Experimental & Clinical Applications of *Ex Vivo* Liver Perfusion

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

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ABSTRACT

Normothermic machine perfusion (NMP) is a novel method of organ preservation that has recently risen in prominence. NMP involves the maintenance of an organ outside the body in a way that seeks to recreate its physiologic milieu, such as by providing oxygen and nutrients and maintaining temperature. NMP of the liver has been shown in clinical trials to improve graft utilization and reduce early allograft dysfunction when used in place of traditional static cold storage (SCS). However, we are only just beginning to understand the variety of ways that NMP can be implemented to solve clinical problems in liver transplantation, as well as address research questions in transplantation and hepatology, more broadly. This thesis presents a mixture of basic science and clinical studies. The former seeks to use NMP to build preclinical models of liver disease, while the latter describes the early clinical implementation of NMP in a mature North American liver transplant program.

The first set of studies are dedicated to building an NMP-based large animal model of donation after circulatory death (DCD) to test the effect of cyclosporine A (CsA) on hepatic ischemia/reperfusion injury (IRI). It starts with a systematic review and meta-analysis to identify evidence for CsA in preclinical models of myocardial IRI (in which it has been most studied). From this, it was found that the most common dose was 10mg/kg and the effect was less pronounced in models using pigs and/or female animals and greater when dosed prior to the onset of ischemia. Also, part of the reason why CsA failed to translate to clinical studies of myocardial IRI was the prolonged duration of ischemia compared to preclinical models, which makes transplantation (with shorter and more predictable ischemic times) potentially better suited to intervention with CsA. The second study in this series tested the effect of CsA and its non-immunosuppressive analog, NIM-811, in a murine model of partial warm hepatic IRI. It was found that animals treated with either 10 or 25mg/kg of CsA or 10mg/kg of NIM-811 had decreased

serum alanine aminotransferase (ALT) at six hours post-reperfusion compared to saline treated controls (1793.5 [1603-2157] U/L, 1823 [1668-2932] U/L, 2375 [1963-2919] U/L vs. 3300.5 [2481-4911] U/L; $p<0.001$, 0.007, and 0.031, for 10mg/kg CsA, 25mg/kg CsA and 10mg/kg NIM-811, respectively). This corresponded to decreased histological injury scores ($p<0.001$, 0.003, 0.043, for 10mg/kg CsA, 25mg/kg CsA and 10mg/kg NIM-811, respectively). The final study tests CsA in an NMP-based DCD model. Pigs were pretreated with 20mg/kg of CsA and subject to either 45 minutes of warm ischemia followed by 2 hours of SCS or 60 minutes of warm ischemia followed by 4 hours of SCS. Their livers were reperfused via an experimental NMP setup. Compared to the livers of animals treated with saline only, livers treated with CsA did not show any difference in markers of injury. Peak values of serum transaminases were no different between groups ($p=0.912$, 0.455 for ALT and aspartate aminotransferase [AST], respectively). Similarly, there was no difference in midpoint histological injury score ($p=0.271$).

The second experimental section focuses on building a model of acute liver failure using an isolated perfused liver. Porcine livers were perfused via NMP and acetaminophen was added as a hepatotoxic agent. Acetaminophen-treated livers received a median dose of 8.93g (8.21-9.75g) of acetaminophen, achieving a peak acetaminophen level of 3780µmol/L (3189-3913µmol/L). Peak values of ALT (76 vs. 105U/L; $p=0.429$) and AST (3576 vs. 4712U/L; $p=0.429$) were not significantly different between treated and untreated livers. However, by the end of perfusion, histology scores were significantly worse in the acetaminophen treated group ($p=0.016$). Injury was confounded by the development of significant methemoglobinemia, with a peak methemoglobin level of 19.3%, compared to 2.0% for control livers ($p=0.004$). *In vitro* experiments demonstrated that the observed methemoglobinemia was caused by the acetaminophen metabolite, p-aminophenol. As methemoglobinemia is not the mechanism by

which acetaminophen is known to cause hepatotoxicity clinically, this was seen as a major limitation of the model.

The final research section reports clinical outcomes of liver recipients transplanted following NMP from one of the first centres in North America to implement NMP. From 2015 to 2021, 79 livers were transplanted following NMP compared to 386 after SCS only. NMP livers were preserved for a median time of 847min compared to 288.5min in the SCS cohort ($p<0.0001$). Despite this, we observed significantly improved 30-day graft survival ($p=0.030$), though no differences in long term patient survival, major complications or biliary or vascular complications.

NMP of the liver is an important technique for studying liver injury in the preclinical setting, both as it relates to transplantation and more broadly to other areas of hepatology. The role of NMP in the clinical setting is still being defined and is likely to expand as it becomes increasingly integrated into modern transplant programs.

The final chapter brings my research contributions into perspective, and updates the current progress in normothermic, hypothermic and now normothermic regional perfusion. It provides an optimistic framework for future endeavors in clinical liver preservation, ensuring more livers of the best quality possible can be transplanted to save lives.

PREFACE

Dear Reader,

This thesis entitled 'Experimental & Clinical Applications of *Ex Vivo* Liver Perfusion' is submitted in partial fulfillment of the requirements for a Doctor of Philosophy in Experimental Surgery at the University of Alberta. The presented work explores the application of liver normothermic machine perfusion (NMP) in both experimental areas of research and clinically in a modern liver transplantation program. This thesis is divided into chapters containing pre-clinical and clinical research, in which the author played the lead role. Each chapter has been published or submitted for publication in a peer-reviewed journal.

Chapter 1 serves as an introduction to the thesis. It is an exploratory review article, which examines evidence for potential applications of liver machine perfusion outside of mere preservation prior to transplantation. This review was published as a first-author publication in journal *Transplantation*, with co-authors B.A. Marfil-Garza, N. Dadheech, and A.M.J. Shapiro. I conducted the literature review and prepared the initial draft of the manuscript. AMJS was responsible for conceptualization. BAMG was responsible for the figures. BAMG, ND, and AMJS all assisted with editing and revision. A short addendum to the original manuscript has been added to the end of this chapter to reflect the advances that have occurred in the field since its publication, and a fuller discussion is now updated in the final thesis chapter.

Chapter 2 is aimed at the development of an NMP model of donation after circulatory death to test cyclosporine A (CsA) in mitigating ischemia-reperfusion injury (IRI). **Part 1** is a systematic review and meta-analysis of preclinical studies using CsA to mitigate myocardial IRI. The purpose of this review was to provide the rationale for testing CsA in hepatic IRI and identify an appropriate dose for testing. This systematic review was published as a first author publication

in the *Annals of Translational Medicine*, with co-authors B.A. Marfil-Garza, S. Campbell, D.H. Freed, and A.M.J. Shapiro. SC, a health science librarian, was responsible for designing the search strategy and conducting the literature search. BAMG and I conceptualized the idea and were responsible for abstract and full text review. I conducted the data extraction, performed the meta-analyses, and wrote the first draft of the manuscript. BAMG, DHF, and AMJS were responsible for revising and editing the manuscript. **Part 2** is original research that describes the initial testing of CsA and its non-immunosuppressive analog, NIM-811, in mitigating hepatic IRI in a murine model. The purpose of this study was to demonstrate efficacy in a simpler model prior to moving to a more complex NMP-based model of hepatic IRI. This study is currently under review in the *Liver Research*, with co-authors R. Pawlick, B.A. Marfil-Garza, A. Thiesen, N. Cuesta-Gomez, S. Hatami, D.H. Freed, C. Karvellas, D.L. Bigam, and A.M.J. Shapiro. DHF, CK, DLB, and AMJS were responsible for conceptualization and design of the experimental plan. I conducted the experimental animal work and performed serum and tissue analysis with the assistance of RP, BAMG, NCG, and SH. AT assisted with pathology review. I wrote the first draft of the manuscript, and it was edited and revised by all of the co-authors. Finally, **Part 3** describes the application of CsA to reduce IRI in a large animal model of donation after circulatory death (DCD) using NMP. This study is currently under revision in the journal *Annals of Transplantation Surgery*, with co-authors S. Hatami, A. Thiesen, M. Wagner, G. Mainardi, S. Himmat, C.J. Karvellas, D.L. Bigam, D.H. Freed, and A.M.J. Shapiro. I conceived of the project with the guidance of CJK, DLB, DHF, and AMJS. I conducted the animal experiments with the assistance of S. Hatami, MW, GM, and S. Himmat. S. Hatami assisted me in completing serum and tissue analysis. AT was responsible for pathology review. I wrote the first draft of the manuscript and all co-authors contributed to its editing and revision.

Chapter 3 explores the development of a preclinical model of acute liver failure (ALF) in an isolated perfused liver. **Part 1** is a review of preclinical models of ALF. It was published as a first author publication in *PeerJ*, with co-authors B.A. Marfil-Garza, R.L. Pawlick, D.H. Freed, C.J. Karvellas, D.L. Bigam, and A.M.J. Shapiro. AMJS was responsible for conceptualization. I conducted the literature review and wrote the first draft of the manuscript, including the tables. BAMG was responsible for the figures. All co-authors contributed to editing and revision of the manuscript. **Part 2** is original research that describes the development of an ALF model in an isolated perfused porcine liver and the limitations encountered. This study was published as a first author publication in *Biomedicines*, with co-authors S. Hatami, A. Thiesen, C. Olafson, K. Dirand, J. Acker, C.J. Karvellas, D.L. Bigam, and A.M.J. Shapiro. CJK, DLB, DHF, and AMJS were responsible for conceptualization and experimental design. SH and I conducted the animal experiments and organ perfusions. I was responsible for the analysis of perfusate and tissue samples, with the assistance of SH, CO, KD, and JA. AT conducted the review of pathology. All co-authors assisted with editing and revision of the manuscript.

Chapter 4 presents an analysis of clinical outcomes following the implementation of NMP prior to liver transplantation at the University of Alberta Hospital in Edmonton, Alberta, Canada. The manuscript is published in the *American Journal of Transplantation*, with co-authors D. Leon-Izquierdo, B.A. Marfil-Garza, G. Meeberg, K. Verhoeff, B. Anderson, K. Dajani, D.L. Bigam, and A.M.J. Shapiro. AMJS was responsible for conceptualization of the study and acquiring funds and resources to conduct the study. BA, KD, DLB, and AMJS performed the liver transplantations included in the study. DLI was the clinical co-ordinator for the study. Data collection was performed by DLI, BAMG, GM, KV, and myself. I performed the data analysis with the assistance of BAMG. All co-authors contributed to the editing and revision of the manuscript.

Chapter 5 serves as the conclusion chapter. It summarizes the current evidence for machine perfusion of the liver, details strategies for its use, and identifies future directions for clinical application and experimental research. It is currently being prepared for submission to *Transplantation Reviews*, with co-authors B.A. Marfil-Garza, K. Verhoeff, Constantine J. Karvellas, David L. Bigam, Darren H. Freed and A.M.J. Shapiro. AMJS and I conceptualized the review. I conducted the literature review and wrote the draft of the manuscript. BAMG contributed to the figures. KV assisted in preparing tables. BAMG, KV, CJK, DLB, DHF, and AMJS contributed to editing and revision.

This thesis is an original work by Joshua Judson Hefler. The research projects composing this thesis received ethical approval from the University of Alberta Ethics Research Board: 'Optimization of Organ Donation' (AUP00002033), 'Improving Liver Preservation in a Large Animal Model by NMP' (AUP00001036), and 'Normothermic Liver Preservation Trial' (Pro00043239).

My sincerest hope is that this work serves to further research in liver NMP and inspire innovative strategies for its clinical implementation.

If the breaking day sees someone proud,
The ending day sees them brought low.
No one should put too much trust in triumph,
No one should give up hope of trials improving.
Clotho mixes one with the other and stops
Fortune from resting, spinning every fate around.
No one has so much devine favor
That they could guarantee themselves tomorrow.
God keeps our lives hurtling on,
Spinning in a whirlwind.

– Seneca, *Thyestes*, 613

ACKNOWLEDGEMENTS

So many people have played a role in the completion of this thesis, and I am grateful to everyone.

First and foremost, I would like to thank my supervisor, Professor James Shapiro. I could not think of a better role model of a surgeon-scientist. His high standards, tireless work ethic and constant availability have provided an invaluable source of motivation that has allowed me to reach this point. Most of all, I appreciate him granting me the independence to learn (and struggle) to develop my own ideas and the support to push through the challenges necessary to grow as a researcher.

I am deeply indebted to my supervisory committee, Drs. Dean Karvellas, David Bigam, and Darren Freed for their support and guidance. I am particularly grateful to Dr. Freed for his assistance in the creation of our experimental NMP model and his patience in troubleshooting technical issue. I value him as a role model of a surgeon-scientist.

I would like to thank the arms-length examiners at my candidacy exam, Winnie Wong and Aldo Montano-Loza, as well as Fred Berry for serving as chair. I appreciate them volunteering their time and challenging me to learn. I am grateful to have Professor Peter Friend serve as my external examiner for my defense. He is a true leader in the field of liver machine perfusion, and I look forward to learning a lot from him.

I have many people to thank from both the Shapiro and Freed labs who have supported my research. From the Shapiro lab, Mariusz Bral, Nidheesh Dadheech, Nerea Cuesta-Gomez, Ila Jasra, Kevin Verhoeff, Haide Razavy all contributed to this work, directly or indirectly, at various stages. In particular, I would like to thank Rena Pawlick. In the chaos that saw the lab transition from islets to stem cells (as well as the off shoot into machine perfusion), she has been the one constant,

the glue that has held the lab together. Also, Braulio Marfil-Garza, my brother in arms, with whom the majority of my PhD overlapped. Despite us having different research focus, Braulio provided me with a tremendous amount of support and guidance and was generous in allowing me to assist him with his clinical research. From the Freed lab, I would like to thank Sayed Himmat, Guilherme Mainardi, Xiao (Sam) Qi, Mitchell Wagner, and Xiuhua (Sue) Wang for their support and assistance. Most of all, I would like to express my deep gratitude and respect for Sanaz Hatami, who taught me everything I know about *ex vivo* organ perfusion and was selfless in sharing her experience and time to get me to this point.

The staff at the Surgical Medical Research Institute (SMRI) – Ryan Edgar, Katie Buswell-Zuk, Greg Olson and Deb Dixon – were invaluable in completing the required porcine experiments. I doubt there is another crew in the country who could match their experience and expertise in large animal surgery.

Last, but not least, I would like to thank my parents for their unwavering support and giving me the freedom to follow my unique, self-determined path.

This work would not have been possible without any of you.

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LIST OF ABBREVIATIONS

90kDa heat shock protein – Hsp90

95%CI – 95% confidence interval

AAGL - *Agrocybe aegerita* galectin

AIH – autoimmune hepatitis

ALF – acute liver failure

ALI – acute liver injury

ALT – alanine transaminase

ANIT – α-naphthyl isothiocyanate

APAP – N-acetyl-para-aminophenol

ARDS – acute respiratory distress syndrome

AST – aspartate transaminase

ATP – adenosine triphosphate

BAL – bioartificial liver

BCL-2 – B-cell lymphoma 2

BMI – body mass index

BSO – buthionine sulfoximine

CAMARADES – Collaborative Approach to Meta Analysis and Review of Animal Data from Experimental Studies

CBD – common bile duct

CCl_4 – carbon tetrachloride

CCHFV – Crimean-Congo hemorrhagic fever virus

CK – creatinine kinase

CK-MB – creatinine kinase myocardial band

CI – cardiac index

CIT – cold ischemia time

CO – cardiac output

ConA – concanavalin A

COR – controlled oxygenated rewarming

COVID-19 – corona virus disease 2019

CPB – cardiopulmonary bypass

CRISPR – clustered regularly interspaced short palindromic repeats

CsA – cyclosporine A

CYP450 – cytochrome P450

d-GAL – d-galactosamine

DAMP – damage-associated molecular pattern

DCD – donation after cardiac/circulatory death

DILI – drug-induced liver injury

DNA – deoxyribonucleic acid

EAD – early allograft dysfunction

EM – electron microscopy

FiO_2 – fraction of inspired oxygen

GGTA1 – α1,3-galactosyltransferase

GSH – glutathione

H&E – hematoxylin and eosin

HAF – hepatic artery flow

HAP – hepatic artery pressure

HAR – hepatic artery resistance

HAV – hepatitis A virus

HBV – hepatitis B virus

HCC – hepatocellular carcinoma

HCV – hepatitis C virus

HE – hepatic encephalopathy

HMP – hypothermic machine perfusion

HOPE – hypothermic oxygenated machine perfusion

HSV-1 – herpes simplex virus type 1

ICP – intracranial pressure

ICU – intensive care unit

IFN(-γ) – interferon (gamma)

IFNAR1 – interferon α/β receptor subunit 1

IL – interleukin

IM – intramuscular

INR – international normalized ratio

IP – intra-peritoneal

IQR – interquartile range

IRI – ischemia/reperfusion injury

IV – intravenous

IVC – inferior vena cava

KC/GRO – keratinocyte chemoattractant / human growth regulated oncogene

L – left

LAD – left anterior descending

LCA – left circumflex artery

LDH – lactate dehydrogenase

LEC – Long Evans cinnamon

LLL – left lateral lobe

LPS – lipopolysaccharide

LSEC – liver sinusoidal endothelial cells

LV – left ventricle

LVEF – left ventricular ejection fraction

MAPC – multipotent adult progenitor cell

MARS – molecular absorbent circulating system

MDA – malondialdehyde

MELD-Na – model of end-stage liver disease-sodium

miRNA – micro-ribonucleic acid

MHV – murine hepatitis virus

ML – median lobe

MP – machine perfusion

mPTP – mitochondria permeability transition pore

MRP2 – multidrug resistance-associated protein 2

MRSA – methicillin-resistant *Staphylococcus aureus*

MSC – mesenchymal stem cell

NAC – N-acetylcysteine

NAPQI – N-acetyl-p-benzoquinone imine

NASH – non-alcoholic steatohepatitis

NICE – National Institute for Health and Care Excellence, NICE

NMP – normothermic machine perfusion

NR – not reported

NRP – normothermic regional perfusion

ns – not significant

NS – normal saline

OCS – Organ Care Systems

OR – operating room

oxLDL – oxidized low-density lipoprotein

PAP – p-aminophenol

PBC – primary biliary cirrhosis

PBS – phosphate buffered saline

PCA – portocaval anastomosis

PCI – percutaneous coronary intervention

PD-1 – programmed cell death protein 1

PERV – porcine endogenous retrovirus

PFU – plaque-forming units

PO – *per os*

POD – post-operative day

PRISMA – Preferred Reporting Items for Systematic Reviews and Meta-Analyses

PSC – primary sclerosing cholangitis

PT – prothrombin time

PVF – portal venous flow

PVP – portal venous pressure

PVR – portal venous resistance

RCT – randomized controlled trial

RHDV – rabbit hemorrhagic disease virus

RLL – right lateral lobe

RNA – ribonucleic acid

RNAi – ribonucleic acid interference

ROS – reactive oxygen species

SC – sub-cutaneous

SCS – static cold storage

SEM – standard error of the mean

SMP/SNMP – sub-normothermic machine perfusion

STEMI – ST-segment elevation myocardial infarction

SYRCLE – Systematic Review Centre for Laboratory Animal Experimentation

TASO – thioacetamide-S-oxide

$TASO_2$ – thioacetamide-S,S-dioxide

TG – triglycerides

TIMI – thrombolysis in myocardial infarction

TLR4 – toll-like receptor 4

TNF-α – tissue necrosis factor alpha

TnI – cardiac troponin I

TnT – cardiac troponin T

Treg – regulatory T cell

TUNEL - terminal deoxynucleotidyl transferase dUTP nick end labeling

UAH – University of Alberta Hospital

UGIB – upper GI bleed

U/O – urine output

UW – University of Wisconsin

VACV – vaccinia virus

WIT – warm ischemia time

CHAPTER 1 – MACHINE PERFUSION OF THE LIVER: APPLICATIONS BEYOND TRANSPLANTATION

1.1 – Machine Perfusion of the Liver: Applications Beyond Transplantation

Review

Machine Perfusion of the Liver: Applications Beyond Transplantation

Joshua Hefler, MD,[1] Braulio A. Marfil-Garza, MD,[1] Nidheesh Dadheech, PhD,[1] and A.M. James Shapiro, MD, PhD[1]

Abstract. Machine perfusion (MP) is at the forefront of innovation in modern liver transplantation. Several approaches, mainly varying the temperature at which the graft is perfused, have shown benefit in preclinical models and nonrandomized clinical trials. Given the recent randomized controlled trial by Nasralla et al demonstrating the efficacy of normothermic MP over static cold storage, MP is likely here to stay for the foreseeable future. We are only beginning to explore the possibilities of this technology, including the prediction of graft function and modification of suboptimal livers. This has the potential to both increase the donor pool and improve the quality of grafts provided to recipients. Beyond transplantation, there may be a role for MP in extracorporeal liver support, cancer research and therapeutics, and pharmaceutical testing. In this review, we provide the rationale and explore the relevant preclinical studies that support the use of ex situ liver perfusion for these extended applications.

