trial, but may limit access to allo-ELC in the future. It may be possible to relax these criteria because the duration of graft function needed for clinical benefit in ELC is much shorter than in transplantation, where the thresholds were defined.

9.3.9 Declined allograft reconditioning during isolated normothermic perfusion phase

Incorporation of donor liver reconditioning techniques during the isolated normothermic perfusion phase may increase the proportion of declined livers that are suitable for ELC. A variety of techniques during normothermic liver perfusion are under investigation to increase the proportion of livers suitable for transplantation, such as defatting steatotic livers to reduce vulnerability to ischaemia-reperfusion injury[315]. A subset of non-transplantable livers may be sufficiently reconditioned by these techniques to make them suitable for ELC but not transplantation.

9.3.10 Declined allograft transition from ELC to orthotopic transplantation

Human donor livers are frequently declined on the basis of donor information or short-duration normothermic perfusion. ELC using declined livers will provide a longer window to assess such livers, and in the context of a specific recipient. Whether the graft recovers from IRI can be assessed in the lower risk setting of ELC compared to orthotopic transplant. Whilst this may not exclude future biliary complications, the additional time could enable more in-depth evaluation of biliary risk. Ultimately, the decision may be taken that the originally-declined graft is suitable for implantation. Meanwhile, the recipient's physiological status may have improved as a result of ELC to better tolerate transplant surgery[20,56,57]. After a period of ELC, the donor liver could be transplanted into the recipient. It is conceivable that, in this way, ELC could be used not only to stabilise the condition of a patient who is unfit for immediate transplantation, but also to test/improve the condition of a donor liver that is unsuitable for immediate transplantation – effectively reconditioning both the patient and the liver before combining the two with a transplant.

9.3.11 Plasma, rather than whole blood, exchange between recipient and *metra* circuit

In the context of xeno-ELC, the mechanism of liver xenograft-associated thrombocytopenia is under active investigation. However, if this proves refractory to further genetic edits (e.g. vWF), pharmacological therapy, or the therapy itself increases bleeding risk (e.g. by inhibiting platelet

activity), a potential solution may be to place a plasma exchange filter membrane between the circulation of the patient and the perfusate of the *metra*. This would prevent direct contact between the recipient's platelets and the endothelium/Kupffer cells of the xenograft.

Therapeutic plasma exchange typically occurs in discrete sessions, exchanging plasma at a rate of 1.5 L/hour, corresponding to 25 mL/minute (equivalent to 42 mL/minute blood)[86,87]. When performing plasma exchange using membrane separation (as opposed to centrifugation), this is achieved using the recommended machine settings: blood flow of about 200 mL/minute with a transmembrane pressure of 100 mmHg[6]. However, this exchange rate is too low for effective hepatic support by ELC. At blood flows of 400 mL/minute and a transmembrane pressure of 120 mmHg, the Prismaflex TPE 2000 plasma membrane filter (Baxter, Deerfield, IL, USA) can achieve a plasma filtration rate of 140 mL/minute[5], which is more similar to the rates achieved with the current *metra*-ELC system. However, there is increased risk of haemolysis and device clotting at higher transmembrane pressures, which may limit the plasma filtration rate below those needed for hepatic support[6]. An alternative approach would involve a bespoke plasma membrane filter designed for counter-current flow of recipient blood and *metra* perfusate either side of the filter with molecular exchange by diffusion; existing commercially-available plasma membrane filters are designed for ultrafiltration only[6]. The effective exchange rate in this setting would depend on the characteristics of individual molecule (e.g. size, charge), which would need to be determined empirically (*figure 71*).

The molecular weight cut-off for plasma exchange filter membranes (1,000 – 3,000 kDa) allows passage of most molecules (except large lipoproteins and ultra-large vWF multimers) but not cells (white cells, red cells, platelets). Although the immunohistochemistry and electron microscopy studies described in *chapters 6* and *7* suggest that the human platelets aggregate within the xenograft, it is possible that they are (also) cleared within the recipient, for example, due to a protein such as vWF secreted into the circulation by the xenograft. The plasma exchange membrane approach would solve the thrombocytopenia issue if platelet clearance occurs specifically within the xenograft, but not if a major fraction occurs within the recipient. Until the mechanism is known, this could be tested empirically in a decedent study by performing ELC using plasma-only exchange.