(*Transplantation* 2020;104: 1804–1812).

INTRODUCTION

Static cold storage (SCS) has been the standard of care for the preservation of liver grafts before transplantation for the past 4 decades. Simply, SCS involves flushing the organ with a preservation solution and keeping it in an ice bath at 4°C.[1] SCS is low cost, effective, and practical, and although it has helped establish transplantation as the only life-saving modality of treatment for end-stage organ failure, preservation remains far from perfect. Indeed, cold ischemia triggers a cascade of cytotoxic pathways, leading to sinusoidal endothelial cell swelling, membrane damage, and injury to other components of the hepatic microvasculature.[2,3] Clinically, it may be associated with early graft dysfunction and limits the time between organ retrieval and implantation.[4,5]

Machine perfusion (MP) techniques are an alternative to SCS, in which a perfusate solution (typically dilute oxygenated blood) is actively circulated through the organ to maintain cellular metabolism and prevent the effects of cold ischemia.[6] MP featured prominently in the early days of liver transplantation, including the first successful human transplants by Starzl, in which a hypothermic, hyperbaric setup with low-flow perfusion by diluted blood was applied.[7,8] It subsequently fell by the wayside, however, partly due to its relative complexity, but more due to the introduction of simple, effective cold storage solutions, most notably Belzer's University of Wisconsin (UW) solution.[9]

There has been a recent resurgence of interest in MP, beginning with preclinical trials of several systems in the early 2000s to its current use in the clinical setting.[10,11] A recently published randomized controlled trial (RCT) by Nasralla et al[12] demonstrated efficacy over SCS using a normothermic MP (NMP) system, by showing lower levels of graft injury despite a lower rate of organ discard and longer preservation times. Similar trials are underway using an alternative hypothermic setup.[13] The aim of these initial clinical investigations was to demonstrate both safety and protective efficacy with preservation of liver grafts, with the hope and expectation that the increasing use of higher risk, marginal liver grafts including donation after circulatory death (DCD), would be afforded with additional protection to reduce patient risk. However, this only begins to scratch the surface of potential uses for this remarkable technology.

This article reviews the rationale and relevant preclinical studies that support the use of ex situ liver perfusion for

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J.H. is responsible for reviewing literature, writing the article, and designing figures. B.A.M.-G. is responsible for writing and editing the article. N.D. is responsible for writing and editing the article. A.M.J.S. is responsible for the conception, writing, and editing the article.

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A version of this section was published as a review in *Transplantation* (Impact Factor 5.385). A short addendum is included at the end of the chapter to highlight more recent advances in the field that have occurred since this review was published, and a more in depth discussion of these advances is provided in the final chapter.

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Title: Machine Perfusion of the Liver: Applications Beyond Transplantation

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1.1.1 – Preface

Machine perfusion (MP) is at the forefront of innovation in modern liver transplantation. Several approaches, mainly varying in the temperature at which the graft is perfused, have shown benefit in preclinical models and non-randomized clinical trials. Given the recent randomized controlled trial by Nasralla et al. demonstrating the efficacy of normothermic machine perfusion over static cold storage, MP is likely here to stay for the foreseeable future. We are only beginning to explore the possibilities of this technology, including the prediction of graft function and modification of sub-optimal livers. This has the potential to both increase the donor pool and improve the quality of grafts provided to recipients. Beyond transplantation, there may be a role for MP in extracorporeal liver support, cancer research and therapeutics, and pharmaceutical testing. In this review, we provide the rationale and explore the relevant preclinical studies that support the use of *ex situ* liver perfusion for these extended applications.

1.1.2 – Introduction

Static cold storage (SCS) has been the standard of care for the preservation of liver grafts before transplantation for the past four decades. Simply, SCS involves flushing the organ with a preservation solution and keeping it in an ice bath at 4°C.[1] SCS is low cost, effective and practical, and while it has helped establish transplantation as the only life-saving modality of treatment for end-stage organ failure, preservation remains far from perfect. Indeed, cold ischemia triggers a cascade of cytotoxic pathways, leading to sinusoidal endothelial cell swelling, membrane damage and injury to other components of the hepatic microvasculature.[2,3] Clinically, it may be associated with early graft dysfunction and limits the time between organ retrieval and implantation.[4,5]

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both safety and protective efficacy with preservation of liver grafts, with the hope and expectation that the increasing use of higher risk, marginal liver grafts including donation after cardiac death (DCD), would be afforded with additional protection to reduce patient risk. However, this only begins to scratch the surface of potential uses for this remarkable technology.

This article reviews the rationale and relevant preclinical studies that support the use of *ex situ* liver perfusion for extended applications adjacent to and beyond the realm of liver transplantation (**Figure 1.1**). This includes the use of MP to assess graft function and treat grafts prior to implantation, for indications such as the eradication of hepatitis C and 'de-fatting' of steatotic livers. Other potential areas of opportunity include use as extracorporeal liver support devices, either with marginal grafts or genetically engineered xenografts, cancer research and therapeutics, and preclinical studies of hepatic toxicology and injury. Many avenues exist for the development and refinement of this technology for use in both clinical and translational research.

1.1.3 – Overview of Machine Perfusion Technology

The main difference between machine perfusion devices is the temperature at which the organ is perfused.[6] With hypothermic machine perfusion (HMP), the graft is kept as cold as 4ºC, similar to SCS.[14] The suggested benefit over SCS is improvement of microcirculation by mobilization and dilution of metabolic waste. HMP does not provide supplemental oxygen, whereas its oxygenated alternative is referred to as hypothermic oxygenated perfusion (HOPE).[15] HOPE typically perfuses at a slightly higher temperature (10-12ºC), though both use preservation solutions, such as UW solution or Vasosol (a modification of UW solution containing additional vasodilatory and antioxidant agents), as the perfusate.[16] The oxygen supplied in HOPE is only that dissolved in the perfusate (i.e. there is no additional oxygen-carrying molecule). A disadvantage

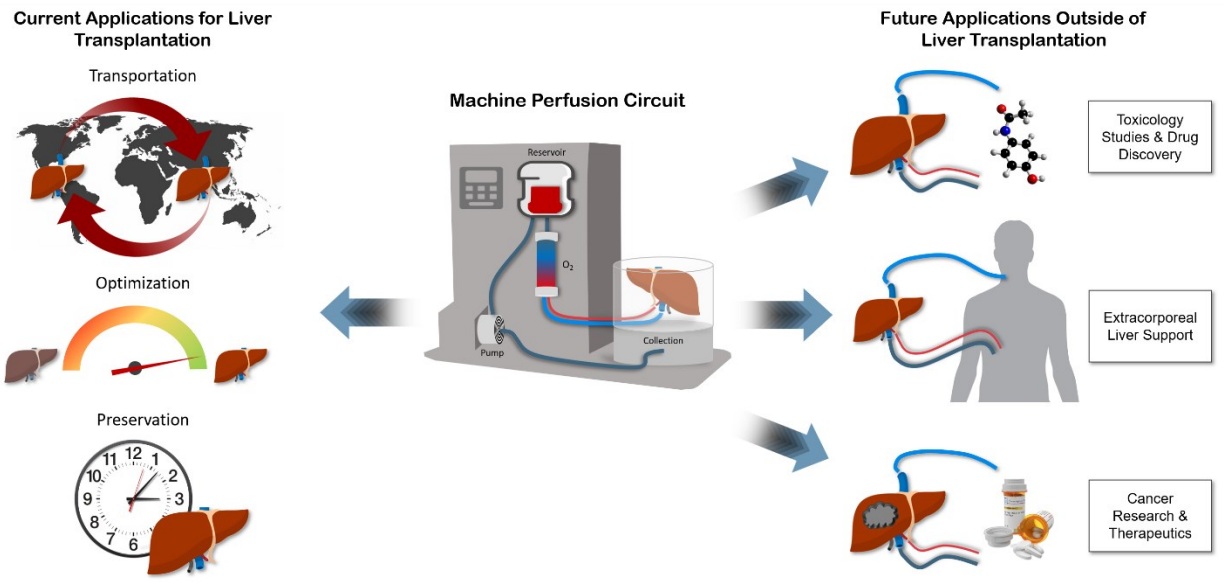

Figure 1.1. Potential future applications of *ex vivo* liver perfusion outside of liver transplantation include extracorporeal liver support, cancer therapeutics and research, and toxicology studies.

of the hypothermic approach is that the low metabolism at these temperatures makes assessment of functional viability more challenging.

Sub-normothermic machine perfusion (SMP) maintains the graft at a warm, but still sub-physiologic temperature, typically between 25-34ºC.[17] Similar to HOPE, the graft is perfused with an oxygenated preservation solution. It essentially attempts to find the middle ground between HOPE and NMP. At this temperature, the graft may still benefit from lower metabolic demand, while retaining enough metabolic function to allow for viability testing. Preclinical studies have demonstrated improved mitochondrial function and replenishment of ATP stores with this approach.[18] A variant of this technique is controlled oxygenated rewarming (COR), in which the perfusion temperature is gradually increased from HMP to SMP or NMP levels, has demonstrated improved efficacy over SMP alone in preclinical studies.[19] The authors hypothesized that the gentler rewarming mitigated the effects of reperfusion injury.

NMP, by contrast, aims to target an environment as close to physiologic as possible. The graft is kept between 35-38ºC and the perfusate contains erythrocytes, either from stored blood products (for human grafts) or diluted whole blood, in the case of animal models. NMP seeks to minimize cold ischemic time and maintain a liver that is fully metabolically active, which allows for accurate functional viability assessments, such as via glucose metabolism and bile production. Each of these approaches have been used clinically, with subsequent transplantation of the perfused livers. NMP is the only approach to have been evaluated in a RCT, as mentioned previously.[12] Non-randomized clinical series have been reported for HMP, HOPE and SMP (in the context of COR), which demonstrated safety and several improved outcomes compared to SCS controls, including lower peak transaminases, fewer biliary complications, and increased graft survival.[20-24] Of note, a non-randomized clinical trial comparing 50 DCD livers transplanted after

HOPE to 50 matched controls from brain dead donors demonstrated similar graft survival at 5 years.[25] Recruitment for larger RCTs involving HOPE (NCT03484455, NCT02584283, NCT03929523) has already been initiated. Commercial adaptations of these devices include the OrganOx metra® and Organ Care SystemTM Liver (by TransMedics), which use NMP, the LifePort® Liver Transporter (by Organ Recovery Systems), which uses now uses HOPE, and the Liver Assist®, which is adaptable to different temperatures. Neither clinical nor preclinical studies have been conducted that compare different machine perfusion strategies against one another.

1.1.4 – Evaluation of Liver Grafts *Ex Situ*

An important strategy to maximize the utility of NMP in liver donation involves assessment and treatment of marginal liver grafts. Marginal grafts refer to organs that are at increased risk of graft failure and/or primary non-function or with an increased potential to transmit diseases based on donor factors.[26] These include grafts obtained from DCD donors, elderly donors, steatotic livers, donors with prolonged stay in the intensive care unit (especially if ionotropic support is required), and donors with positive viral serology, malignancy or active bacterial infections. In the previously mentioned RCT, Nasralla et al. demonstrated that the use of NMP decreased the rate of organ discard by 50% as compared to SCS, without compromising graft quality and function.[12]

With SCS, assessment of the graft itself is limited to biopsy, which is considered in the context of donor characteristics and laboratory tests. Conversely, NMP (and SMP) allows for additional evaluation, which can be as simple as monitoring physiologic parameters and basic laboratory investigations (pH, lactate, transaminases, etc.) to more complicated scoring systems. The validation of viability markers on NMP will assist in making the decision to transplant the

graft. As well, these markers could be used to justify deferment of transplantation to daylight hours and in prolonged transport from regional hospitals to transplantation centres.

Many potential biomarkers have been proposed for evaluation of graft function on NMP, including bile production, transaminase levels, ATP content, and lipid metabolism.[27] Analysis of bile composition has been suggested as a means to predict ischemic-type biliary lesions in particular. Preclinical studies have associated increased bile pH and bicarbonate concentration with decreased biliary epithelial injury.[28,29]

Mergental et al. published a clinical series, in which previously discarded grafts were successfully transplanted after undergoing assessment on NMP.[30] They judged graft viability based on lactate concentration in the perfusate, bile production, vascular flow, and overall appearance (e.g. macroscopic evidence of fibrosis, cirrhosis, steatosis, etc.). The same group has already initiated a non-randomized, prospective clinical trial (NCT02740608).[31] In this trial, livers are considered viable if they meet two or more of the following criteria within the first four hours of perfusion – lactate in the perfusate ≤2.5mmol/L, any evidence of bile production, metabolism of glucose, hepatic artery flow ≥150mL/min and portal vein flow ≥500mL/min, pH ≥7.30 or homogeneous perfusion (i.e. all segments of the liver appear to be perfused equally). Since this trial makes use of NMP, it is unclear if and how these criteria could be applied to an alternative setup, such as hypothermic oxygenated machine perfusion (HOPE).[13]

As this area of research progresses, both experimentally and clinically, it may be necessary to develop two sets of criteria: one that is used to determine donor suitability prior to procurement and a second used to judge graft viability, once it has been applied to MP, prior to implantation. The former would likely be a modification of existing donor criteria, informed by preclinical and clinical experience with MP. Of the many potential candidates for the latter, the optimal solution

should ultimately be one that combines predictive ability with clinical feasibility. An ideal scoring system should integrate donor and organ factors simultaneously.

1.1.5 – Machine Perfusion for Optimization of Liver Grafts

Using MP as a platform for optimization of liver grafts has not yet progressed to clinical applications. Several approaches have been explored in preclinical models, though there is still much research to be done. Among the most promising techniques include 'de-fatting' of steatotic livers, gene therapy, and eradication of hepatitis C virus.

Treatment of steatotic grafts or 'de-fatting' is a promising application in liver transplantation, given the impact of steatosis on graft viability. A study of data from European transplant centres found high grade hepatic steatosis to be the reason for transplant cancellation in 63.6% of cases in 2016.[32] A preclinical study by Nagrath et al. details the development of a 'de-fatting cocktail', which they tested during NMP of steatotic rat livers.[33] Their solution contained a combination of amino acids, visfatin (an adipocyte hormone that promotes glucose uptake and cell proliferation, among other functions), forskolin (a plant derivative used to raise levels of cAMP), and several nuclear receptor ligands, acting on PPARα, PPARδ, PXR, and CAR. The Nagrath group were able to show a 50% reduction in intracellular lipid content after 3 hours of NMP. Boteon et al. validated the same combination in human grafts that had been discarded for steatosis.[34] They found a reduction in tissue triglycerides (TG) and macrovesicular steatosis by nearly 40% within 6 hours compared to controls. This was also associated with enhanced metabolic function, lower vascular resistance, lower levels of transaminases in the perfusate, and higher bile production. As hepatic steatosis is primarily associated with TG accumulation, the challenge is to increase TG breakdown via lipolysis, while preserving structural and functional lipids important

to normal liver function.[35] Another consideration is the removal of by-products of de-steatification from the closed system, though this could conceivably be achieved by the addition of a dialysis circuit or a novel, targeted filtration system. Interestingly, Jamieson et al. showed a 50% reduction in the size of lipid deposits after 48 hours of NMP alone (i.e. with no lipolytic additives) in a porcine model of mild hepatic steatosis.[36] In this case, however, the length of perfusion required is clearly prohibitive and it is unclear how representative the porcine livers are of discarded steatotic grafts.

MP may also be a suitable platform for the eradication of viral infections from donor livers, such as hepatitis C virus (HCV). This has been shown in porcine livers undergoing NMP by Goldaracena et al., who used Miravirsen® to inhibit viral replication via sequestration of miRNA-122, on which HCV replication is dependent.[37] A comparatively simple approach for inactivation of HCV by circulating perfusate around a lamp producing germicidal ultraviolet light was shown to be successful in *ex situ* lung perfusion.[38] However, this approach may not be effective in livers, given that HCV replication primarily occurs intracellularly in hepatocytes.[39]

Beyond the simple addition of pharmaceutical compounds to the MP circuit, of which there are any number of reasonable candidates (anti-apoptotic agents, antioxidants, vasoactive drugs, etc.), more advanced therapies could be used to modify the graft on a genetic level. Gene therapy, with the aim of either improving graft quality or suppressing immunogenicity, has been employed to modify several types of machine perfused organs. Proof of concept has been recently demonstrated in large animal *ex situ* heart models, with the successful integration of the reporter gene luciferase.[40] Machuca et al., working with a porcine *ex situ* lung model, were able to show successful delivery of human IL-10, with decreased inflammation and improved gas exchange post-transplantation.[41] A study by de Roos et al. demonstrated the successful transfection of *ex situ*

rat livers using the two reporter genes (E. coli lacZ and firefly luciferase).[42] However, we failed to find any gene therapy studies using MP of livers from large animals. The advent of CRISPR/Cas9 may allow for more sophisticated and reliable gene editing techniques.[43] Depending on the intended duration of effect, techniques, such as RNA interference, could be used to downregulate whole pathways. Suggested targets are mainly aimed at preventing apoptosis caused by ischemia-reperfusion injury include p53, FAS, caspase-3, and complement component C3.[44] Proof of concept was recently reported by Gillooly et al. in isolated rat livers, in which they used lipid-based nanoparticles to achieve hepatocyte uptake of small interfering RNAs designed to target FAS receptor expression.[45] Cellular therapy is an additional avenue that merits further investigation. Verstegen et al. have recently reported the successful delivery of human mesenchymal stem cells at therapeutic doses to porcine liver grafts undergoing HOPE, including the retention of paracrine signaling function.[46]

1.1.6 – Extracorporeal Liver Support Using Machine Perfused Livers

The first potential application of MP, which extends beyond its direct use in liver transplantation, involves an integrative approach using livers perfused *ex situ* to provide metabolic support for patients in liver failure. This would involve using a healthy liver, most likely a xenograft, on a MP circuit and connecting it with the patient's circulation, either directly or separated by a filter (**Figure 1.2**). The *ex situ* liver would compensate for the patient's failing liver, allowing it time to recover while providing normal liver functions, such as glucose regulation, drug metabolism, and synthesis of albumin and clotting factors. This could potentially be applied to several groups of patients, including those awaiting a liver transplant, and patients presenting with acute or acute-on-chronic liver failure who are not immediate transplant candidates. It is even

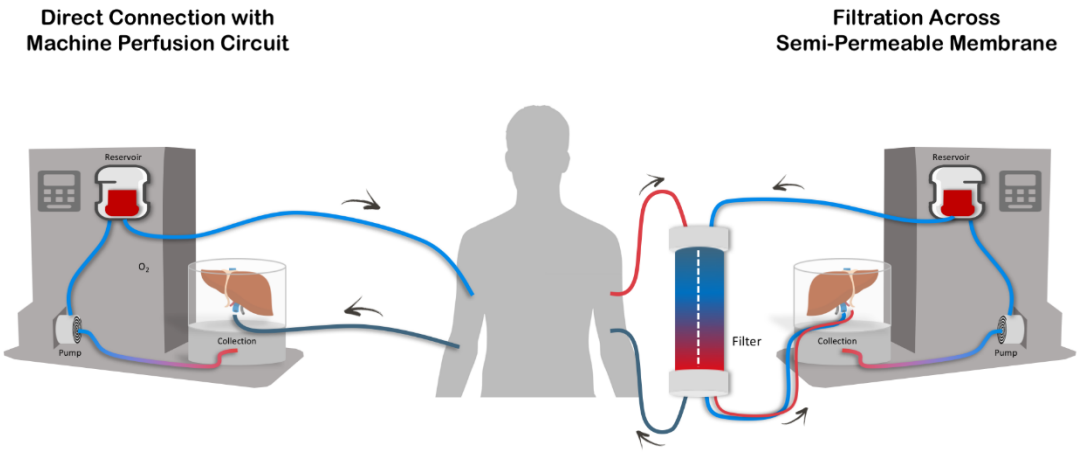

Figure 1.2. Alternative strategies for extracorporeal liver support using machine perfusion – via direct perfusion or across a semi-permeable membrane.

conceivable that it could be used for routine treatment of chronic liver failure patients, akin to renal dialysis, with the goal of prolonging or improving quality of life. However, this would require significant investment in supporting infrastructure. The recent study by Eshmuminov et al., demonstrating viability of a liver graft after 7 days of *ex situ* perfusion, supports the feasibility of this idea, as a single liver could be used to support multiple patients within this time period.[47]

The idea behind application of MP for liver support stems from research on bioartificial livers (BALs). Clinical reports of BALs appear in the literature as early as 1987, where one was used at a Japanese centre to treat hepatic failure in a patient with inoperable cholangiocarcinoma.[48] BALs have been used in many individual cases as a bridge therapy to transplant, but a benefit in mortality has not been convincingly shown. A recent systematic review and meta-analysis by He et al. identified 2 RCTs, 16 non-randomized clinical trials, and 12 pre-clinical large-animal studies.[49] The pooled analysis from the RCTs included 195 patients (97 treated with BAL) and showed a trend towards improved mortality that was not statistically significant. The preclinical studies all reported benefit in terms of mortality and metabolic function.

The main limitation with BALs has been the functional mass of hepatocytes. It has been estimated that 10 to 40 billion functional hepatocytes are required to provide adequate metabolic support.[50] Hepatocytes used in BALs have come from four main sources: primary human hepatocytes, hepatoblastoma cell lines, human-induced hepatocytes, and porcine hepatocytes.[51] Each source comes with its own challenges. Human hepatocytes are difficult to maintain in culture. They are also limited in availability and often of inconsistent quality. Interestingly, MP has been proposed as a platform to enhance the yield of isolated hepatocytes.[52] Cancer and animal cells lines are typically less metabolically active than normal human hepatocytes and with animal hepatocytes there is risk of zoonoses, such as porcine endogenous retrovirus (PERV). Induced hepatocytes (i.e.

from pluripotent stem cells) can function well, but are expensive, time-consuming, and require special expertise to produce.[53]

Novel techniques have been employed to enhance hepatocyte function, such as the use of bio-scaffolds and 3D tissue culture.[54] The general premise behind these innovations is that enhanced hepatocyte survival and function can be achieved by better mimicking the in vivo microenvironment. Specifically, extracellular biomatrices derived from porcine liver, cryogel matrix, hepatocyte spheroids, and co-culture with sinusoidal endothelial and stellate cells have been successfully employed to improve the functional capacity of BALs.[55-58] Some groups have even made use of decellularized human livers from discarded grafts.[59] *Ex situ* may offer a solution to this problem, though it comes with its own challenges of organ availability and procurement.

There are several preclinical studies that directly support the application of MP to extracorporeal liver support. A study by Nakamura et al., published in 1999, connected a healthy porcine liver on a NMP circuit directly to the circulation of a second animal, in which ischemic hepatopathy had been induced by 2 hours of hepatic artery and portal vein clamping.[60] Their study included both positive and negative controls. The former consisted of an animal without liver injury attached to the NMP circuit, while the latter was made up of animals with ischemic hepatopathy, to which no treatment was given. They found evidence that the *ex situ* liver assumed some of the physiological functions in the injured animal, including the production of ketone bodies, clearance of bilirubin, and regulation of glucose (all of the control animals died of hypoglycemia before 24 hours). Carraro et al. reported a similar study in 2007, with a more severe hepatic injury induced by complete hepatic artery ligation in a porcine model.[61] They were able to successfully maintain perfusion for 6 hours, though the animal eventually succumbed to lactic acidosis. Unfortunately, they did not provide any controls for comparison. Naruse et al. developed a xenogeneic model

using a canine recipient that relied on separate leukocyte- and immunoglobulin-adsorbent filtration columns. They were able to perfuse the animal for 3 hours without acute rejection and demonstrated reduction in ammonia levels.[62] The sole clinical report was described by Levy et al. in 2000, in which they used MP of a transgenic porcine liver undergoing to treat two patients with acute liver failure awaiting transplantation.[63] They were able to demonstrate safety and feasibility over a total perfusion time of 16.5 hours, including the lack of zoonotic transmission. No similar studies have been reported in the literature since, presumably due to the complexity of the models and the number of technical and logistical hurdles to be overcome before they could be applied clinically.

A simpler extracorporeal liver support device, dubbed L.E.O.NARDO, which provides oxygenation directly via the portal vein, has been described by Nardo et al.[64] They were able to demonstrate benefit in a porcine model of acute liver failure (ALF) induced by sub-total hepatectomy, with the treatment group showing lower serum transaminases, improvement of coagulopathy, and increased 7 day survival (75% vs. 0%). They subsequently published a clinical case report using this device in a patient who underwent an extended hepatectomy for colorectal metastases.[65] Although it appears quite resource-intensive (requiring application in the operating room for 6 consecutive days), the patient reportedly had an uneventful post-operative course and was discharged home on post-operative day 10. Additionally, it is unclear whether this treatment would be effective outside of ischemic liver injury.

A potential concern with the use of MP for extracorporeal support is the immunologic effect of the *ex situ* perfused liver. The most likely source would be large animal xenografts. Discarded human grafts could potentially be used, which would be less immunogenic, but would also be limited by their sporadic availability and potentially decreased function (for the same

reason they were discarded). Immunosorbant filters (i.e. filters capable of absorbing immunoglobulins) used in BALs have been shown to mitigate imunogenic hepatocyte injury when treating across species.[66]

More promising is the extensive research that has been done in the genetic engineering of animals with the express purpose of xenotransplanting various organs, from large solid organs, like liver and kidney, to heart and lungs to smaller volume tissues, like corneas and pancreatic islets.[67] There has been remarkable success in xenotransplantation between animals. Of note, a model of orthotropic heart transplantation from a genetically engineered pig into a baboon has been reported, which survived for up to 195 days.[68] Swine are often the animal of choice for such models, given their similarity to humans in terms of size and metabolism. Typically, porcine models of xenotransplantation rely on the knockout of α1,3-galactosyltransferase (GGTA1) with or without the addition of human CD46.[69] GGTA1 is the enzyme responsible for the synthesis of α1,3Gal epitopes on the cell surface.[70] These are present in most mammals, with the exception of apes (including humans) and old world monkeys. These epitopes were identified in early research in xenotransplantation as playing a major role in hyperacute rejection in pig-to-human transplants.[71] CD46 is a membrane-bound protein that plays an inhibitory role in the complement system.[72] Though CD46 is the most widely studied in this capacity, other human complement inhibitors, such as CD55 and CD59, have also been investigated for their utility in models of xenotransplantation.

Porcine livers from animals genetically engineered to be less immunogenic could serve as the basis for extracorporeal liver support via MP. There has been success in preclinical studies with the transplantation of such organs between animals and, because liver support would be of relatively short duration, immunogenicity would potentially be less of a concern.

Immunosuppressive agents could still be given during therapy to mitigate any residual or idiosyncratic response. The use of CRISPR/Cas9 gene editing technology could greatly benefit the further development of suitable transgenic animals for liver support.[73] The gene editing technology has already successfully been used to inactivate PERV in porcine models.[74]

1.1.7 – The Role of Machine Perfusion in Cancer Research & Therapeutics

Machine perfusion of livers and other organs has the potential for benefits in the field of oncology, both with regard to research and therapeutics. Both systemic and regional therapies could be tested for efficacy on *ex situ*-perfused livers, both healthy and diseased. Organs could come from discarded grafts or even from resected specimens from cancer patients. Alternatively, researchers could potentially test therapies on animal livers that had been xenografted with human tumours.[75] MP is unlikely to be a feasible platform to guide personalized cancer therapeutics (i.e. using patient derived xenografts), but this is a conceivable possibility.

One study was identified in the literature that made use of an NMP circuit along these lines. Cyzmek et al. used an *ex situ* liver model to establish dose-response relationships with electrochemical ablative techniques.[76] The effects of other regional therapies, such as radiofrequency ablation or transarterial chemoembolization could likewise be studied in this way. Systemic chemotherapy could also be tested for efficacy within the circuit. *Ex situ* liver perfusion limits animal suffering compared to in vivo models, while maintaining representative physiology. MP could potentially play a role in the treatment of patients, such as with high-dose chemotherapy with or without the addition of radiotherapy, with goal being to maximize the therapeutic window for exposure to the liver while minimizing systemic exposure.[77] This would be similar to in vivo isolated hepatic perfusion, which has been studied clinically. With in vivo isolated hepatic

perfusion, a closed circuit is made between an extracorporeal perfusion pump, with inflow via the hepatic arterial system and outflow via the inferior vena cava (IVC).[78] Vascular clamps are applied to occlude other inflow and outflow tracts and a second circuit is required to shunt blood from the infrarenal IVC back to the heart. Applying this idea *ex situ* perfusion, the patient's liver could be resected, attached to the circuit, treated, and then re-implanted. The advantage of *ex situ* over in vivo hepatic perfusion would be the avoidance of systemic leakage, as well as the potential for more targeted techniques. Currently, however, the literature does not support the use of in vivo isolated hepatic perfusion for the treatment of malignancies. A phase I clinical trial using high dose melphalan to treat colorectal metastases showed only a 29% response rate, with only one complete response among 24 patients.[79] The same treatment has been reported for hepatic metastases of uveal melanoma, with better, but still variable, response rates.[80] Effectiveness of the chemotherapeutic agent would have to be firmly established prior to investigating this application of *ex situ* MP clinically.

A second application of MP in relation to oncologic therapies would be to use this technology for otherwise unresectable tumours. With the exception of hepatocellular carcinoma, which is regularly treated with liver transplantation, palliative treatments are often the only options for surgically unresectable hepatic tumours.[81] Similar to the high-dose chemotherapy treatment described above, it would require hepatectomy, resection of the tumour, while the organ undergoes MP, followed by autotransplantation. Gringeri et al. described a preclinical model using MP at 20^{o}C, in which they demonstrate the feasibility of this technique.[82] A partial hepatectomy was performed *ex situ* during MP, which lasted 2 hours. The liver remnant was re-implanted and the animals were recovered. They reported peak transaminases and lactate levels at 3 hours post-reperfusion and negligible histologic damage upon examination of the re-implanted segment. A

clinical series by Forni and Meriggi details four cases of *ex vivo* tumour resection, one of whom underwent *ex situ* hypothermic perfusion at 4ºC.[83] The evidence for *ex vivo* liver resection, in general, is limited to case reports and series. It is mainly used in cases requiring complex vascular or biliary reconstruction, such as tumours adjacent to the inferior vena cava or involving the porta hepatis.[84-86] Success is variable, though tumour free survival has been reported as far out as 17 months.[87] The application of MP to this technique could mitigate ischemia and reperfusion injury of the remnant liver, potentially reducing the high morbidity and mortality in the early post-operative period.

1.1.8 – Toxicology Studies Using Machine Perfused Livers

A variety of models exist that are routinely employed for the preclinical evaluation of hepatoxicity.[88] These include in vitro techniques from basic hepatocyte culture, to co-culture with non-parenchymal cells, to liver slices to 3D cell culture and BALs, in addition to in vivo studies.[89] Isolated perfusion of rat livers was developed as a model for hepatotoxicity in the 1950's.[90] However, the increased setup and finesse required may not be justified given the differences in rat metabolism, including resistance to acetaminophen toxicity.[91,92] It is generally assumed that studies in higher order species are more representative of humans, with rodent studies being less relevant than porcine or canine studies, which are less relevant than studies in non-human primates. Clinical correlation with animal models can often be disappointingly low, with concordance of approximately 40% for rodent models alone.[93,94] Though the addition of a second animal model can raise concordance rates to over 70%, this still results in a number of unpredicted effects. A review of FDA reports found hepatotoxicity to be the primary reason for the withdrawal of a drug

from the market in 32% of cases from 1975 to 2007.[95] This was second only to cardiovascular side-effects.

MP could offer an alternative platform for hepatotoxicity testing, serving as a bridge between preclinical and clinical studies. It would make use of porcine grafts, whose hepatic metabolism is generally more comparable with humans than that of other animal models, or potentially cadaveric grafts, subject to availability.[96] Studies investigating drug metabolism as a markers of graft viability have shown *ex situ* perfused grafts to be metabolically active. Linares-Cervantes et al. recently demonstrated metabolism of rocuronium in an *ex situ* liver on a NMP circuit.[97]

There is the possibility of further refinement using genetically engineered animals, such as a model expressing human cytochrome p450 genes, the enzymes responsible for metabolism of up to 55% of drugs.[98] Compared to an in vivo model, this approach reduces animal suffering and reduces the need for care and husbandry, while maintaining representative physiology. A longer duration of perfusion, such as the 7 days demonstrated by Eshmuminov et al., may prove better suited for this purpose.[47] Though, it would be limited to the use of intravenous formulations. Beyond hepatotoxicity, MP could potentially be developed into models of specific diseases, such as acute liver failure.

1.1.9 – Other Proposed Uses

The main potential for MP of the liver outside of transplantation involves research and clinical applications of novel therapeutic agents. However, some have suggested a role in surgical education. Liu et al. recently published a report of a laparoscopic training system using an *ex situ* perfused porcine liver.[99] This is an innovative use of a novel technology, though it is not clear that

this use would offer much advantage over live animals. The animal may not have to be maintained under anesthesia for a prolonged period, but it would still need to undergo a hepatectomy. This setup would also offer lower fidelity than laparoscopy in a live pig and would still be single use. Perhaps if multiple parts of the animal were needed simultaneously for teaching purposes or if organs were obtained from an abattoir at the time of commercial sacrifice this could be a viable option.

1.1.10 – Conclusion

The past two decades have seen the rise of remarkable technology for the preservation of whole organs outside of the body. Machine perfusion of the liver has entered the clinical sphere as a tool to improve organ preservation between removal and implantation, in an effort to reduce complications of ischemia and make efficient use of the limited organ supply. Recently completed and ongoing randomized controlled trials make it likely that machine perfusion will remains a part of liver transplantation for the foreseeable future. The assessment and modification of externally perfused grafts is at the frontlines of research in this field. Beyond transplantation, this technology has the potential to be applied towards other problems in healthcare, including the use of extracorporeal liver support, oncologic research and therapeutics, and toxicology testing.

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1.1.12 – Addendum

The field of liver machine perfusion has advanced considerably since the publication of this review article in 2020. There are currently 5 different products that have been commercially developed for liver perfusion. The OrganOx metra® and Organ Care Systems (OCS™) Liver are designed to perfuse the organ at normothermic temperatures (NMP), while the Liver Assist (XVIVO) is capable at perfusing at a range of temperatures (from 12-37ºC), though it has been most commonly used for hypothermic oxygenated machine perfusion (HOPE). Bridge to Life's Vitasmart™ is being marketed as a more simply designed device for HOPE, while Organ Recovery Systems' LifePort® Liver Transporter was designed to be as transportable as possible using a hypothermic machine perfusion strategy (i.e., without providing continuous oxygen).

The OrganOx metra® was the first device to undergo a large, adequately powered randomized controlled trial (RCT), as outlined in the previous review paper.[1] It achieved regulatory approval in the United Kingdom (National Institute for Health and Care Excellence, NICE) on this basis in early 2019 and was approved for up to 24 hours of continual use. The OrganOx has subsequently been granted premarket approval by the Federal Drug Administration (FDA) in the United States, but interestingly limited for use restricted to under 12 hours which is half the duration approved in the UK and Europe due to the relatively small number of cases in which the liver was perfused beyond 12 hours. A RCT of the OrganOx metra® was completed (with summary data accessible from the FDA website) across 15 US sites included 383 randomized livers, 266 of which were transplanted. Similar to the European study, the US trial demonstrated reduction in post-reperfusion syndrome (5.9% in the NMP group vs. 14.6% in the SCS group), though significance was not reported. However, there was no difference in the primary endpoint of early allograft dysfunction (EAD) when analysed as intention-to-treat (20.5% in the NMP group

vs. 22.8% in the SCS group, p=0.275).[2] The OrganOx metra® is currently under active review by Health Canada, and a request for ongoing clinical use through Health Canada's Special Access program is pending currently. Data from our centre has been recently published, as detailed in *Chapter 4*.[3] In summary, we found significant decrease in 30-day graft failure (p=0.03), with no differences in longer term outcomes, despite a much more prolonged pre-perfusion cold ischemic phase in our study compared to the UK-European experience. The prolonged cold ischemia was due to the broader geographical distribution and our 'back-to-base' approach, where livers are transported by small planes rather than road transportation) compared to the European and American RCTs.

Organ Care Systems (OCS) trial of their normothermic machine perfusion device for the liver has also been completed, with results published in early 2022.[4] Markmann *et al.* randomized 476 livers across 20 US centres, 300 of which were transplanted. They demonstrated 45% reduction in EAD in the NMP group by intention-to-treat analysis (p=0.01). This was reflected by pathological assessment, where livers treated with NMP showed a lower proportion of lobular inflammation compared to controls (p=0.04). They also reported an impressive 72% reduction in ischemic biliary complications (defined as non-anastomotic ischemic strictures or bile leaks) at 12 months (p=0.02) – a finding that was not evident in the Organox experience. The main difference between the OCS Liver and the OrganOx, which may explain the reduction in ischemic biliary complications, is the use of pulsatile perfusion in the former versus continuous flow in the latter. This has also been suggested as important by other groups and is used by the Liver Assist device, with the proposition that it may reduce microvascular thrombi, though this has not been investigated in detail on a mechanistic level.[5] TransMedics, the company that markets the OCS, has recently been promoting a comprehensive approach to surgical procurement, NMP and

transportation across the US using their own commercial planes and dedicated expert surgical teams. Interestingly, this model is being adopted and supported by third party payers in the US despite a substantial cost to this package.