Plasma-only ELC using wild-type pig livers in a circuit similar to *figure 71* (*left*) was trialled in the 1990s, although details of the exchange parameters (flows, transmembrane pressure) are not described (*table 3*). Accepting the small sample size, plasma-only exchange between recipient and xenograft did not attenuate immune-mediated damage of the graft, suggesting, as expected, that the immediate response is primarily enacted by the humoral immune system (complement, immunoglobulin). The impact on platelet count was unfortunately not reported[102].

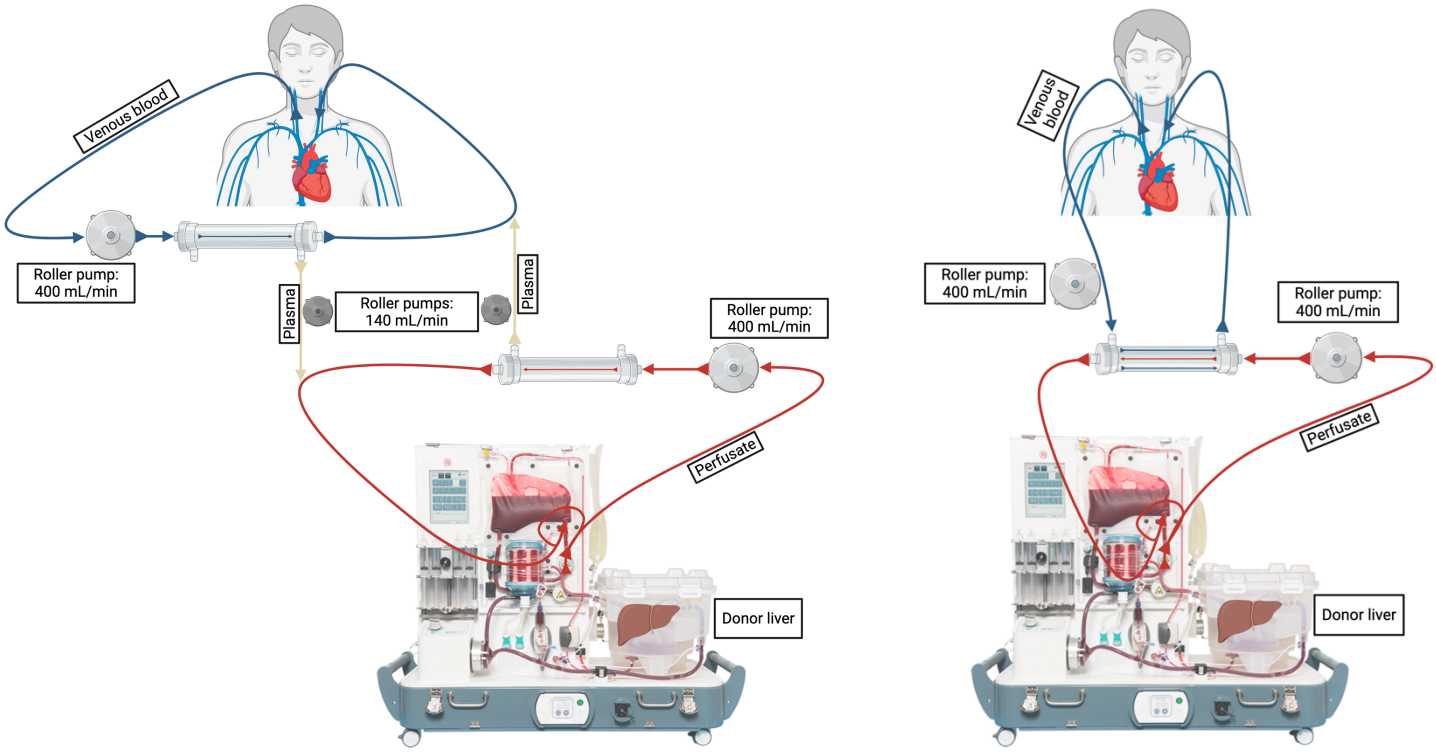


Figure 71: hypothetical circuits to enable plasma-exchange extra-corporeal liver cross-circulation.
Left: reciprocal plasma exchange of donor liver perfusate and recipient central venous blood using two Prismaflex TPE 2000 plasma filters[5] operating in ultrafiltration mode with a transmembrane pressure of about 120 mmHg. *Right*: counter-current flow of donor liver perfusate and recipient central venous blood either side of a single plasma filter membrane. Existing plasma filters are designed to function in ultrafiltration mode only and so do not permit counter-current flow in a manner analogous to a dialysis filter[6]: a bespoke filter would need to be manufactured for this application. The quoted flows are a guide and would require determination through empirical experimentation.