A multicentre randomized trial applying HOPE to livers following donation after circulatory death (DCD) was published in early 2021.[6] Van Rijn *et al.* included 78 livers perfused with the Liver Assist device for a minimum of two hours prior to transplantation compared to the same number of controls. They demonstrated a marked reduction in non-anastomotic biliary strictures, which occurred in 6% of perfused livers, compared to 18% of controls (p=0.03). They also found a reduction in EAD (26% in the HOPE group vs. 40% in controls). Results from a second HOPE trial using the Liver Assist for DBD livers were recently published.[7] Schlegel *et al.* included 85 perfused livers compared to 85 SCS controls. They found no difference in their primary outcome of postoperative complications Clavien-Dindo grade 3 or greater (51.8% of HOPE livers vs. 54.1% of controls; p=0.76), but did find that recipients of control livers had more severe complications (≥ Clavien-Dindo grade 3; p=0.027) and a greater proportion of graft loss due to liver-related complications (0% of HOPE livers vs. 7% of controls; p=0.0004). The Liver Assist device is currently under expedited review with the FDA as part of their Breakthrough Device program.[8] The HOPE system creates a conundrum as outcomes have been surprisingly positive with more marginal/DCD livers, but the cooler non-physiologic perfusion temperature precludes pre-emptive and predictive testing for discard or use of higher risk grafts – clearly a drawback of that approach despite the excellent clinical outcomes. An editorial entitled "Warming up to cold perfusion" nicely encapsulated this debate.[9]

The VitaSmart™ device, similarly intended for HOPE, is approved for use in most of Europe and is actively recruiting for trials in the United States.[10] A single centre RCT was

published using the VitaSmart™ device that included 55 HOPE livers compared to 55 controls. This study found a lower incidence of EAD in the HOPE group, (13% vs. 35%; p=0.007), which corresponded with a lower rate of retransplantation within the follow up period (0% vs. 11%; p=0.027). At the time of writing, clinical trial results for the LifePort® Liver Transporter, based on the early studies with HMP by Guarera *et al.*, have yet to be released and are eagerly anticipated.[11]

While many of the possibilities for liver machine perfusion discussed in our review remain unrealized, some are being actively pursued, with promising inroads being made in some areas. As discussed previously, the development of a NMP device capable of maintaining a liver for 7 days (Liver4Life), facilitated by the Swiss technology accelerator Wyss Zurich and professor Pierre Clavien, is a technological achievement that may pave the way for more extensive reconditioning of grafts.[5] This has the potential to allow for such approaches as regeneration of a liver segment for autotransplantation or reseeding severely injured livers with fresh hepatocytes, and could potentially facilitate global sharing of livers internationally as there would be time for planning and transportation. In early 2022, a report was published of the first liver transplanted using this device.[12] The liver had been rejected by other centres due to the presence of an undiagnosed tumour in segment 1 and multi-resistant intra-abdominal infection in the donor. It was perfused for 3 days, allowing for resection and definitive pathological diagnosis of the tumour (a benign perivascular epithelioid tumour) and broad-spectrum antimicrobial treatment. Having also passed viability criteria, including responsiveness to vasoactive drugs, satisfactory bile production, preservation of tissue architecture on histology, and subsidence of injury markers (aminotransferases, pro-inflammatory cytokines), the graft was transplanted into the recipient with

good success. According to the report by Clavien *et al.*, the recipient was discharged after 12 days and remained well at 1-year follow up.

Much research has also been done with regards to liver splitting during machine preservation. Technical success has been demonstrated using both NMP and HOPE.[13,14] Liver splitting during machine perfusion can reduce cold ischemia during the splitting process (which typically takes at least 90 minutes).[15] It also has the potential to enable the transplantation of both hemi-livers by the same team in sequence (i.e., the second portion of the graft continues to be perfused while the first is transplanted), which could allow for increased use of split grafts. Lau *et al.* reported an NMP-based technique to split 10 discarded livers into left lateral and extended right grafts.[16] They demonstrated successful perfusion of each graft separately for 24 hours, with only one hemi-liver lost due to technical failure, though none of these were transplanted so the findings are somewhat artificial. Successful transplantation of 16 hemi-livers into eight adult and eight pediatric recipients following splitting during HOPE has also been reported by Rossignol *et al.*, with no graft loss or death over a median follow-up of 7.5 months.[17]

Adjacent to the field of machine perfusion, there has also been a recent surge of interest in normothermic regional perfusion (NRP) for DCD organ procurement. This approach involves perfusion of the abdominal organs *in situ* before retrieval and can be done in combination with or separate from the intra-thoracic organs (i.e., heart and lungs).[18] It is currently standard practice for some European countries; however, uptake in North America has been slow and there are concerns that it may conflict with standards for determining death, which can vary between states and provinces.[19] NRP has the potential to replace machine perfusion in certain settings, particularly when machine perfusion is not intended for transport, and may be technically simpler. Comparing NRP with NMP and SCS, Gaurav *et al.* found that both NRP and NMP reduced EAD compared

to SCS ($p<0.0001$).[20] NRP livers additionally showed a trend toward improved 6-month graft survival (90% vs. 76% in the SCS group; $p=0.006$) and none developed non-anastomotic strictures whereas 11% of the NMP and 14% of the SCS livers did.

Still, NRP may be augment rather than supplant machine perfusion. Patrono *et al.* were able to achieve outcomes for DCD livers subject to NRP followed by HOPE comparable to donation after brain death donors (DBD) with 1-year graft survival of 90% versus 95% in the DBD cohort ($p=0.82$).[21] NRP has alternatively been used in conjunction with NMP to achieve ischemia-free liver procurement, as described by Zhao *et al.*, whereby cannulation and initial perfusion was done via the hepatic artery and portal vein *in situ* as the liver was resected, after which it was transitioned to the organ chamber.[21] Guo *et al.* published a series of 38 ischemia-free liver transplants and found that this approach resulted in a significant reduction in EAD ($p<0.001$) compared to a cohort of livers procured in the standard fashion.[22] One-month graft survival was also higher (97.4% vs. 90.0%) though it did not achieve statistical significance ($p=0.195$).

Our understanding of what machine perfusion can do, as well as some of its limitations have become clearer over the last several years. It is likely to remain an important part of the transplant surgeon's toolkit going forward, particularly as the gap between organ demand and supply continues to expand and use of extended criteria donors is increasingly relied on. Future developments will see an even greater role for machine perfusion in the recovery and modification of otherwise unusable grafts.

1.1.13 – References (for Addendum)

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CHAPTER 2 – CYCLOSPORINE A FOR THE PREVENTION OF ISCHEMIA REPERFUSION INJURY

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PART 1 – PRECLINICAL SYSTEMATIC REVIEW & META-ANALYSIS OF CYCLOSPORINE FOR THE TREATMENT OF MYOCARDIAL ISCHEMIA REPERFUSION INJURY

2.1 - Preclinical Systemic Review & Meta-Analysis of Cyclosporine for the Treatment of Myocardial Ischemia Reperfusion Injury

Original Article

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Preclinical systematic review & meta-analysis of cyclosporine for the treatment of myocardial ischemia-reperfusion injury

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Background: Though best known for its immunosuppressant effects, cyclosporine A (CsA) has also been studied as a treatment to mitigate ischemia-reperfusion injury (IRI) by its inhibition of the mitochondria permeability transition pore (mPTP). Despite numerous preclinical studies supporting its benefit in reducing infarct size following myocardial IRI, large randomized controlled clinical trials have been unable to show a beneficial effect. Exploring existing preclinical data can give us the opportunity to revisit some the assumptions that may have led to the failure of these studies to translate clinically. Herein, we present a systematic review of preclinical studies testing CsA to attenuate myocardial IRI (PROSPERO CRD42020159620).

Methods: We conducted a systematic search of health research databases Ovid MEDLINE, Ovid EMBASE, Web of Science BIOSIS, and Scopus, as well as Cochrane and PROSPERO systematic review databases, on March 9, 2022 for non-human *in vivo* animal studies of myocardial IRI, using CsA as a treatment that reported clinically relevant outcomes. Bias was assessed using the Systematic Review Centre for Laboratory Animal Experimentation's risk of bias tool and a modified Collaborative Approach to Meta Analysis and Review of Animal Data from Experimental Studies checklist. Sub-group meta-analyses were conducted to identify potential factors influencing outcomes.

Results: We identified 71 studies, 59 of which were studies of coronary occlusion. Overall, 75% of studies reported a clear positive effect of CsA in mitigating myocardial IRI by some clinically relevant parameter (e.g., infarct size). A meta-analysis including 43 coronary occlusion studies showed an overall reduction in infarct size with CsA treatment (16.09%; 95% CI: −18.50% to −13.67%). Subgroup meta-analyses identified species, age, timing of administration, and duration of ischemia as factors potentially affecting the efficacy of CsA in the setting of myocardial IRI.

Conclusions: Our systematic review and meta-analysis identifies questions that have yet to be answered by preclinical studies, highlighting important differences between these and clinical studies that should be addressed prior to proceeding with any further clinical studies using CsA to treat IRI in the heart or other

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Title: Preclinical Systematic Review & Meta-Analysis of Cyclosporine for the Treatment of Myocardial Ischemia-Reperfusion Injury

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2.1.1 – Preface

Background: Though best known for its immunosuppressant effects, cyclosporine A (CsA) has also been studied as a treatment to mitigate ischemia/reperfusion injury (IRI) by its inhibition of the mitochondria permeability transition pore. Despite numerous preclinical studies supporting its benefit in reducing infarct size following myocardial IRI, large randomized controlled clinical trials have been unable to show a beneficial effect. Exploring existing preclinical data can give us the opportunity to revisit some the assumptions that may have led to the failure of these studies to translate clinically. Herein, we present a systematic review of preclinical studies testing CsA to attenuate myocardial IRI (PROSPERO CRD42020159620).

Methods: We conducted a systematic search of health research databases Ovid MEDLINE, Ovid EMBASE, Web of Science BIOSIS, and Scopus, as well as Cochrane and PROSPERO systematic review databases, on March 9, 2022 for non-human *in vivo* animal studies of myocardial ischemia-reperfusion injury, using CsA as a treatment that reported clinically relevant outcomes. Bias was assessed using the Systematic Review Centre for Laboratory Animal Experimentation's risk of bias tool and a modified Collaborative Approach to Meta Analysis and Review of Animal Data from Experimental Studies checklist. Sub-group meta-analyses were conducted to identify potential factors influencing outcomes.

Results: We identified 71 studies, 59 of which were studies of coronary occlusion. Overall, 75% of studies reported a clear positive effect of CsA in mitigating myocardial IRI by some clinically relevant parameter (e.g., infarct size). A meta-analysis including 43 coronary occlusion studies showed an overall reduction in infarct size with CsA treatment (16.09%; 95%CI -18.50% to -13.67%). Subgroup meta-analyses identified species, age, timing of administration, and duration of ischemia as factors potentially affecting the efficacy of CsA in the setting of myocardial IRI.

Discussion: Our systematic review and meta-analysis identifies questions that have yet to be answered by preclinical studies, highlighting important differences between these and clinical studies that should be addressed prior to proceeding with any further clinical studies using CsA to treat IRI in the heart or other organs. We also use the example of CsA to highlight general considerations for researchers attempting to translate animal studies into the clinical setting.

2.1.2 – Introduction

Cyclosporine A (CsA) was first isolated in 1970 at Sandoz laboratories from a fungus found in a Norwegian soil sample (1). Sandoz had been trying to identify novel antibiotic compounds, but, in screening this compound, discovered that it had the ability to neutralize cytotoxic T cell activity *in vitro* (2). Subsequent *in vivo* studies further demonstrated its ability to suppress both antibody- and cell-mediated immunity (2). By the late 1970's, CsA had been shown to promote graft survival in animal models of heart and kidney transplantation (3,4). This quickly led to clinical trails, which found similar benefits in human transplant recipients (5). This, combined with its low toxicity, led to CsA become the immunosuppressant drug of choice in the early days of solid organ transplantation and enabled the expansion of transplant programs worldwide.

The immunosuppressant effect of CsA is a result of calcineurin inhibition (6). Calcineurin is a phosphatase, whose activation of certain transcription factors leads to the upregulation of interleukin-2 and other cytokines important for initiating the T cell response. A secondary effect of CsA on mitochondria membrane permeability was later described by researchers trying to understand the mechanism behind CsA nephrotoxicity (7). It was discovered that CsA can bind to cyclophilin D, part of the mitochondria permeability transition pore (mPTP), preventing its opening during times of increased oxidative stress, which could otherwise lead to mitochondrial swelling, disruption of the electron transport chain, and eventual rupture (8).

Researchers soon realized that this property of CsA could mitigate ischemia-reperfusion injury (IRI) caused by transient loss of blood flow to an organ or tissue. This was demonstrated in animal models involving various organs, including the heart and kidney (9,10). There was

particular interest in using CsA to protect the myocardium from IRI following revascularization, such as after coronary artery thrombosis.

Despite promising studies in animals, attempts to translate findings into the clinical realm produced mixed results. An initial pilot randomized controlled trial (RCT) of 58 patients conducted across several hospitals in France found that CsA given at 2.5mg/kg at the onset of reperfusion in patients undergoing percutaneous coronary intervention (PCI) led to smaller infarct size and decreased creatinine kinase (CK) levels (11). However, the subsequent larger trial involving 970 patients failed to show any clinical benefit and found that CsA did not reduce the risk of adverse left ventricular remodeling at one year (12).

In order to better understand the failure of CsA to translate clinically, it is worthwhile to return to the preclinical studies that informed clinical trials. The purpose of this review is to summarize evidence in the preclinical literature for the benefit of CsA in IRI. In addition to elucidating possible reasons for the failure of preclinical studies to translate into the clinical realm, we sought to identify gaps that should be addressed before moving forward with any further clinical studies aiming to use CsA to mitigate IRI in the heart or other organs. Our search included models of IRI in any organ; however, in this article we will summarize only cardiac studies to allow for a more in-depth analysis. We present the following article in accordance with Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist.

2.1.3 – Methods

Database & Literature Search Strategies

The proposed systematic review was prospectively registered in the online international registry PROSPERO (www.crd.york.ac.uk/prospero/) with the unique ID CRD42020159620.

Searches were conducted on March 9, 2022 by a health librarian/expert searcher (SC) of the health research databases Ovid MEDLINE, Ovid EMBASE, Web of Science BIOSIS, and Scopus, as well as the Cochrane Database of Systematic Reviews and the PROSPERO database of systematic review protocols. Keywords and controlled vocabulary (e.g., MeSH, EMTREE) were used to identify studies related to the concepts: "reperfusion injuries" and "cyclosporin". *In vitro* studies were excluded. No other limits were applied. Searches were adjusted appropriately for each database. Results were exported to Covidence review management software (www.covidence.org) and duplicates were automatically removed. A detailed search strategy is included in the Supporting Materials.

Eligibility Criteria

The primary aim of the review was to include all non-human *in vivo* animal studies of ischemia-reperfusion injury, using CsA as a treatment. There was no exclusion of studies based on species, language, date of publication or type of publication (e.g., paper, brief communication, abstract). Non-experimental publications, as well as *in vitro* and *ex vivo* (i.e., isolated perfused organs) studies were excluded. Studies were excluded if they did not have an appropriate control group for comparison (i.e., ischemia-reperfusion alone) or if CsA was used for another indication (e.g., at high doses to cause nephrotoxicity). Studies not reporting clinically relevant outcomes were also excluded. Clinically relevant outcomes were taken as routine serum biochemistry (e.g., troponin, creatinine, lactate, liver transaminases), infarct size, histological assessment of injury, organ function, and survival. Human studies were excluded last, with the intention for them to be analyzed separately if appropriate. Different publications presenting identical data (e.g., conference abstracts and full-length papers by the same authors) were excluded, however,

publications presenting non-identical data from the same authors were included to minimize the risk of publication bias.

Studies were reviewed in two stages. First, a title and abstract review was conducted independently by two reviewers (JH and BMG). This was followed by a full text review, applying the same inclusion/exclusion criteria. Conflicts were resolved by means of consensus between the two reviewers.

Data Extraction

Data was retrieved from selected studies by a single reviewer (JH). Data extracted included animal characteristics (species, strain, sex, age, number per group), experimental characteristics (dose of CsA, timing of drug administration, duration of ischemia, blood vessel occluded), and animal outcomes (infarct size, biochemical markers of injury, histological evidence of injury, markers of organ function, survival). Data was extracted manually from graphs if it was not listed explicitly.

Quality Assessment

Bias was assessed using the Systematic Review Centre for Laboratory Animal Experimentation's (SYRCLE) risk of bias tool, as well as a modified Collaborative Approach to Meta Analysis and Review of Animal Data from Experimental Studies (CAMARADES) checklist (13,14). Though any type of publication was included in our review, only full-length articles were assessed for bias, as this was impractical for conference abstracts and brief reports due to their lack of detail.

Statistical Analysis

Meta-analysis was conducted using RevMan 5 software (Cochrane). Only coronary occlusion studies reporting infarct size were included, as this was the most common study design and most common reported outcome. Results were reported as weighted mean differences since all studies used the same unit measure (percentage of area at risk). Results from abstracts were included in the analysis only if there were no subsequent full-length publications of the same study (to avoid duplication of results). Different treatment groups within the same study were treated separately. A random effects model was chosen due to the statistical and methodological heterogeneity of the studies. Subgroup meta-analyses were planned based on age, sex, species, dose, timing of administration, and ischemia duration, if appropriate.

2.1.4 – Results

Study Inclusion

The PRISMA diagram for the systematic review is presented in **Figure 2.1.1**. Our initial search yielded 2,070 unique records. At the abstract review phase, the *kappa* score between reviewers was 0.98, indicating almost perfect agreement. After abstract screening, 625 studies remained for full text review, 164 of which were ultimately included as preclinical studies. The full text of 67 studies could not be found despite extensive searching through online databases and physical records. The majority of these were either conference abstracts (37/67) or from smaller, non-English language journals (12/67). The numbers of included studies were further broken down by organ. Given the high number of records identified, this article will deal with only cardiac IRI, which includes 71 total studies (see Supplements Appendix for full list of included articles).

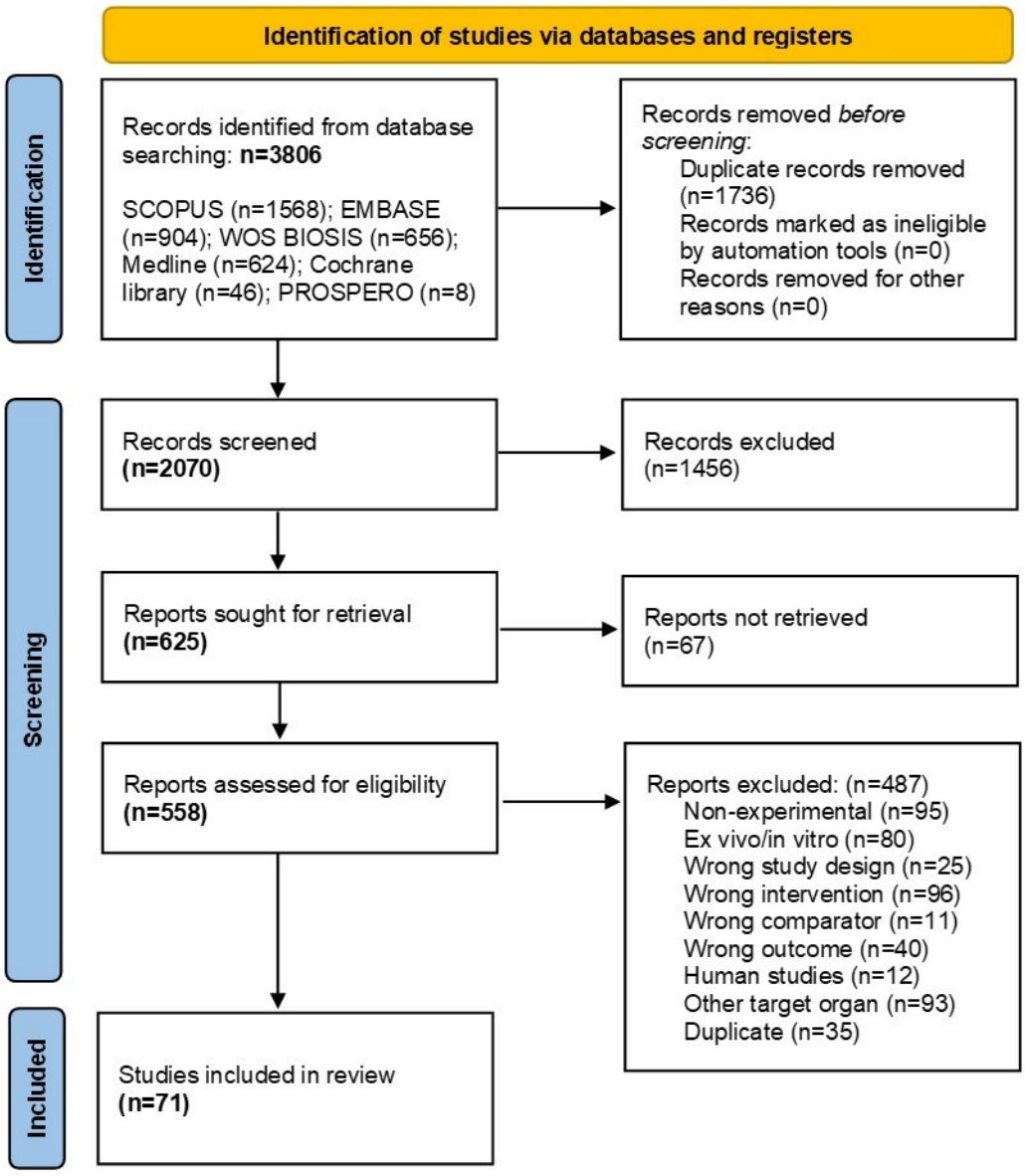

Figure 2.1.1. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) diagram for systematic review of preclinical studies of cyclosporin A for the treatment of ischemia/reperfusion injury.

Risk of Bias Assessment

Risk of bias was assessed using SYRCLE's risk of bias tool, as well as a modified CAMARADES checklist. Using SYRCLE's tool, the majority of categories had either high or unclear risk of bias across studies (**Figure 2.1.2A**). The risk of bias being unclear was mainly due to studies lacking sufficient detail about procedures, such as randomization, allocation concealment, and handling of baseline characteristics. We found low risk of bias related to selective outcome reporting. Though no study had a prespecified protocol available, we did find that the majority of studies were consistent in reporting all outcomes described in the methods. We also found that studies were largely free of other important sources of bias, such as contamination, unit analysis error or the inappropriate influence of funders. Results from the CAMARADES checklist (**Figure 2.1.2B**) additionally highlighted other potential areas of bias, such as lack of sample size calculation, unclear conflicts of interest, and confirmation of ischemia.

Study Characteristics

Of the 71 cardiac studies identified, 13 (18%) were conference abstracts, while 58 (82%) were full-length articles. The majority of cardiac studies (59/71, 83%) used a model of coronary artery occlusion, most commonly occlusion of the left anterior descending artery (36/59, 61%) (**Supplementary Table 2.1.1**). Other models included cardiac arrest, cardiopulmonary bypass, hypoxia, and one study of CsA for the treatment of IRI in cardiac transplantation (**Supplementary Table 2.1.2**).

Studies employed a variety of animals, including mice, rats, rabbits, pigs, and sheep. However, rats were the most common animal, used in 42% of studies (30/71). CsA was most commonly administered as a single dose, intravenously or intraperitoneally, though several studies

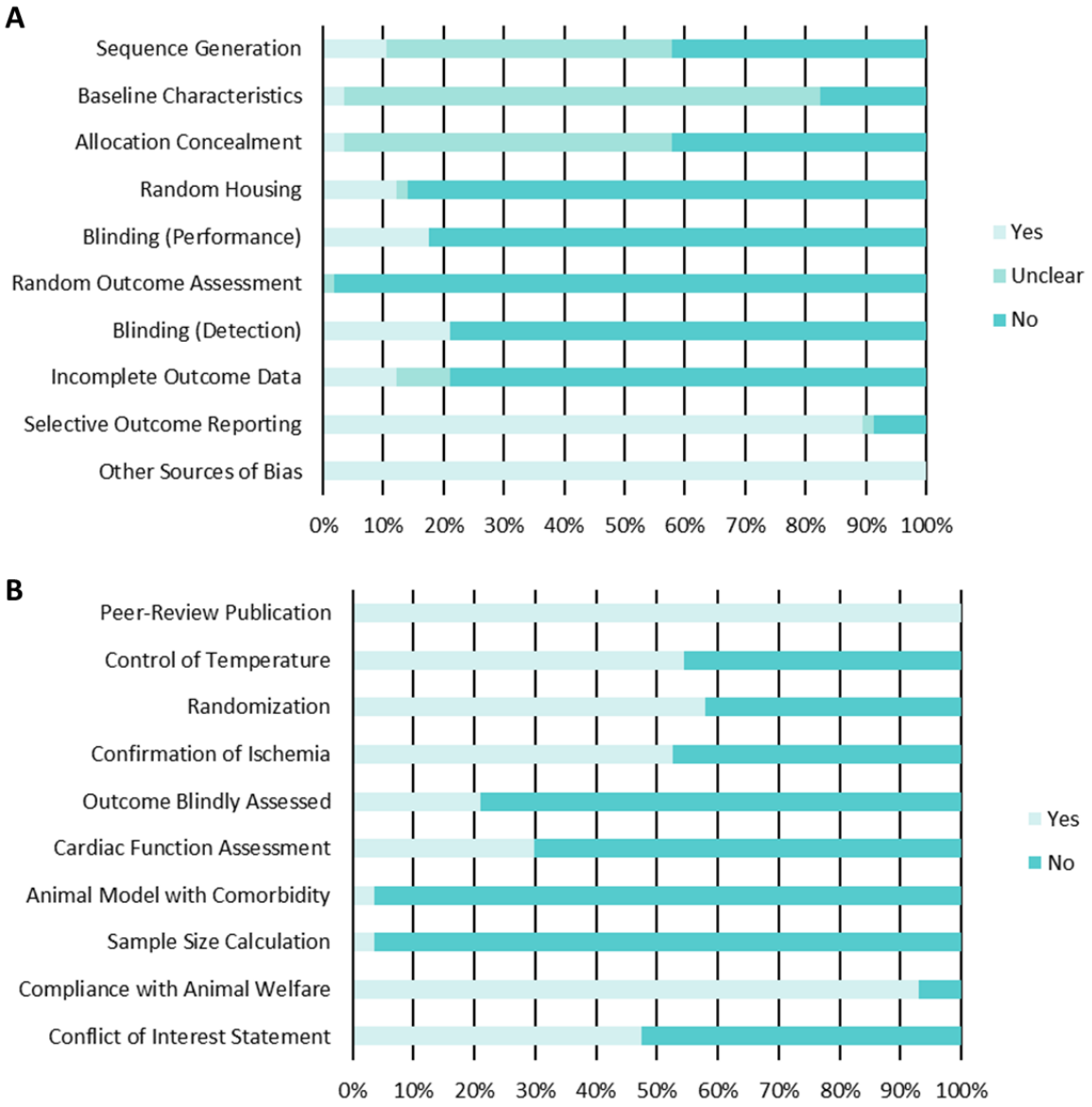

Figure 2.1.2. Risk of bias (ROB) assessment of full-length articles. **A.** ROB assessment using the Systematic Review Centre for Laboratory Animal Experimentation's (SYRCLE) risk of bias tool **B.** ROB assessment using a modified Collaborative Approach to Meta Analysis and Review of Animal Data from Experimental Studies (CAMARADES) checklist.

either pretreated the animal with CsA for several days prior or continued dosing CsA up to 24 hours post-ischemia. Doses ranged from 0.25 to 40mg/kg, with 10mg/kg being the most common, used in 51% (36/71) of studies. Regarding studies of myocardial IRI through coronary artery occlusion, the most common duration of ischemia was 30 minutes (59% [35/59] of studies), though ischemic times ranged from 5 to 90 minutes. The majority of studies administered CsA during the period of myocardial ischemia (58% [34/59]). Fewer studies administered CsA prior to myocardial ischemia (22% [13/59]) or following reperfusion (i.e., post-ischemia; 12% [7/59]), while a minority administered CsA both before and after myocardial ischemia (multiple doses) or during and after myocardial ischemia (multiple doses or continuous infusion).

Study Outcomes

Overall, 75% (53/71) of the studies reported a clear positive effect of CsA in mitigating myocardial IRI by some clinically relevant parameter, such as infarct size, serum troponin, or cardiac function parameters (e.g., cardiac output, cardiac index). However, some studies testing multiple doses reported no positive effect with the lowest tested dose. Coronary artery occlusion studies most commonly reported infarct size (reported in 93% [55/59] of studies), given as the percentage of the myocardium at risk. Of these, 80% (44/55) reported a reduction in infarct size with CsA. Serum troponin and/or CK or cardiac function parameters (e.g., cardiac output or cardiac index) were less commonly reported with coronary occlusion studies. Only 2 of the 4 studies testing CsA in cardiac arrest showed positive effects on cardiac parameters following resuscitation and only 2 of the studies testing CsA in cardiopulmonary bypass (CPB) reported post-CPB cardiac output, with no observed benefit. Three studies of anoxia in a piglet model reported a positive effect of CsA in post-hypoxia cardiac function and troponin, though they were all

published by the same group. A minority of studies (4%, 3/71) reported histologic findings exclusively.

Meta-analysis of Coronary Artery Occlusion Studies

Combining the results of all suitable coronary artery occlusion studies (43/59), we found an overall positive effect of CsA administration in reducing infarct size, with a combined reduction of 16.09% (95%CI 13.67% to 18.50%) of infarct size as a percentage of the area at risk (**Figure 2.1.3**). Statistical heterogeneity between studies, however, was found to be high (I^2=89%), suggesting this effect may be due to study differences rather than a true effect of the treatment. This is similarly reflected in the funnel plot (**Figure 2.1.4**), whose asymmetry may be explained, in part, by statistical heterogeneity.

Subgroup analysis was undertaken to uncover the potential effects of various study differences. We performed meta-analyses grouping studies by species, sex, age, dose, and ischemia time. A similar overall effect was seen between mouse, rat, and rabbit studies (**Supplementary Figure 2.1.1**). However, the effect of CsA became non-significant (p=0.08) when considering only porcine studies (**Figure 2.1.5A**). The five porcine studies also had lower statistical heterogeneity (I^2=48%) compared to the other species subgroups. Similarly, the effect of CsA disappeared when considering studies that included only female animals (p=0.88), though this subgroup included only three studies (**Supplementary Figure 2.2.2**). Combining studies including older animals (rodents 20-24 months) likewise showed a non-significant effect (p=0.14), though this was not statistically different from the combined effect seen in studies containing young animals (p=0.48) (**Figure 2.1.5B**).

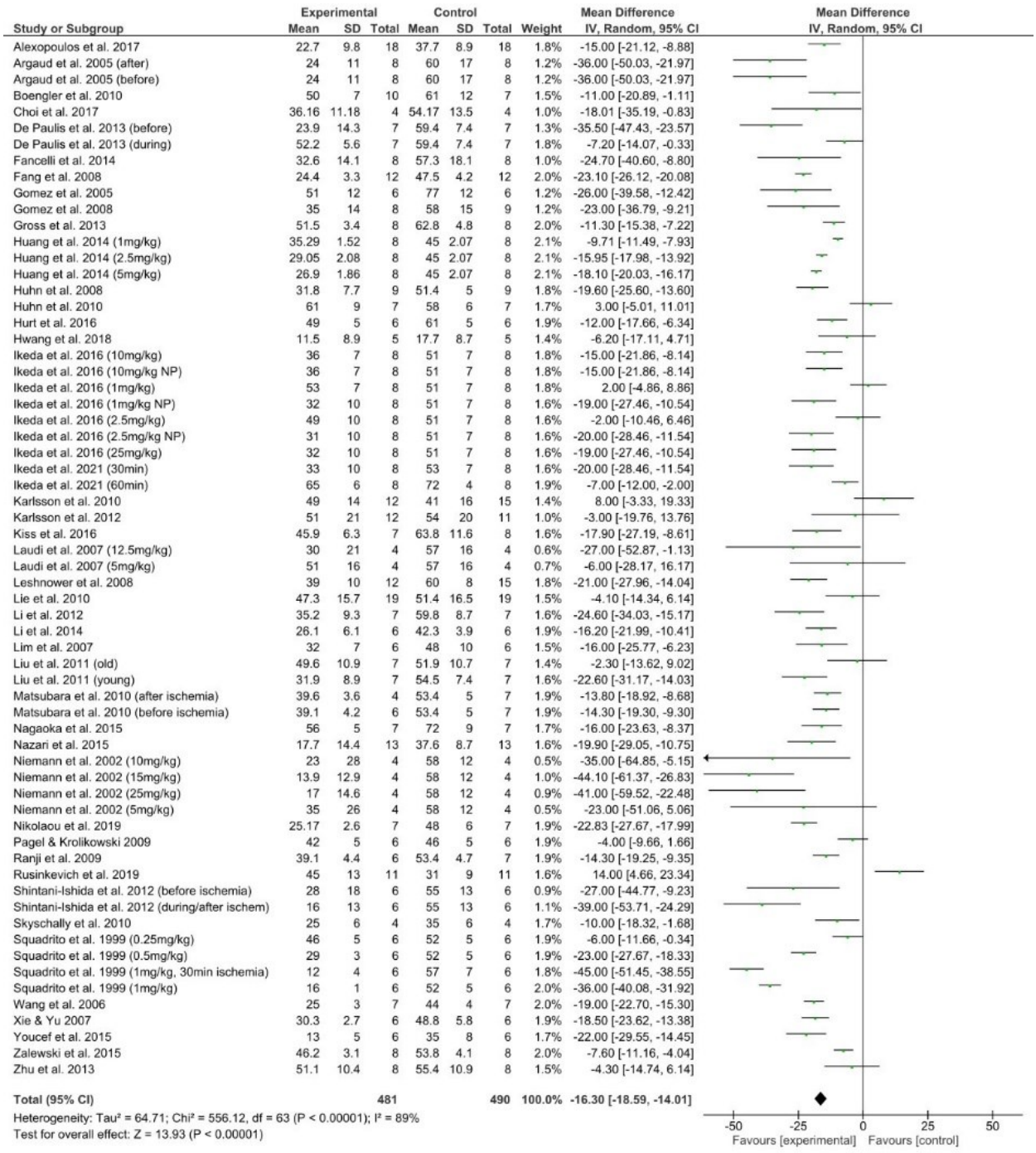

Figure 2.1.3. Forrest plot showing the effect of cyclosporine A treatment on infarct size.

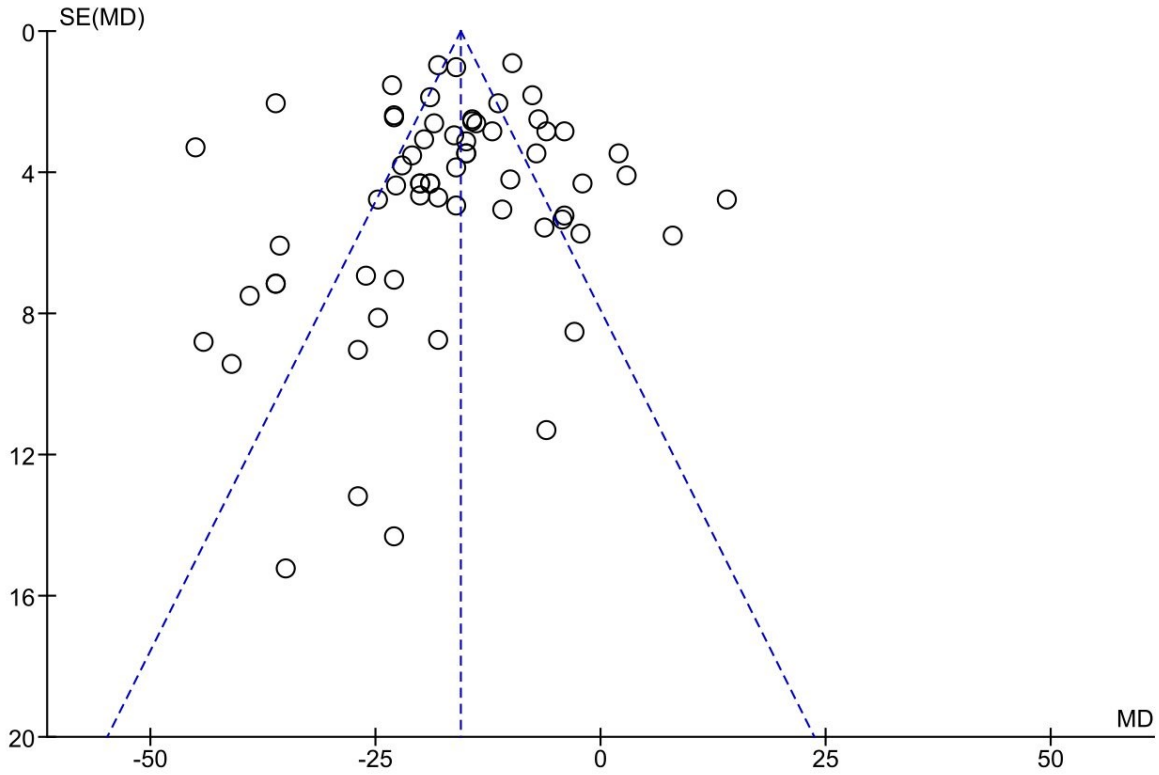

Figure 2.1.4. Funnel plot of coronary artery occlusion studies reporting the effect of cyclosporine A on infarct size, which represents the effect estimates from individual studies against its standard error.

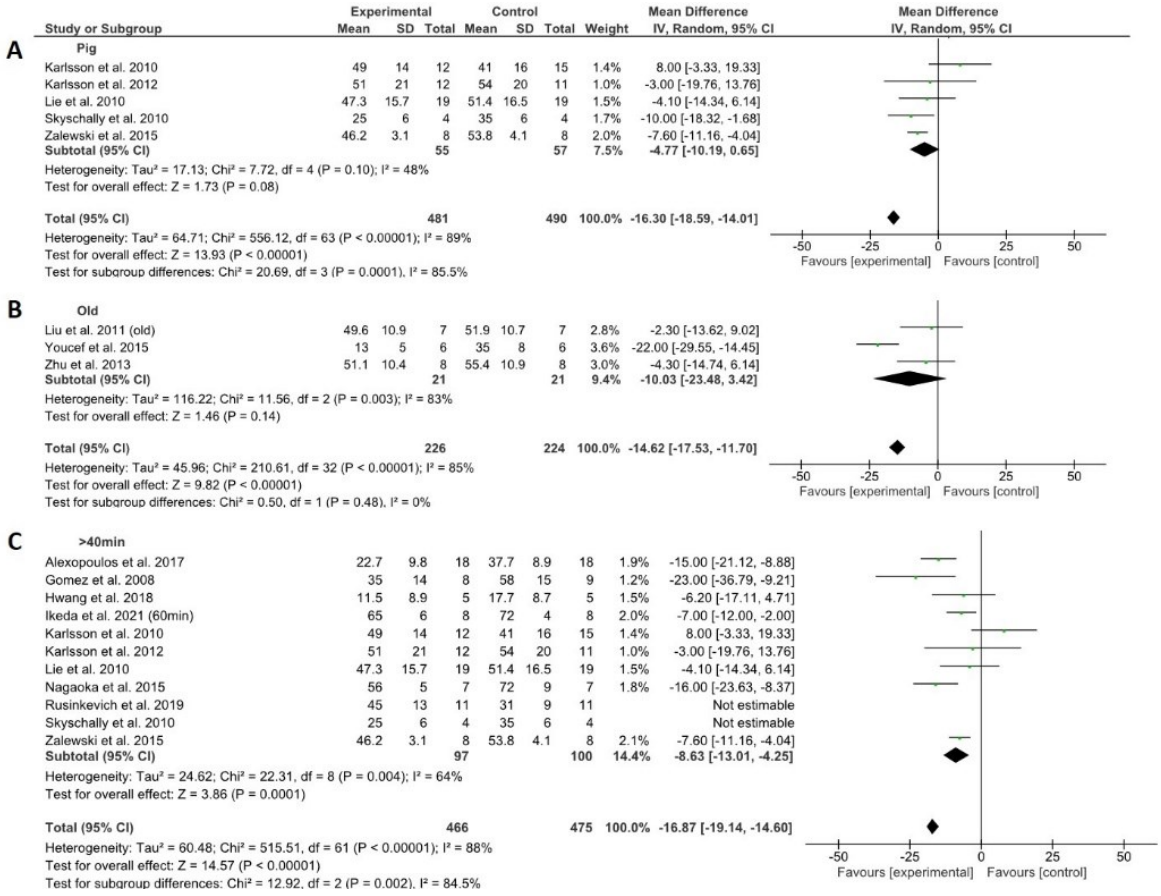

Figure 2.1.5. Combined effect of cyclosporine A (CsA) on infarct size for coronary occlusion studies of different subgroups compared with the combined effect from all coronary occlusion studies (for which the subgroup variables were known). **A.** Effect of CsA on infarct size in studies using porcine models. **B.** Effect of CsA on infarct size in studies using old animals. **C.** Effect of CsA on infarct size in studies in which duration of ischemia was greater than 40 minutes.