9.4 Duration of ELC

In *chapter 5*, allo-ELC was tested in a porcine ALF model for 24 hours. In *chapter 7*, xeno-ELC was tested in the decedent model for 72 – 84 hours. The phase-1 allo- and xeno-ELC trials will evaluate ELC up to 72 and 120 hours, respectively.

Safe extension of perfusion duration is facilitated by the fact that the graft can be continuously monitored, and immediately disconnected if concern arises regarding the function of the graft. Graft monitoring includes vascular flows; bile production; impact on recipient biochemistry; indocyanine green clearance; and gradient of ammonia, lactate, and bilirubin across the *metra*-ELC system. If there is concern, the macroscopic appearance of the liver can be inspected by opening the lid under sterile conditions.

The duration of ELC required for clinical benefit will be specific to each recipient. The duration for consistent benefit in the majority of patients is not yet known.

For patients who do not receive a transplant, the median time-to-death in non-survivors is 2.5 – 4.5 days in ALF (paracetamol-induced *versus* non-paracetamol)[54] and 5 – 11 days in ACLF (grade III *versus* II)[20,446]. For survivors, the median ICU stay is 13 – 14 days in both ALF and ACLF[447].

For patients listed for emergency transplant, the median time-to-transplant is 2 – 3 days in both ALF and ACLF[20,24]; the median time-to-death in emergency listed patients who do not receive a transplant is 3 days in ALF[54] and 7 days in ACLF[20].

These data suggest that 3 days of effective ELC would bridge at least half of emergency transplant waitlisted ALF and ACLF patients to transplant. Estimating the duration required to bridge most patients to transplant-free survival is challenging because the impact of physiological stabilisation by ELC on recovery of the native liver and other failing organ systems is unknown. An estimate for the upper limit might be the median ICU stay of transplant-free survivors (i.e. 13 – 14 days). The lower limit is difficult to predict. In ALF and severe ACLF, many patients are either transplanted or die within 3 – 7 days. In ACLF, survivors usually reach their maximum grade within the first week and severity on days 3 – 7 predicts outcome better than at diagnosis[75,349,448]. These observations suggest that an equilibrium state may be reached within this time in patients that recover. 7 days of effective temporary hepatic support might therefore be sufficient for most patients. Ultimately, this lower limit needs to be determined empirically in clinical trials.

Previous clinical trials of artificial, bio-artificial liver support systems, and plasma exchange mostly provided therapy over 2 – 3 days, although the MARS, Prometheus, and ELAD studies continued for longer. Except for ELAD, these systems are used in discrete (usually daily) sessions rather than

continuously: the average total duration on device in these studies ranged from 8 to 46 hours (*table 2*). The studies of ELC in the 1990s reported mean ELC durations between 5 and 55 hours (*table 3*).

The planned phase-1 allo- and xeno-ELC trials aim to consistently deliver ELC for (at least) 72 hours and measure its clinical impact. Once sufficient safety data have been generated and in-depth understanding of graft function over this period developed, a subsequent trial may assess longer durations if the phase-1 trials suggest this would be beneficial. For xeno-ELC at least, this could require more comprehensive immunosuppression than methylprednisolone. However, even for longer ELC durations, the immunosuppressed state of liver failure[111,449], the immuno-privileged nature of the liver graft[450], and the temporary nature of the therapy will enable milder immunosuppression regimens than those used for kidney and heart xenotransplantation[194,197].