Studies administering CsA prior to ischemia showed a greater reduction in infarct size (22.86%; 95%CI 17.73% to 27.98%) compared to those administering CsA during or after ischemia (**Supplementary Figure 2.1.3**). The test for subgroup differences was statistically significant (p=0.01). A subgroup meta-analysis of studies by dose likewise showed a greater reduction in infarct size with doses ≥12.5mg/kg (22.36%; 95%CI 17.26% to 27.46%), however this effect was not statistically different from other subgroups (p=0.09) (**Supplementary Figure 2.1.4**). The overall effect of CsA on infarct size reduction was lower in studies with ischemic times greater than 40 minutes (8.63%; 95%CI 4.25% to 13.01%) compared to other studies (p=0.002), but still remained positive (p=0.0001) (**Figure 2.1.5C, Supplementary Figure 2.1.5**). Despite differences observed between subgroups, heterogeneity within most subgroups remained high (I^2>70%).

2.1.5 – Discussion

In this systematic review of preclinical studies administering CsA to mitigate myocardial IRI, we found that the majority of studies reported a clearly positive effect on various clinically relevant parameters. A meta-analysis of 43 studies utilizing coronary artery occlusion demonstrated an overall reduction in infarct size with the use of CsA (**Figure 2.1.3**). In stark contrast, several clinical studies have been conducted with weak or non-effective benefit (12,15). Understanding this discrepancy between positive results in small and large animals and negative results in clinical practice is vital. Importantly, subgroup meta-analyses suggest that the effect of CsA may differ based on species, sex, age, timing of administration, and ischemia duration.

The findings of these multiple studies contrast with clinical trials, which have shown mixed results at best. The largest trial, published by Cung *et al.* in 2015, included 970 patients with presenting with anterior ST-elevation myocardial infarction (STEMI) undergoing PCI randomized

to receive 2.5mg/kg of CsA or placebo immediately prior to reperfusion (12). They found that CsA conferred no benefit on multiple clinical parameters, including death, worsening heart failure, and left ventricular remodeling. The concurrently run trial (using the same dose), published by Ottani *et al.* in 2016, which included 410 STEMI patients undergoing PCI, similarly found no difference in multiple cardiac-specific outcomes, including ST-segment resolution, serum troponin, and left ventricular ejection fraction (15).

There are notable differences between these trials and the preclinical studies identified by our search, such as animal age, health, CsA dose, duration of ischemia, and timing of dose, as well as species differences (i.e., humans vs. research animals) that could potentially explain the discrepancies in outcomes. Animal age was not commonly reported for rabbits or pigs, but most rodent studies used animals between 8 and 12 weeks old, which is roughly equivalent to a young adult or even adolescent human (16). In both of the large RCTs testing CsA in reperfusion following STEMI, the average age of patients was close to 60 (12,15).Only three studies were identified that used older animals (rodents aged 20-24 months), two of which showed no effect of CsA on infarct size (17-19). While Cung *et al.* did include a subgroup analysis of patients older and younger than 75, which showed no difference in clinical outcomes, patients in the younger group were still quite a bit older, relative to the animals used in preclinical studies(12). Furthermore, the animals used in these studies were typically disease free. Only one study was identified that tested the ability of CsA to reduce infarct size in a co-morbid animal (pre-diabetic, obese Zucker rats) and found no effect on infarct size (20). In contrast, participants in the clinical trials by Cung *et al.* and Ottani *et al.* were often co-morbid, with type 2 diabetes and hypertension being common, as well as being overweight (12,15). While young, healthy animals may be appropriate for initial investigations, moving toward an animal model that is more representative

of the clinical population to which the intervention would likely be applied should be considered prior to proceeding with costly clinical trials.

Our meta-analysis of subgroups divided by species suggested that CsA could be less effective in pigs (**Figure 2.1.4**). This is somewhat confounded by the fact that all of the pig studies used mixed sex or female only animals. However, it does highlight the importance of considering species differences when interpreting preclinical studies. For pharmacological interventions, particular attention should be paid to the specific pathways of metabolism for the drug of interest. CsA is metabolised by the cytochrome P450-3A family of enzymes (21). Not only does the kinetic activity of cytochrome P450 (CYP450) enzymes differ between animals and humans, it appears that there is no one animal whose CYP450 enzymatic activity best matches that of humans across multiple metabolites (22). This does not even take into consideration differences between individuals, which is likely more pronounced in human populations than the inbred animal strains used for most biomedical research. Seeing a consistent effect across a variety of species and strains increases confidence that the intervention will work in human studies.

Grouping studies by duration of ischemia, we found that the effect of CsA in reducing infarct size was significantly reduced for ischemic times longer than 40 minutes. Average ischemic times in the studies by Cung *et al.* and Ottani *et al.* were 4.5 and 3 hours, respectively (12,15). In both studies, more than 80% of patients had no flow through the occluded vessel (i.e., thrombolysis in myocardial infarction [TIMI] score of 0), as was the case in all but one of the animal studies identified. In clinical practice, it is rare to have ischemia of such short duration in acute coronary thrombosis, given the time that is taken for patients to present, diagnosis to occur, and treatment to be initiated. Though a target of 90 minutes from presentation to PCI is recommended by the American Heart Association, shorter time to reperfusion (e.g., less than 60 minutes) has been

shown to be associated with decreased mortality (23). Similarly, we did find a significant difference between subgroups divided by timing of administration, with dosing prior to ischemia being more effective at reducing infarct size. This is relevant, as it would be impossible to administer CsA prior to unexpected ischemia as occurs in the setting of MI, but CsA could be given prior to known periods of ischemia, such as during cardiac surgery or transplantation.

It is worth noting that our subgroup meta-analysis did not suggest an effect based on dose. All clinical trials of CsA have used doses of 2.5mg/kg, while preclinical studies tended to use higher doses (with 10mg/kg being most common). The overall effect from studies using doses of 12.5mg/kg or more showed greater reduction in infarct size, however, this was not significantly different from other subgroups. This was true even after eliminating studies using nanoparticle formulations, which tended to show greater benefit with lower doses (24,25). It may be that for this particular drug the effect on mitochondria is not gradational, but rather exhibits more of a threshold effect, below which it is ineffective (or at least a very narrow range in which increased doses will result in increased effect).

As alluded to previously, the goal of this systematic review is largely hypothesis generating. The suggestions gleaned from meta-analyses of subgroups should be understood within certain limitations. An important caveat for interpreting the results of the meta-analyses is the high degree of statistical heterogeneity observed between studies, which remained largely unchanged despite grouping studies according to several different methodological considerations. It does not appear that the heterogeneity can be entirely explained by dose, timing of administration, duration of ischemia or species (though the heterogeneity for porcine studies was low). It may be a result of a combination of these factors. Methodology is another consideration to explain heterogeneity in results, especially given the high degree of variation in their results

compared to others. The majority of studies purported to be measuring infarct size by injecting Evans blue dye, which is a well-established technique, though may lead to variability in unskilled hands. Particularly with preclinical studies, there is always the concern for publication bias, which can contribute to heterogeneity. As well, selective reporting of results (i.e., omitting negative results) could also be a factor and is not easily detectable in preclinical studies.

Another important limitation is the high risk for bias seen in these studies. Animal studies are typically far less diligent in following standard practices that are commonly used to minimize bias in clinical trials (e.g., randomization, allocation concealment, blinding during analysis) (26). They are also less detailed in their description of methods taken to minimize bias. For instance, while several studies indicated that they randomized animals, they did not include sufficient detail to judge whether this was properly done (e.g., using a random number table or generator, as opposed to assigning every other animal to a group). It is important to encourage the implementation of these bias-reducing methods in preclinical studies, as this will, not only increase confidence in study results, but reduce the chance of obtaining false positive results.

We would like to acknowledge a systematic review posing a similar question, published by Lim *et al.* in 2012 (27). They similarly found an overall positive effect, while commenting on several discrepancies, such as between species. In addition to updating and broadening the search results, which resulted in the addition of 23 studies for meta-analysis, we have added extended subgroup meta-analyses. As well, we now have the opportunity to interpret the findings of our systematic review and meta-analysis in light of the data from several large clinical trials.

Overall, our systematic review identified multiple preclinical studies that tested CsA for the treatment of myocardial IRI. Their indication of an overwhelmingly positive effect is in contrast with the results from clinical studies. Our meta-analysis identified several factors that

potentially contributed to these discrepancies. It may not be worthwhile to further explore these in animal studies of myocardial ischemia, given that the clinical trials have already been conducted. However, our findings highlight the potential pitfalls of translating the results of preclinical studies that should be considered prior to initiating clinical trials.

2.1.6 – References

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2.1.7 – Supplementary Material

Detailed Search Methods

Ovid MEDLINE(R) ALL <1946 to March 08, 2022>

#	Search Statement	Results
1	exp Reperfusion Injury/	46662
2	(((reperfus* or IR or hypoxi* ischemi*) adj3 (injur* or damag* or necrosis or necrotic or hemorrhag* or haemorrhag* or (free adj2 radical*))) or ((hypox* or hemorrhagic) adj3 shock)).mp. or exp Shock, Hemorrhagic/ [mp=title, abstract, original title, name of substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	81057
3	1 or 2	81955
4	exp Cyclosporine/ or cyclosporin.ti,ab. or cyclosporine.ti,ab.	57197
5	("csa neural" or csaneoral or "cya nof" or "ol 27 400" or "ol 27400" or sandimmun).ti,ab.	344
6	("adi 628" or adi628 or equa or "cgc 1072" or cgc1072 or ciclomulsion or cicloral or cipol or consupren or cyclasol or cyclokat or "de 076" or de076 or deximune or equoral or gengraf or ikervis or iminoral or implanta or imusporin or "lx 201" or lx201 or "c2 03" or mc203 or "mtd 202" or mtd202 or neoral or neuro-stat or neurostat or "nm 0133" or "nm 133" or nm0133 or "nm133" or "nova 22007" or nova22007 or ol27400 or "olo 400" or olo500 or "opph 088" or opph088 or opsisporin or "otx 101" or otx101 or "p 3072" or p3072 or padciclo or papilock or pulminiq or restasis or restaysis or sanciclo or sanciclo or sandimmune or sandimun or sandimune or "sang 35" or sang35 or sangcya or "sp 14019" or "sti 0529" or sti0529 or "t 1580" or t1580 or verkazia or vekacia).ti,ab.	1418
7	4 or 5 or 6	57371
8	3 and 7	698
9	(invitro or "in vitro").mp. or Invitro Techniques/	1622947
10	9 not (invivo or "in vivo").mp. [mp=title, abstract, original title, name of	1144101

	substance word, subject heading word, floating sub-heading word, keyword heading word, organism supplementary concept word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	
11	8 not 10	624
12	remove duplicates from 11	624

Embase <1974 to 2022 March 04>

#	Search Statement	Results
1	(((reperfus* or ir or hypoxi* ischemi*) adj3 (injur* or damag* or necrosis or necrotic or hemorrhag* or haemorrhag* or (free adj2 radical*))) or ((hypox* or hemorrhagic) adj3 shock)).mp. or exp hemorrhagic shock/	106648
2	exp reperfusion injury/	65519
3	1 or 2	106790
4	exp Cyclosporine/ or cyclosporin.ti,ab. or cyclosporine.ti,ab.	84079
5	("csa neural" or csaneoral or "cya nof" or neural or "ol 27 400" or "ol 27400" or sandimmun).tn.	2547
6	("adi 628" or adi628 or equa or "cgc 1072" or cgc1072 or ciclomulsion or cicloral or cipol or consupren or cyclasol or cyclokat or "de 076" or de076 or deximune or equoral or gengraf or ikervis or iminoral or implanta or imusporin or "lx 201" or lx201 or "c2 03" or mc203 or "mtd 202" or mtd202 or neoral or neuro-stat or neurostat or "nm 0133" or "nm 133" or nm0133 or "nm133" or "nova 22007" or nova22007 or ol27400 or "olo 400" or olo500 or "opph 088" or opph088 or opsisporin or "otx 101" or otx101 or "p 3072" or p3072 or padciclo or papilock or pulminiq or restasis or restaysis or sanciclo or sanciclo or sandimmune or sandimun or sandimune or "sang 35" or sang35 or sangcya or "sp 14019" or "sti 0529" or sti0529 or "t 1580" or t1580 or verkazia or vekacia).tn.	6795
7	4 or 5 or 6	87630
8	3 and 7	978
9	(invitro or "in vitro").mp. [mp=title, abstract, heading word, drug trade name,	2257265

	original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	
10	9 not (invivo or "in vivo").mp. [mp=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword heading word, floating subheading word, candidate term word]	1598454
11	8 not 10	920
12	remove duplicates from 11	904

SCOPUS Searched March 8, 2022 Results = 1568

(((TITLE-ABS-KEY ((reperfus* OR ir OR "Hypoxi* ischemi*") W/3 (injur* OR damag* OR necrosis OR necrotic OR hemorrhag* OR haemorrhag*))) OR (TITLE-ABS-KEY ((reperfus* OR ir OR "Hypoxi* ischemi*") W/3 ("free radical*" OR "free oxygen radical*"))) OR (TITLE-ABS-KEY (((hypox* OR hemorrhagi* OR haemorrhagi*) W/3 shock)))) AND (TITLE-ABS-KEY ("adi 628" OR adi628 OR equa OR "cgc 1072" OR cgc1072 OR ciclomulsion OR cicloral OR cipol OR consupren OR "csa neural" OR "cya nof" OR cyclasol OR cyclokat OR cyclosporine OR cyclosporin OR "de 076" OR de076 OR deximune OR equoral OR gengraf OR ikervis OR iminoral OR implanta OR imusporin OR "lx 201" OR lx201 OR "c2 03" OR mc203 OR "mtd 202" OR mtd202 OR neoral OR neuro-stat OR neurostat OR "nm 0133" OR "nm 133" OR nm0133 OR "nm133" OR "nova 22007" OR nova22007 OR "ol 27 400" OR "ol 27400" OR ol27400 OR "olo 400" OR olo500 OR "opph 088" OR opph088 OR opsisporin OR "otx 101" OR otx101 OR "p 3072" OR p3072 OR padciclo OR papilock OR pulminiq OR restasis OR restaysis OR sanciclo OR sanciclo OR sandimmun OR sandimmune OR sandimun OR sandimune OR "sang 35" OR sang35 OR sangcya OR "sp 14019" OR "sti 0529" OR sti0529 OR "t 1580" OR t1580 OR verkazia OR vekacia))) AND NOT ((TITLE-ABS-KEY (invitro OR "in vitro")) AND NOT ((TITLE-ABS-KEY (invitro OR "in vitro")) AND (TITLE-ABS-KEY ((invivo OR "in vivo")))))

WOS BIOSIS Searched March 9, 2022 Results

Indexes=BIOSIS Previews Timespan=All years

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#2 DS=Reperfusion Injury 36,628

#3 #1 or #2 59,219

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#5 cr=59865-13-3 28,691

#6 #4 OR #5 68,460

#7 #3 AND #6 691

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#9 TS=((invitro or "in vitro") and (invivo or "in vivo")) 453,102

#10 #8 Not #9 1,154,443

#11 #7 Not #10 656

Cochrane Library Searched March 8, 2022

(Cochrane Database of Systematic Reviewes Results =0)

(Cochrane Central Register of Controlled Trials Results =46)

ID Search Hits

#1 MeSH descriptor: [Reperfusion Injury] this term only 606

#2 MeSH descriptor: [Shock, Hemorrhagic] this term only 113

#3 (((hypox* or hemorrhagic) Near/3 shock)):ti,ab,kw 387

#4 ((reperfus* or ir or "hypoxi* ischemi*") NEAR/3 (injur* or damag* or necrosis or necrotic or hemorrhag* or haemorrhag*)):ti,ab,kw 2952

#5 ((reperfus* or ir or hypoxi* ischemi*) NEAR/3 (free NEAR/2 radical*)):ti,ab,kw 61

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#7 MeSH descriptor: [Cyclosporine] this term only 2826

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#10 #7 or #8 7709

#11 #6 and #10 46

#12 (exvivo OR "ex vivo") NOT ((exvivo OR "ex vivo") AND (invivo OR "in vivo")) 2916

#13 #11 NOT #12 46

PROSPERO Searched March 9, 2022

Line Search for Hits

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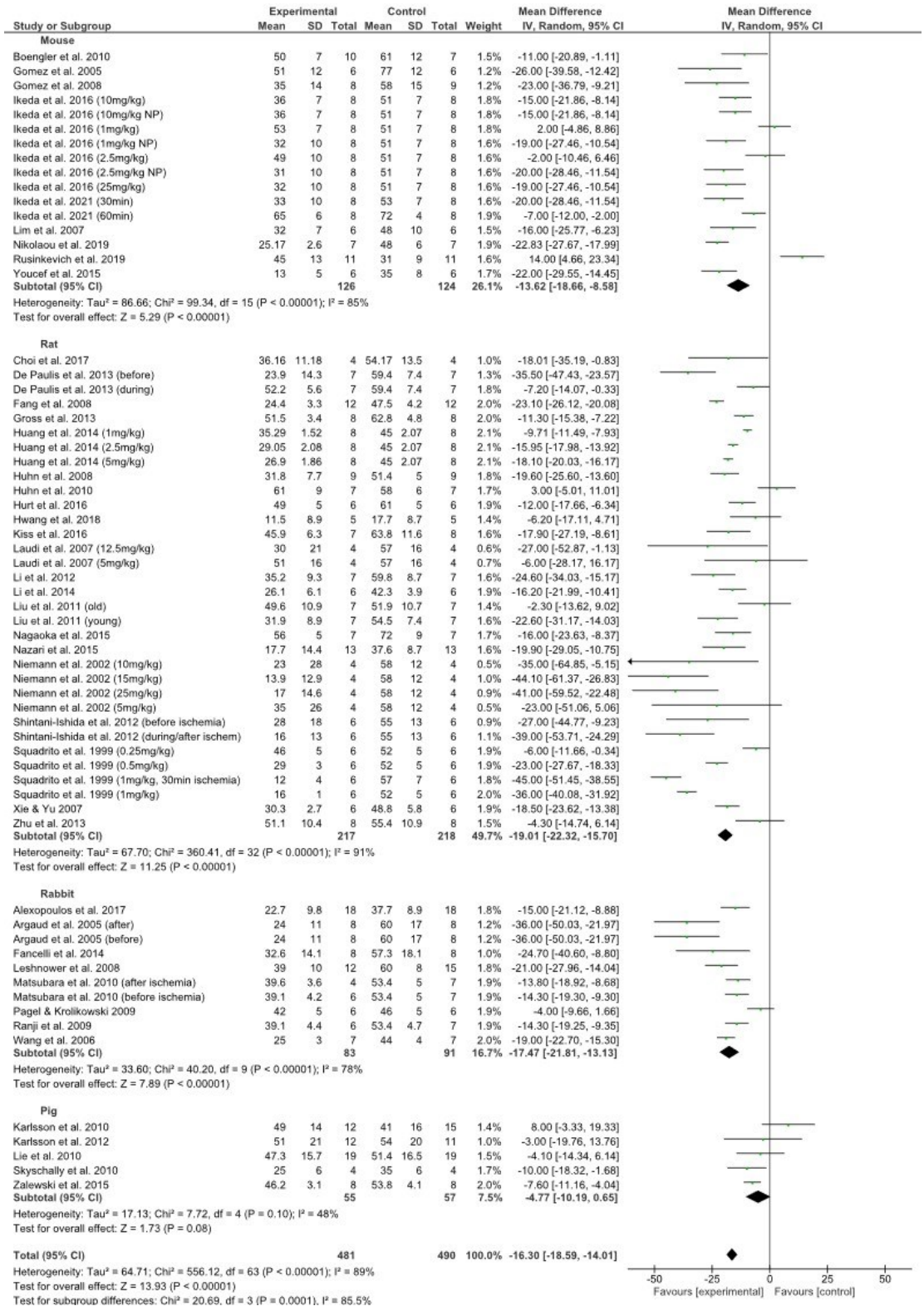
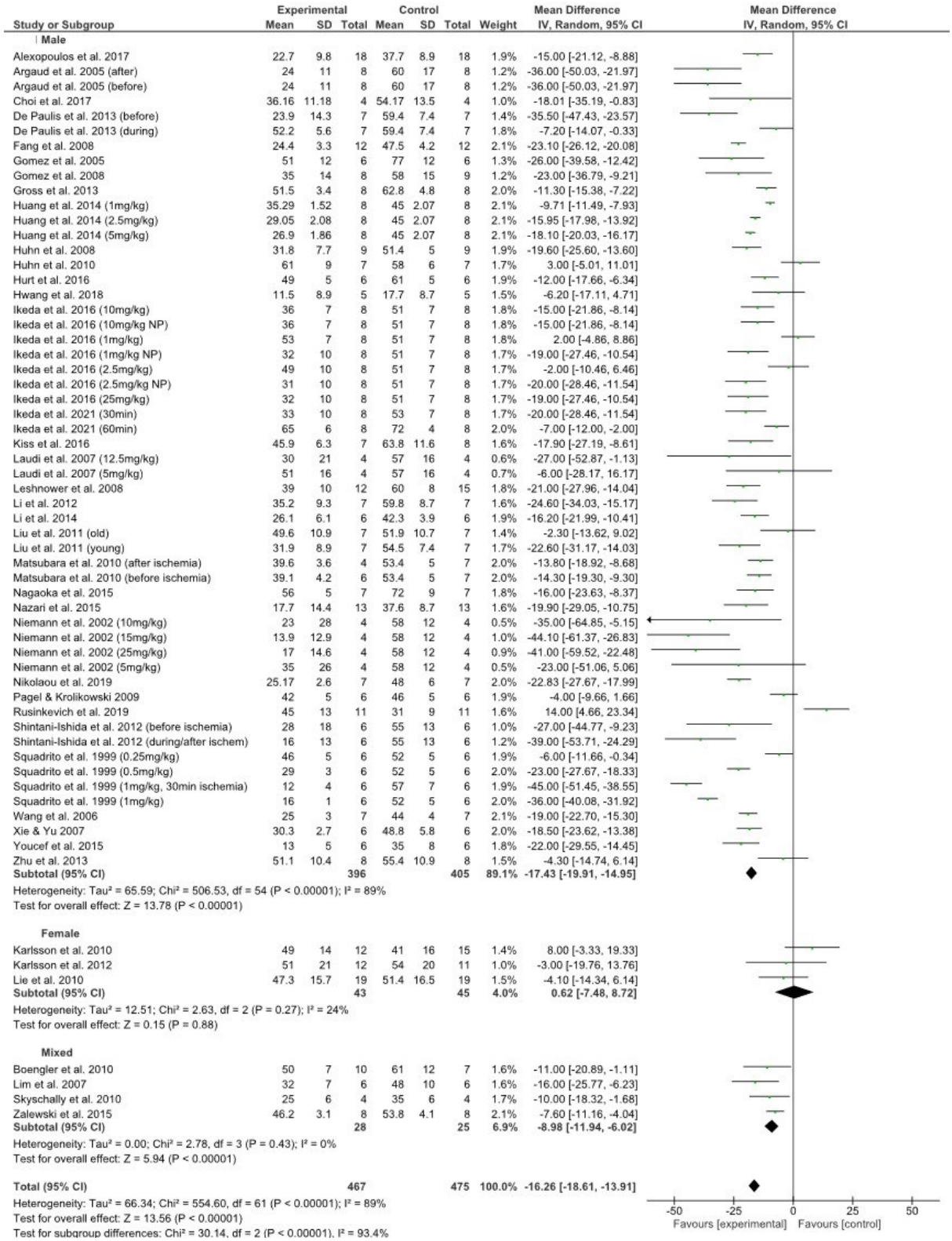
olo500 or "opph 088" or opph088 or opsisporin or "otx 101" or otx101 or "p 3072" or p3072 or padciclo or papilock or pulminiq or restasis or restaysis or sanciclo or sanciclo or sandimmun or sandimmune or sandimun or sandimune or "sang 35" or sang35 or sangcya or "sp 14019" or "sti 0529" or sti0529 or "t 1580" or t1580 or verkazia or vekacia) 236