9.5 Decision to disconnect

For the phase-1 trials, due to the underlying disease severity, non-transplant-listing, and relatively short duration of ELC, it is not anticipated that patients will recover to the point that premature elective discontinuation of ELC is indicated. However, in the future, as ELC durations are extended and a greater range of recipients are treated, clinical decision-making could incorporate consideration of whether a patient's native liver has recovered sufficiently to warrant de-listing or elective discontinuation of ELC. This requires differentiation of the function of the native liver from the donor liver. Several dynamic tests of liver function exist that may be useful.

A modified HepQuant SHUNT V2.0 test[339] assessed clearance of ^{13}C-cholate in the decedent xeno-ELC study in *chapter 7*. ^{13}C-cholate was administered intravenously and the concentration measured at the 4 sites show in *figure 52* at 5-, 20-, and 60-minutes post-administration. Whilst the standard SHUNT V2.0 test measures the differential clearance of intravenously administered ^{13}C-cholate and enterally administered D_4-cholate to provide information on liver function and portal haemodynamics, inconsistent gastrointestinal absorption in the brain-dead decedents meant that analysis was limited to the intravenous dose. Measurement from these 4 sites differentiated clearance by the native liver from

the donor liver without disconnecting the recipient from ELC. However, ^{13}C-cholate measurement requires mass spectrometric analysis that is not currently available in a hospital setting.

Indocyanine green clearance by the liver has been used clinically pre-hepatectomy to inform decision-making regarding the minimum required future liver remnant[451,452]. Indocyanine green is cleared exclusively by hepatocytes[453,454], although clearance is impacted by hepatic haemodynamics[455], inflammation[454,456], and hyperbilirubinaemia[453,457]. The hepatic extraction ratio (0.7) and half-life (3 – 4 minutes) of indocyanine green are comparable to cholate[458,459]. Indocyanine green could be measured at the same sites as the modified HepQuant SHUNT V2.0 test (*figure 52*) to differentiate native and donor liver function. Indocyanine green is measured using a photometric assay[458] that provides results more readily than HepQuant.

Since indocyanine green is excreted in bile without biotransformation[453,460], it would be theoretically possible to measure how much is excreted in the donor liver bile and use this to differentiate native from donor liver clearance. However, the time course of indocyanine green appearance in bile is substantially delayed relative to the plasma concentration decay curve and the low bile production rate makes precise measurement of bile clearance over time challenging[461].

A third approach would measure the plasma disappearance rate of indocyanine green clearance in the patient: first during ELC and then after temporary disconnection. The donor liver would be perfused in isolation whilst the second indocyanine green test is performed. ELC would be resumed once the second test is complete and the results are being analysed. This would quantify native liver clearance directly and determine the contribution of the *metra*-ELC system by subtraction. Transcutaneous pulse dye densitometry devices (LiMon, Pulsion Medical Systems, Feldkirchen, Germany; DDG 2001, Nihon Kohden, Tokyo, Japan) that measure real-time blood indocyanine green levels could provide immediate results[458,459,462].

A potential limitation to the use of indocyanine green with the *metra*-ELC system is that it may interfere with the function of the shunt sensor that is an integral component of the *metra* device (see *2.3*). The CDI510H shunt sensor works on the basis of optical fluorescence to monitor perfusate levels of pO_2, pCO_2, pH, and potassium[463]; this may be affected by circulating indocyanine green due to its absorption

peak at 805 nm[458,464]. Determination of the relevance of this will require empirical evaluation in an isolated normothermic liver perfusion model. This work is currently being planned.

This 'test-disconnect-test' approach could alternatively be employed with the CE-marked LiMaX test[465] (Humedics, Berlin, Germany), which measures conversion of ^{13}C-methacetin by CYP1A2 to $^{13}CO_2$[466]. $^{13}CO_2$ generated in this process is measured in exhaled air. Whilst usually performed in conscious patients, the test can be adapted for mechanically-ventilated patients[467,468]. The same enzymatic step was assessed in the xeno-ELC studies in *chapter 7* using a research assay that measured blood, rather than exhaled, $^{13}CO_2$ levels (Metabolic Solutions, Nashua, NH, USA). Activity was augmented in the presence of the xenograft suggesting that porcine livers possess CYP450 enzymes with comparable function to human CYP1A2. The LiMaX test has been used to assess CYP450 activity during *ex situ* liver perfusion by measuring $^{13}CO_2$ excretion via the oxygenator gas outlet[99]. However, this would not enable the 4-point discrimination during ELC that was possible with the HepQuant test: i.e. it would not differentiate native liver and graft activity, necessitating the 'test-disconnect-test' method described for indocyanine green clearance.

Of note, if a patient is receiving continuous RRT, it should be paused whilst these tests are performed to prevent clearance of the test substances via the dialysis filter.

There is evidence, although in relatively small numbers of patients, that indocyanine green clearance[469-471] and LiMaX[467,472-474] correlate with severity and prognosis in ALF. Only indocyanine green clearance has been assessed in the context of (hepatitis B-associated) ACLF[475]. HepQuant has not been evaluated in either condition. The use of these assays to assess recovery of liver function during ELC will be exploratory in the first instance. In the phase-1 trials, the plan is that indocyanine green clearance will be measured pre-ELC, daily during ELC, and post-disconnection. This is dependent upon the aforementioned experimental plan to determine the impact of indocyanine green on the function of the shunt sensor in the *metra* device.

In the future, these tests could be combined to assess different domains of hepatic function: HepQuant and indocyanine green clearance test excretory function, whilst LiMaX assesses detoxification. The trend in native and donor liver function could guide decision-making regarding ELC discontinuation

and transplant de-listing. As clinical experience accrues, it may be possible to define thresholds of native liver function that predict transplant-free survival and donor liver function that predict perfusion failure.

9.6 Acute *versus* acute-on-chronic liver failure

The planned phase-1 allo- and xeno-ELC trials will evaluate these therapies in patients with ACLF rather than ALF. The incidence of ACLF is about 10 times that of ALF (approximately 2,500 *versus* 250 per year in the UK[1,45-47,59-63]). Moreover, the phase-1 trials will only recruit patients not listed for transplant. Fewer patients with ACLF are listed for emergency transplant: about 1% of ACLF patients receive emergency wait-list prioritisation[20] *versus* 35% of ALF patients[48,54]; 9% of ACLF patients receive a transplant within 28 days[1], whereas 23% of ALF patients are transplanted at a median of 3 days[24]. ACLF has been chosen for the phase-1 trials to support an appropriate recruitment rate and because this represents the largest group of patients who stand to benefit from the therapy, in terms of absolute numbers and the proportion without access to an alternative (i.e. transplantation). However, treating patients with ACLF carries challenges.

The pathophysiology of ACLF comprises chronic liver disease with acute hepatic decompensation leading to failure of at least one organ system (e.g. cerebral, cardiovascular, renal). There is a relative deficiency of hepatic function induced by liver-directed injury (e.g. alcoholic hepatitis) or a broader physiological hit (e.g. infection, gastro-intestinal bleed). ACLF is a heterogenous condition with a variety of potential triggers and phenotypes[1,2,66,68-74]. The hypothesis is that ELC will restore a state of adequate hepatic function, support the body's response to the physiological hit, and provide additional time for the native liver to recover. ELC may be more effective in cases where the primary driver is deficiency of hepatic function rather than, for example, renal dysfunction. To ensure a more homogeneous cohort of patients more likely to benefit from ELC, the phase-1 trial inclusion criteria require raised bilirubin and/or evidence of hepatic encephalopathy.

In the absence of transplantation, ELC for patients with ACLF does not address their underlying chronic liver disease; they are vulnerable to portal hypertension-related variceal haemorrhage during ELC and relapse of ACLF if they recover from the acute episode. The risk of variceal bleed will be minimised

through case selection guided by endoscopic assessment of varices and strict management of anticoagulation. Whilst 61% of ACLF patients that survive to hospital discharge are alive and transplant-free after 1 year[76], 44% are re-admitted with ACLF at median of 38 days[476]. There is a need to address the underlying liver disease for patients who are supported to recovery by ELC. One route is that ELC, by improving physiological status and allowing more time for pre-transplant work-up, may increase access to elective transplantation for patients who are initially not listed[20]. Follow-up of these aspects will be an important outcome for the phase-1 trials (allo-ELC trial: over 90 days; xeno-ELC trial: over 365 days).