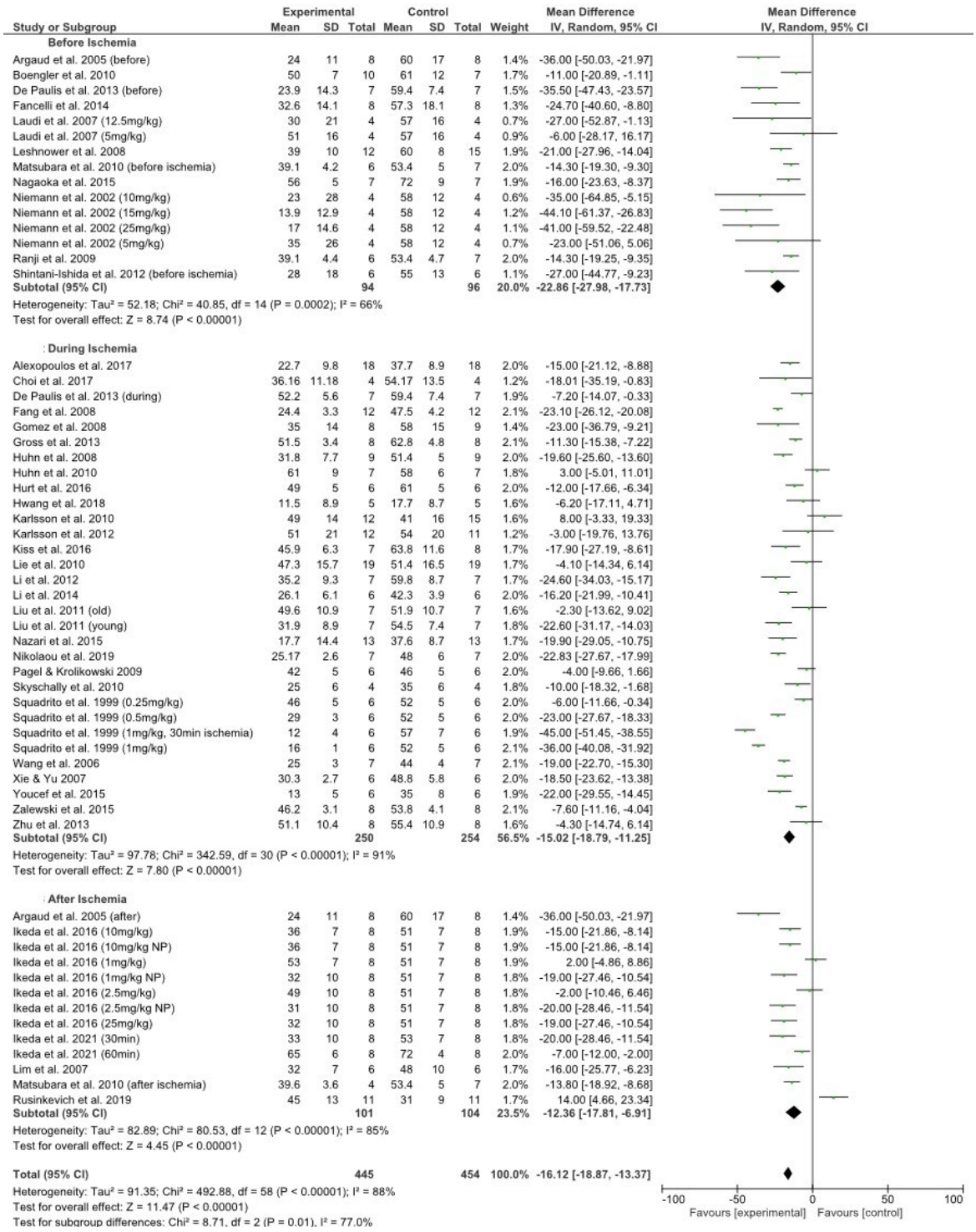
#3 #1 and #2 8



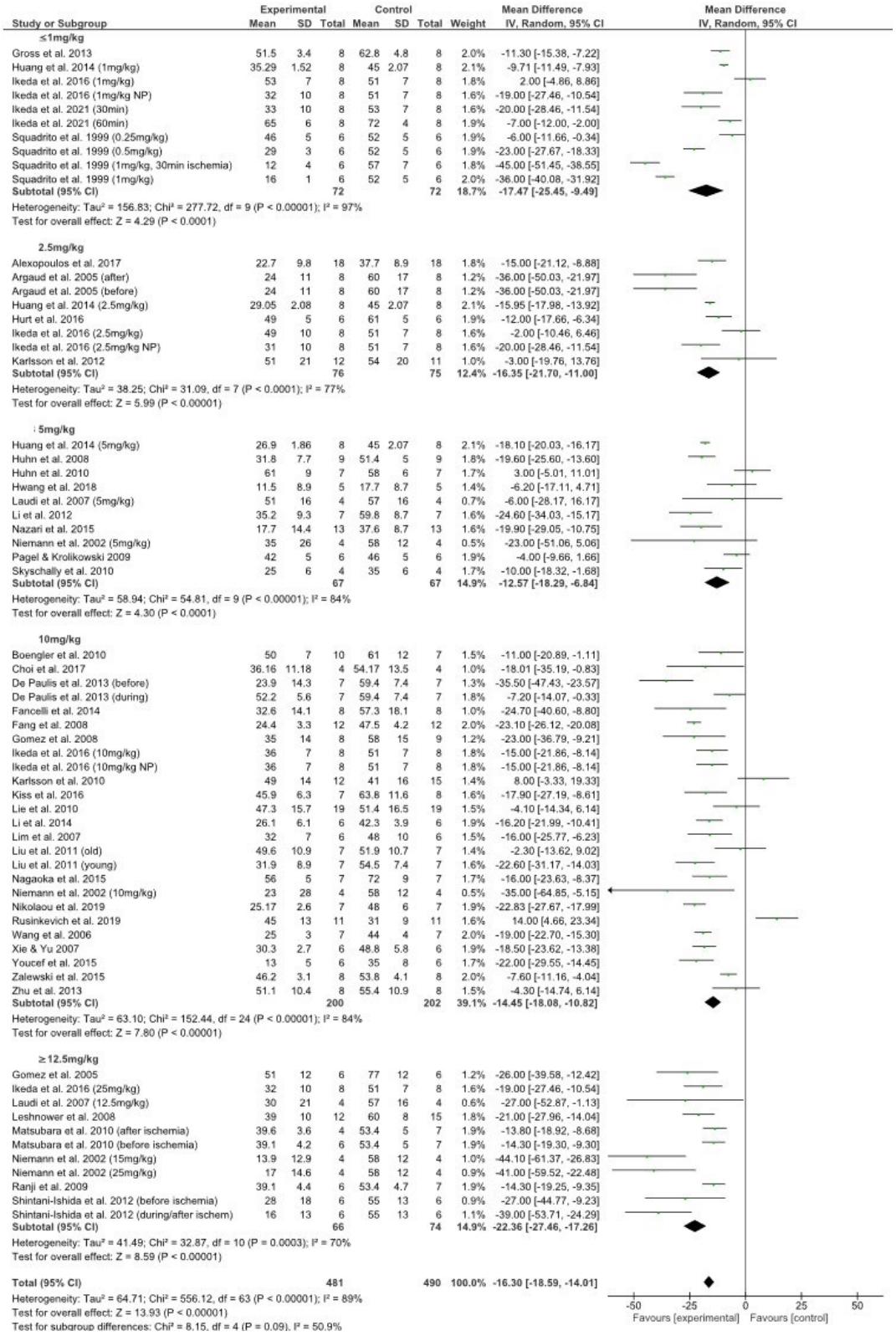
Supplementary Figure 2.1.1. Subgroup meta-analysis of coronary occlusion models of myocardial ischemia/reperfusion injury treated with cyclosporine a, stratified by species.



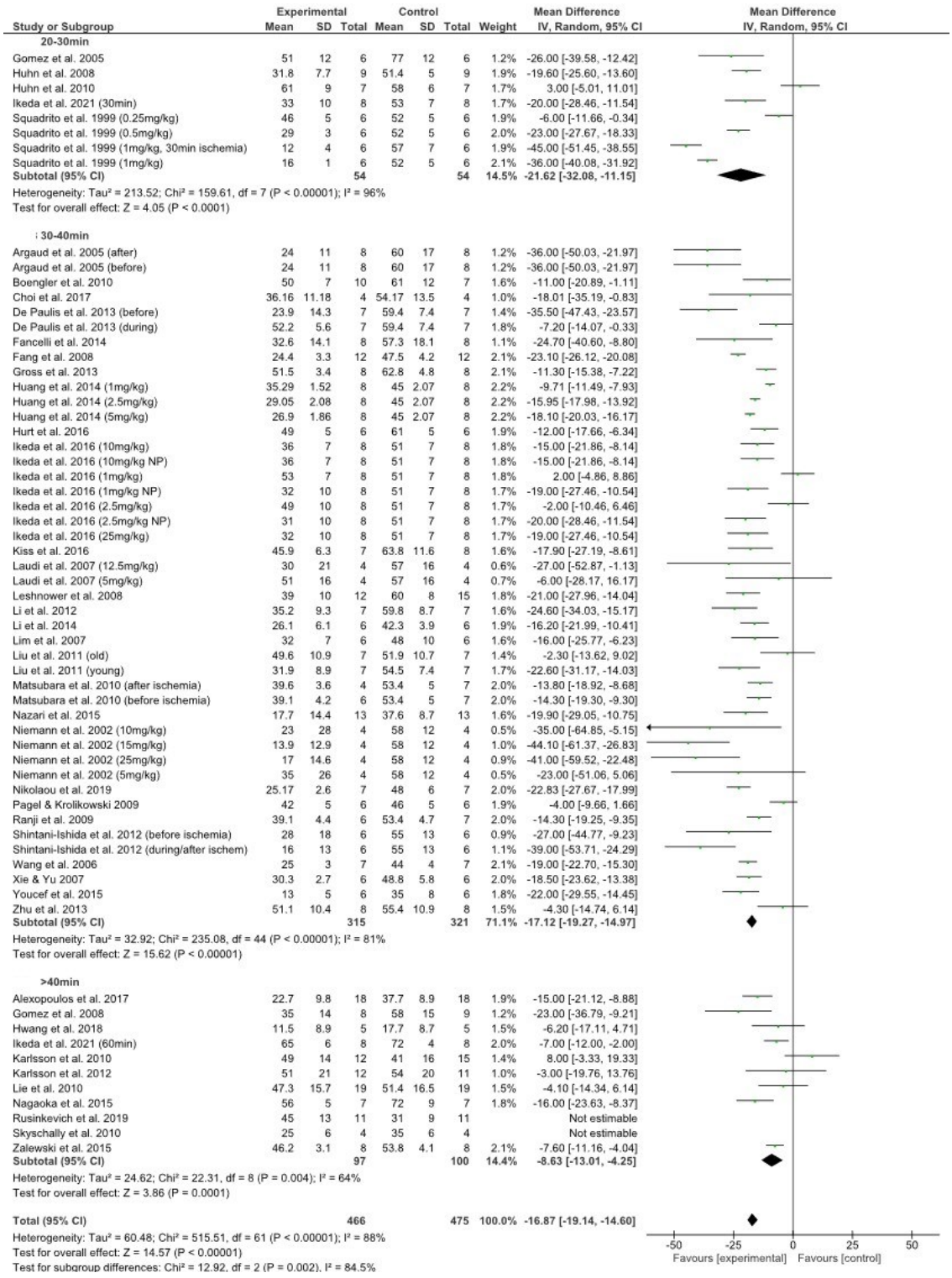
Supplementary Figure 2.1.2 Subgroup meta-analysis of coronary occlusion models of myocardial ischemia/reperfusion injury treated with cyclosporine a, stratified by sex.



Supplementary Figure 2.1.3. Subgroup meta-analysis of coronary occlusion models of myocardial ischemia/reperfusion injury treated with cyclosporine a, stratified by timing of treatment.



Supplementary Figure 2.1.4. Subgroup meta-analysis of coronary occlusion models of myocardial ischemia/reperfusion injury treated with cyclosporine a, stratified by dose.



Supplementary Figure 2.1.5. Subgroup meta-analysis of coronary occlusion models of myocardial ischemia/reperfusion injury treated with cyclosporine a, stratified by duration of ischemia.

Supplementary Table 2.1.1. Summary of myocardial ischemia/reperfusion injury studies using temporary coronary artery ligation and testing cyclosporine A.

	Species (strain, sex, age, n = control/experimental group)	Dose (mg/kg; route)	Duration of Ischemia (min; artery ligated)	Infarct Size (%AAR ±SEM)	Additional Clinically Relevant Outcomes
Before ischemia					
Boengler *et al.* (2010)	mouse (C57Bl/6, ♂/♀, 8wk, 7/10)	10 (IV)	30 (LAD)	61±5% vs. 50±2% ($p<0.05$)	NR
Arteaga *et al.* (1992)	rat (Wistar, ♀, NR, 5/9)	20 (IV)	5 (LCA)	NR	• CK 2728U/L vs. 801U/L[*] • Interstitial edema & loss of striation of myocardial in control group on histology
Niemann *et al.* (2002)	rat (Sprague-Dawley, ♂, 6mo, 4/4/4/4/4)	5 x3 (PO)	30 (LCA)	58±6% vs. 35±13% ($p>0.03$)	NR
		10 x3 (PO)		vs. 23±14% ($p<0.03$)	
		15 x3 (PO)		vs. 13.9±6.5% ($p<0.03$)	
		25 x3 (PO)		vs. 17.0±7.3% ($p>0.03$)	
Laudi *et al.* (2007)	rat (Sprague-Dawley, ♂, 8-10wk, 4/4/4)	5 x3 (PO)	30 (LAD)	57±8% vs. 51±8%[†]	• LVEF 55.0±7.3% vs. 45.5±8.1% (ns) • 14d survival 16.0% vs. 31.6% (ns)
		12.5 x3 (PO)		vs. 30±10%[†]	• LVEF 55.0±7.3% vs. 54.0±11.3% (ns) • 14d survival 16.0% vs. 55.6% ($p=0.017$)
Shintani-Ishida *et al.* (2012)	rat (Sprague-Dawley, ♂, 8wk, 6/6)	25 (IP)	30 (LAD)	55±5% vs. 28±7% ($p<0.05$)	NR
De Paulis *et al.* (2013)	rat (Wistar, ♂, NR, 6-8/6-8)	10 (IV)	30 (LAD)	59.4±2.8% vs. 23.9±5.4% ($p<0.05$)	NR
Nagaoka *et al.* (2015)	rat (Sprague-Dawley, ♂, NR, 7/7)	10 (IV)	45 (LAD)	72±4% vs. 56±2% ($p<0.05$)	NR
Argaud *et al.* (2005)	rabbit (New Zealand white, ♂, NR, 8/8)	2.5 (IV)	30 (left marginal)	60±6% vs. 24±4% ($p<0.0001$)	NR