ALF, by contrast, is a liver-specific injury that occurs in the absence of pre-existing liver disease[42-44]. Temporary replacement of hepatic function may address the underlying pathophysiology more directly than in ACLF. For those who are transplanted, the duration of ELC is likely to be short (median waiting time: 3 days[24]), which will favour xeno- over allo-ELC to avoid the delay associated with waiting for a suitable declined liver. For those that recover without a transplant, long-term prognosis is excellent: long-term survival is 96% for patients surviving 90 days from ICU admission without a transplant (median follow-up 7.5 years)[477]. Though the number of patients with ALF that may benefit from ELC is much lower than ACLF, the efficacy may be greater. Patients with ALF should be included, but analysed separately, in a subsequent, large, multi-centre efficacy trial.

9.7 Health economic considerations

In addition to impact on survival, health economic analysis of a new therapy must consider impact on quality of life. For patients with ACLF, those who do not receive transplants may return to a state of compensated cirrhosis. Quality of life in compensated cirrhosis is impaired compared to healthy controls[478,479] but significantly greater than in decompensated cirrhosis[480], which in turn exceeds that in ACLF[481]. Transplantation in ACLF is associated with improved quality of life[482]. In ACLF, bridging to recovery of native liver function or transplant would be associated with improved quality of life from baseline. For patients with ALF, long-term survivors (both transplant recipients and transplant-

free survivors) report a modest reduction in quality-of-life compared to age- and sex-matched controls[483,484].

Cost-utility analysis must incorporate effect on hospital and ICU stay, downstream costs, transplantation rate and, for allo-ELC, the opportunity benefit of an otherwise unused resource (non-transplantable livers). The comparator for ELC is ICU standard-of-care. For ACLF, the median cost per ICU admission is £17,800[64,485] in the UK and $97-127,000 in the USA[486]. The cost of hospitalisation for ALF in the US is similar: $83,000 – 119,500 per case[487].

The incremental cost of allo-ELC is estimated to be £15 – 20,000 per patient in the UK. To achieve the NICE threshold of £20 – 30,000 per quality-adjusted life year (QALY)[488], each treated patient would need to gain 0.5 – 1.0 QALYs on average. The poor prognosis and quality of life associated with ACLF, combined with the effect of *metra*-ELC seen in pre-clinical studies, suggests that this may be feasible. For comparison, the incremental cost of the MARS system was €11,000 – 19,800 two decades ago[489,490]. The incremental cost of xeno-ELC will include the additional costs of generating, rearing, housing, screening, and retrieving livers from transgenic porcine donors. Initially, this would substantially increase costs, but would fall as production volume rises.

Health economic pathway analysis will be undertaken as a component of the planned phase-1 trials to define the cost of intervention; full health economic analysis will require large RCTs with extended follow-up of health outcomes including quality of life.

9.8 Bio-engineered livers

Bio-engineered livers are a potential future alternative to temporary hepatic support by ELC using declined allografts and xenografts. The latest generation of bio-engineered livers use a decellularised porcine liver to provide native extra-cellular matrix architecture, which is then recellularised.

Decellularised porcine livers re-endothelialised with human umbilical vein endothelial cells maintained viable perfusion after heterotopic implantation in pigs up to 15 days without systemic anticoagulation; the re-endothelialised vascular network supported whole blood perfusion[491]. Decellularised porcine livers were recellularised with both human umbilical vein endothelial cells and primary porcine

hepatocytes, then implanted into pigs with liver failure. The bio-engineered livers contributed to ammonia clearance, but other liver functions were not assessed. The bio-engineered livers weighed 260 ± 40.8 g with flows 120 – 410 mL/minute; implantation was complicated by small-for-size syndrome and portal hypertension despite a porto-caval shunt[492]. In a subsequent study, the porcine liver scaffolds were recellularised with human umbilical vein endothelial cells and 5 x 10^9 human hepatocytes. These demonstrated ammonia clearance and α1-antitrypsin synthesis in culture medium, and were perfused up to 3 hours with heparinised whole blood (ACT 400 – 600 seconds) with flows 350 mL/minute[493]. In all studies, the scaffold was recellularised with endothelial cells via the portal vein only; inflow perfusion was exclusively via the portal vein[491-493].

The miroliver*ELAP* device (Miromatrix Medical, United Therapeutics, Eden Prairie, MN, USA) has developed from these studies. The bio-engineered liver, recellularised with human umbilical vein endothelial cells and hepatocytes from declined human donor livers, is perfused with patient's blood using a modified Baxter PrisMax dialysis system as the blood exchange system[494]. The concept is similar to ELC, with a bio-engineered liver as the graft. A phase-1 trial assessing the use of the miroliver*ELAP* system to treat non-transplant candidates with ALF for 48 hours opened to recruitment in October 2024 (clinicaltrials.gov: NCT06285253[495]); the first patient was treated in June 2025[496].

Publicly available data regarding the functional capacity of the miroliver*ELAP* system *in vivo* are limited. By allowing whole blood perfusion and retaining the underlying architecture of the liver, the aim is for bio-engineered livers to reproduce native liver function more closely than the hepatocyte cultures of bio-artificial liver support systems. By using human cells, bio-engineered livers have the potential to avoid the obstacles of xenotransplantation such as functional incompatibility, the xeno-immune response, and zoonotic transmission. This extends to xenotransplantation of other organs (heart, kidney), for which outcomes in the medium-to-long-term are currently limited despite advances in genetic engineering and immunosuppression. In the future, this approach may enable bio-engineered organs to be tailored to the recipient (e.g. HLA compatibility, recipient cell-derived) reducing or abolishing the immunosuppression burden and facilitating transplantation for highly sensitised individuals.

However, outstanding questions related to the miroliverELAP system include:

i. will the hepatocyte mass be sufficient to support a patient with liver failure? An adult human liver typically contains 2.8 x 10^{11} hepatocytes[497] and weighs 1.6 kg[498], versus the 5 x 10^{9} hepatocytes and 0.26 kg grafts described in the pre-clinical miroliver studies[492,493].

ii. will hepatocytes-alone provide comprehensive replacement of hepatic function (i.e. in the absence of Kupffer cells, cholangiocytes etc)? It is not clear if bile is produced by the miroliver: bile production is not reported in the previous studies[492,493]. The purpose of re-endothelialisation with umbilical vein endothelial cells is to support whole blood perfusion[491] rather than to replicate the specialised role of liver sinusoidal endothelial cells (e.g. with respect to their phagocytic function).

iii. will the recellularised hepatic lobule reflect the specialised zonal architecture of a healthy liver (particularly in the context of portal vein-only perfusion)[213,499]?

iv. will there be sufficient manufacturing capacity to provide bio-engineered livers to patients in a timely manner? The manufacturing process in the pre-clinical study took 11 – 19 days (3 days decellularization, 8 – 16 days recellularisation), followed by 6 days of hepatic function[493]. The manufacturing time is too long for a patient with ALF or ACLF to wait and the functional period is short relative to this. Unless these periods are adjusted, it will be necessary to have pre-manufactured bio-engineered livers continuously available (and wasted when not used).

These limitations suggest that bioengineered livers may not yet provide definitive temporary hepatic support. Nonetheless, there have been major advances in bio-engineering in the past two decades; the phase-1 miroliver*ELAP* trial will provide initial answers to these questions and inform future refinement as the underlying technology continues to develop.

9.9 Concluding remarks

Within the next twelve months, simultaneous recruitment to phase-1 trials of ELC using declined allografts, xenografts, and bio-engineered livers, as well as a phase-2 trial of DIALIVE (artificial liver support system) is anticipated. Each therapy takes a different approach to temporary hepatic support, associated with specific advantages and disadvantages. To fully characterise each intervention, analysis

should incorporate rigorous study of feasibility and logistics, as well as a consistent approach to data collection and endpoints. These trials have the potential to deepen insight into the physiological considerations of supporting a patient with a failing liver. Despite substantial advances, this remains poorly understood. Mechanistic analysis should extend beyond standard clinical measures of liver function to include the broader impact on the proteome, metabolome, lipidome, and inflammatory response. Whilst the natural history of these conditions with standard-of-care is well-defined, the impact of effective hepatic support is not; the optimal timing, dose, and duration of support are unknown. Combining what is learned in these trials will guide future efforts towards a therapy that consistently bridges patients with liver failure to either transplant or recovery of native function.

10 References

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