Ranji *et al.* (2007)‡	rabbit (NR, NR, NR, 5/5)	NR	30 (NR)	55.9±1.7% vs. 39.7±2.1% (p<0.05)	NR
Leshnower *et al.* (2008)	rabbit (New Zealand white, ♂, NR, 15/12)	25 (IV)	30 (left marginal)	60±2% vs. 39±3% (p<0.001)	• 53±12% vs. 20±7% disrupted mitochondria on EM
Ranji *et al.* (2009)	rabbit (New Zealand white, NR, NR, 7/6)	25 (IV)	30 (left marginal)	53.4±1.8% vs. 39.1±1.8% (p<0.0001)	• 53.31±12% vs. 19.71±7% disrupted mitochondria on EM
Matsubara *et al.* (2010)	rabbit (New Zealand white, ♂, NR, 7/6)	25 (IV)	30 (left marginal)	53.4±1.9% vs. 39.1±1.7% (p<0.001)	• 53±16% vs. 20±9% disrupted mitochondria on EM
Fancelli *et al.* (2014)	rabbit (New Zealand white, NR, NR, 8/8)	10 (IV)	30 (LAD)	57.3±6.4% vs. 32.6±5.0% (p<0.01)	NR
Before/after ischemia					
Gomez *et al.* (2004)‡	mouse (NR, NR, NR, 6/6)	40 x3 (IP)	25 (NR)	72±4% vs. 56±4% (p<0.05)	NR
Gomez *et al.* (2005)	mouse (C57Bl/6, NR, 8-10wk, 6/6)	40 x3 (IP)	25 (LAD)	77±5% vs. 51±5% (p<0.01)	NR
He *et al.* (2010)	rat (Sprague-Dawley, ♂, NR, 10/10	2 x2 (IP)	30 (LAD)	NR	• TnI 12.38±0.66ng/mL vs. 9.26±0.56ng/mL (p<0.01) • CK-MB 123.22±2.10U/L vs. 100.87±2.23U/L (p<0.01)
During ischemia					
Gomez *et al.* (2007)‡	mouse (NR, NR, NR, 9/9)	10 (IV)	60 (NR)	56±5% vs. 36%* (p<0.05)	NR
Gomez *et al.* (2008)	mouse (C57Bl/6, ♂, 8-10wk, 9/8)	10 (IV)	60 (LAD)	58±5% vs. 35±5% (p<0.05)	NR
Youcef *et al.* (2015)	mouse (C57Bl/6, ♂, 22mo, 5-7/5-7)	10 (IV)	30 (LAD)	35±3% vs. 13±2% (p<0.05)	NR
Nikolaou *et al.* (2019)	mouse (C57Bl/6, ♂, 8-12wk, 7/7)	10 (IV)	30 (LAD)	48±2% vs. 25.17±1.0% (p<0.0001)	NR
Squadrito *et al.* (1999)	rat (Sprague-Dawley, ♂, NR, 6/6/6/6/6/6, NR)	0.25 (IV)	20 (LCA)	52±2% vs. 46±2% (p>0.05)	NR
		0.5 (IV)		vs. 29±1% (p<0.05)	
		1 (IV)		vs. 16±0% (p<0.005)	
		1 (IV)	30 (LCA)	57±3% vs. 12±2% (p<0.01)	
Xie & Yu (2007)	rat (Sprague-Dawley, ♂, NR, 6/6)	10 (IV)	30 (LAD)	48.8±2.2% vs. 30.3±1.1% (p<0.05) (%total LV area)	• Less vacuolar degeneration & no swelling of mitochondria in CsA group on EM

Fang *et al.* (2008)	rat (Sprague-Dawley, ♂, NR, 12/12)	10 (IV)	30 (LAD)	47.5±1.2% vs. 24.4±1.0% (p<0.01)	• 2.09±0.03 vs. 0.97±0.03 (p<0.01) mitochondria score on EM
Huhn *et al.* (2008)	rat (Wistar, ♂, NR, 9/9)	5 (IV)	25 (LCA branch)	51.4±1.7% vs. 31.8±2.6% (p<0.05)	NR
Huhn *et al.* (2010)	rat (Zucker obese, ♂, 10wk, 7/7)	5 (IV)	25 (LCA branch)	58±2% vs. 61±3% (p>0.05)	NR
Liu *et al.* (2011)	rat (Fischer 344, ♂, 3-5mo, 7/7)	10 (IV)	30 (LAD)	54.5±2.8% vs. 31.9±3.4% (<0.01)	NR
	rat (Fischer 344, ♂, 20-24mo, 7/7)			51.9±4.0% vs. 49.6±4.1% (>0.05)	
Li *et al.* (2012)	rat (Sprague-Dawley, ♂, NR, 7/7)	5 (IV)	30 (LAD)	59.8±3.3% vs. 35.2±3.5% (p<0.001)	• dP/dt_{max} 686mmHg/s* vs. 1286±147mmHg/s (p<0.001)
De Paulis *et al.* (2013)	rat (Wistar, ♂, NR, 6-8/6-8)	10 (IV)	30 (LAD)	59.4±2.8% vs. 52.2±2.1% (p>0.05)	
Gross *et al.* (2013)	rat (Sprague-Dawley, ♂, NR, 6-10/6-10)	1 (IV)	30 (LAD)	62.8±1.7% vs. 51.5±1.2% (p<0.05)	NR
Zhu *et al.* (2013)	rat (Fischer 344, ♂, 22-24mo, 8/8)	10 (IV)	30 (LAD)	54±4% vs. 51±4% (p>0.05)	NR
Li *et al.* (2014)	rat (Sprague-Dawley, ♂, NR, 6/6)	10 (IP)	30 (LAD)	42.3±1.6% vs. 26.1±2.5% (p<0.05)	• CK-MB 692±22U/L vs. 346±22U/L (p<0.05) • Decrease in vacuolar degeneration & lack of swelling in mitochondria on EM in CsA group
Choi *et al.* (2015)‡	rat (Sprague-Dawley, NR, NR, 4/4)	10 (NR)	35 (NR)	33.51±4.65% vs. 14.88±5.74% (p=0.3143)	NR
Nazari *et al.* (2015)	rat (Wistar, ♂, NR, 13/13)	5 (IV)	30 (LAD)	37.6±2.4% vs. 17.7±4.0% (p<0.0001)	• CK-MB 279±29U/L vs. 188±19U/L (p>0.05)
Hurt *et al.* (2016)	rat (Sprague-Dawley, ♂, 8-10wk, 6/6)	2.5 (NR)	30 (LAD)	61±2% vs. 49±2% (p<0.01)	NR
Kiss *et al.* (2016)	rat (Wistar, ♂, NR, 8/7)	10 (IV)	30 (LAD)	63.8±4.1% vs. 45.9±2.4% (p<0.05)	NR
Choi *et al.* (2017)	rat (Sprague-Dawley, ♂, 8wk, 4/4)	10 (IV)	35 (LAD)	54.17±6.75% vs. 36.16±5.59% (p=0.0041)	NR
Hwang *et al.* (2018)	rat (Sprague-Dawley, ♂, 8wk, 5/5)	5 (IP)	45 (LAD)	17.7±3.9% vs. 11.5±4.0% (p>0.05)	• LVEF 47.2±1.7% vs. 48.2±1.7% at 3d (p>0.999),

				(%total LV area)	43.3±3.2% vs. 47.7±2.9% at 7d (p=0.949), 44.6±1.9% vs. 46.7±3.0% at 14d (p>0.999) • 19±3% vs. 11±4% (p>0.05) area of necrotic myocardium & 64±3% 31±4% (p<0.05) necrotic cardiomyocytes on histology
Zhang *et al.* (2019)[§]	rat (Sprague-Dawley, ♂, NR, NR)	2.5 (IV)	30 (LAD)	46±5% vs. 36±4% (p>0.01)	• TnI 350±30ng/mL vs. 270±20ng/mL (p<0.01) • CK-MB 350±21U/L vs. 320±21U/L (p<0.01)
		2.5 (nanoparticle)		vs. 19±4% (p<0.01)	• TnI 350±30ng/mL vs. 210±10ng/mL (p<0.01) • CK-MB 350±21U/L vs. 170±10U/L (p<0.01) • Near normal histological features compared to large area of necrosis, structural disarray & inflammatory infiltrate in control tissue
Krolikowski *et al.* (2005)[§]	rabbit (New Zealand white, ♂, NR, NR)	5 (IV)	30 (left marginal)	42±7% vs. 43±6% (p>0.05)	NR
		10 (IV)		vs. 21±4% (p<0.05)	
Wang *et al.* (2006)	rabbit (New Zealand white, ♂, NR, 7-8/7-8)	10 (IV)	30 (LAD)	44±1% vs. 25±1% (p<0.05)	NR
Pagel & Krolikowski (2009)	rabbit (New Zealand white, ♂, NR, 6/6)	5 (IV)	30 (LAD)	46±2% vs. 42±2% (p>0.05)	NR
Paillard *et al.* (2009)	rabbit (New Zealand white, ♂, NR, 8/8)	5 (IV)	30 (left marginal)	NR	• Preservation of myofibril organization & mitochondrial structure in CsA group on EM
Alexopoulos *et al.* (2017)	rabbit (New Zealand white, ♂, NR, 18/18)	2.5 (IV)	40 (LCA or branch)	37.7±2.1% vs. 22.7±2.3% (p<0.05)	• TnI 159.2±10.4ng/mL vs. 101.7±10ng/mL (p<0.05)
Karlsson *et al.* (2010)	pig (Swedish Landrace, ♀, NR, 15/12)	10 (IV)	45 (LAD)	41±4% vs. 49±4% (p>0.05)	NR
Lie *et al.* (2010)	pig (mixed Danish Landrace/Yorkshire, ♀, NR, 19/19)	10 (IV)	40 (LAD)	51.4±3.8% vs. 47.3±3.6% (p>0.05)	• TnT 6.4±0.7ng/mL vs. 9.7±1.1ng/mL (p>0.05)

					• CO at 180min after reperfusion 3.8±0.2L/min vs. 3.8±0.2L/min (p>0.05)
Skyschally *et al.* (2010)	pig (Göttinger minipigs, ♂/♀, NR, 4/4)	5 (IV)	90 (LAD hypoperfusion)	35±3 % vs. 25±3% (p<0.05)	• dP/dt_{max} at 120min after reperfusion 1222±174mmHg/s vs. 946±111mmHg/s (p>0.05)
Karlsson *et al.* (2012)	pig (mixed Swedish/Pigham/Yorkshire, ♀, NR, 11/12)	2.5 (IV)	40 (left marginal)	54±6% vs. 51±6% (p=0.75)	NR
Zalewski *et al.* (2014)‡	pig (NR, NR, NR, 8/8)	NR	60 (NR)	54±1% vs. 44±2% (p=0.017)	• LVEF (%Δ) -15.6±3.7% vs. -7.9±2.2% (p=0.015)
Zalewski *et al.* (2015)	pig (NR, ♂/♀, NR, 8/8)	10 (IV)	60 (LAD)	53.8±1.4% vs. 46.2±1.1% (p=0.016)	• LVEF 38.9±2.0% vs. 46.3±1.2% (p<0.05) • CO 42.9±2.3mL/s vs. 42.6±2.7mL/s (p>0.05) • Increased edema with reduced myocyte density on histology in both groups
Kloner *et al.* (2011)‡	sheep (NR, NR, NR, NR)	NR	60 (NR)	<10% reduction (p>0.05)	NR
During/after ischemia					
Shintani-Ishida *et al.* (2012)	rat (Sprague-Dawley, ♂, 8wk, 6/6)	10 (IV)	30 (LAD)	55±5% vs. 16±5% (p<0.05)	NR
After ischemia					
Lim *et al.* (2007)	mouse (B6Sv129F1, ♂/♀, 8-10wk, 6/6)	10 (NR)	30 (LAD)	48±4% vs. 32±3% (p<0.05)	NR
Horstkotte *et al.* (2011)	mouse (dtTomato, NR, NR, 6/6)	10 (IV)	90 (LAD)	NR	• dP/dt_{max} 19000±3000mmHg/s vs. 18000±4000mmHg/s (p>0.05)
Ikeda *et al.* (2016)	mouse (C57Bl/6, ♂, 10-12wk, 8/8/8/8/8/8/8/8/8)	1 (IV)	NR (left marginal)	51±3% vs. 53±3% (p>0.05)	• LVEF 33.0±2.0% vs. 32.0±2.6% (p>0.05)
		1 (nanoparticle)		51±3% vs. 32±3% (p<0.001)	• LVEF 33.0±2.0% vs. 49.0±2.0% (<0.05)
		2.5 (IV)		51±3% vs. 49±3% (p>0.05)	
		2.5 (nanoparticle)		51±3% vs. 31±3% (p<0.001)	
		10 (IV)		51±3% vs. 36±3% (p<0.05)	• LVEF 33.0±2.0% vs. 43.2±2.0% (p<0.05)

		10 (nanoparticle)		51±3% vs. 36±3% (p<0.01)	
		25 (IV)		51±3% vs. 32±3% (p<0.01)	
Rusinkevich *et al.* (2019)	mouse (C57Bl/6, ♂, 12-14wk, 11/11)	10 x5 (IP)	90 (LAD)	31±3% vs. 45±4% (p<0.05) (%total LV area)	• LVEF 35±2% vs. 27±2% at 7d (p<0.05); 35±2% vs. 28±2% at 14d (p<0.05; 35±2% vs. 30±2% at 28d (p>0.05)
Ikeda *et al.* (2021)	mouse (C57Bl/6, ♂, 10-12wk, 8-9/8-9/8-9/8-9	1 (nanoparticle)	30 (LAD) 60 (LAD)	53±2% vs. 33±3% (p<0.0001) 72±1% vs. 65±2% (p<0.001)	NR
Argaud *et al.* (2005)	rabbit (New Zealand white, ♂, NR, 8/8)	2.5 (IV)	30 (left marginal)	60±6% vs. 24±4% (p<0.0001)	NR
Matsubara *et al.* (2010)	rabbit (New Zealand white, ♂, NR, 7/4)	25 (IV)	30 (left marginal)	53.4±1.9% vs. 39.6±1.8% (p<0.001)	• 53±16% vs. 18±7% disrupted mitochondria on EM
Not reported					
Ikeda *et al.* (2014)‡	mouse (NR, NR, NR, 8/8)	(nanoparticle)	NR	52±4% vs. 32±9% (p<0.05)	NR
Ikeda *et al.* (2015)‡	mouse (NR, NR, NR, NR)	1mg/kg (nanoparticle)	NR	52±4% vs. 32±6% (p<0.05)	NR
Ikeda *et al.* (2016)‡	mouse (NR, NR, NR, 8/8)	1mg/kg (nanoparticle)	30 (NR)	52±5% vs. 31±6% (p<0.05)	NR
Huang *et al.* (2014)	rat (Sprague-Dawley, ♂, NR, 8/8/8/8)	1 (NR)	30 (LAD)	45.00±0.73% vs. 35.29±0.54% (p<0.05)	• TnI 12.98±0.46ng/mL vs. 9.38±0.38ng/mL (p<0.05) • CK-MB 125.38±2.07U/mL vs. 109.79±1.51U/mL (p<0.05)
		2.5 (NR)		vs. 29.05±0.74% (p<0.05)	• TnI 12.98±0.46ng/mL vs. 8.53±0.30ng/mL (p<0.05) • CK-MB 125.38±2.07U/mL vs. 99.83±0.46U/mL (p<0.05)
		5 (NR)		vs. 26.90±0.66% (p<0.05)	• TnI 12.98±0.46ng/mL vs. 8.35±0.30ng/mL (p<0.05) • CK-MB 125.38±2.07U/mL vs. 98.24±1.63U/mL (p<0.05
Gu *et al.* (2020)‡	rat (NR, NR, NR, 5/5)	2.5 (NR)	NR	46.8%* vs. 42.6%* (p=0.682)	NR

* standard error not reported; † p-value not reported; ‡ conference abstract; § results presented with standard deviation; CK-MB=creatinine kinase myocardial band, CO=cardiac output, CsA=cyclosporine A, EM=electron microscopy, IP=intraperitoneal, IV=intravenous, L=left, LAD=left anterior descending, LCA=left

coronary artery, LV=left ventricle, LVEF=left ventricular ejection fraction, NR=not reported, ns=not significant, PO=per os, SEM=standard error of the mean, TnI=cardiac troponin I, TnT=cardiac troponin T

Supplementary Table 2.1.2. Summary of myocardial ischemia/reperfusion injury studies testing cyclosporine A, using methods other than coronary artery occlusion.

	Species (strain, sex, age, n = control/experimental group)	Dose (mg/kg; route)	Duration of Ischemia (min; method)	Cardiac Function (SEM)	Additional Clinically Relevant Outcomes
Cardiac arrest					
Before/during ischemia					
Ayoub *et al.* (2017)	rat (Sprague-Dawley, ♂, NR, 6/12)	10 (NR)	10 (electricity)	CI 62±8 mL/min/kg vs. 63±4 mL/min/kg at 120min (ns), 58±6 mL/min/kg vs. 59±3 mL/min/kg at 240min (ns), 52±4 mL/min/kg vs. 46±5 mL/min/kg at 360min (ns)	• TnI 130±76ng/mL vs. 210±61ng/mL (ns)
During ischemia					
Huang *et al.* (2011)*	rat (Wistar, ♂, 8wk, NR)	10 (IV)	8.5 (asphyxia)	CO 80.7±20.0mL/min vs. 87.6±22.6mL/min (p=0.58)	• 72hr survival 16.7% vs. 58.3% (p=0.016)
Huang *et al.* (2012)	rat (Wistar, ♂, 8wk, 10/10)	10 (IV)	8.5 (asphyxia)	CO 22±3mL/min vs. 71±10mL/min at 1hr, 22±1mL/min vs. 76±11mL/min at 2hr, 31±3mL/min vs. 49±3mL/min at 3hr, 36±3mL/min vs. 53±3mL/min at 4hr (p<0.01)	• Mitochondrial injury score 1.5±0.2 vs. 0.6±0.2 on EM (p<0.01) • 72hr survival 18.2% vs. 53.8% (p=0.046)
Cour *et al.* (2014)	rabbit (New Zealand white, NR, NR, 24/18)	5 (IV)	5-7 (asphyxia)	CO 60±6 mL/min vs. 90±6 mL/min (p<0.05)	• TnI 34±10ng/mL vs. 10±2ng/mL (p<0.05) • Survival 67% vs. 89%‡
After ischemia					
Huang *et al.* (2012)	rat (Wistar, ♂, 8wk, 10/10)	10 (IV)	8.5 (asphyxia)	CO 18±1mL/min vs. 22±3mL/min at 1hr, 27±1mL/min vs. 36±4mL/min at 2hr,	• Mitochondrial injury score 1.5±0.2 vs. 1.3±0.2 on EM (p>0.01)

				49±8mL/min vs. 44±6mL/min at 3hr, 58±7mL/min vs. 49±3mL/min at 4hr (p=0.690)	• 72hr survival 20% vs. 30% (p=0.829)
Cardiopulmonary bypass					
Oka *et al.* (2008)	pig (NR, NR, 2wk, 5/5)	10 (IV)	60 (cardioplegia)	NR	• Preservation of cristae architecture & intermembrane space in CsA-treated group compared to controls on EM
Hoyer *et al.* (2016)*	pig (Landrace, NR, NR, 6/6)	1.2mg/L (cardioplegia)	90 (cardioplegia)	NR	• No difference in cross striation (p=0.917), eosinophil infiltration (p=0.661), loss of cell boundaries (p=0.362) or myocardial edema (p=0.998) on histology
Hoyer *et al.* (2019)	pig (Landrace, NR, 4-5mo, 10/10)	1.2mg/L (cardioplegia)	90 (cardioplegia)	CO 5.2±0.5L/min vs. 4.7±0.4L/min (ns)	NR
Hoyer *et al.* (2021)	pig (German Sattle, NR, NR, 10/10)	1.2mg/L (cardioplegia)	90 (cardioplegia)	CO 5.2±0.5L/min vs. 4.7±0.4L/min (ns)	• No difference in cross striation (p=0.845), eosinophilia (p=0.510), myocardial edema (p=0.596), cellular infiltration (p=0.279), visible bleeding (p=0.876) or loss of cell boundaries (p=0.510) on histology
Hypoxia					
Gill *et al.* (2012)a	pig (NR, NR, 1-4d, 8/8/8)	10 (IV, 5min after reoxygenation)	120 (ventilation with FiO_2 0.11-0.15)	CI 62±5% vs. 95±4% of baseline (p<0.05)	• Lactate 6.1±0.4mM vs. 4.9±0.4mM at 2hr (p>0.05), 4.4±0.8mM vs. 2.8±0.2mM at 6hr (p>0.05)
		10 (IV, 120min after reoxygenation)		CI 62±5% vs. 79±6% of baseline (p=0.1)	• Lactate 6.1±0.4mM vs. 7.0±0.7mM at 2hr (p>0.05), 4.4±0.8mM vs. 4.2±0.9mM at 6hr (p>0.05)
Gill *et al.* (2012)b	pig (mixed, NR, 1-4d, 8/8/8/8)	2.5 (IV)	120 (ventilation with FiO_2 0.10-0.15)	CI 57±8% vs. 88±8% of baseline (p<0.05)	• TnI 1.2±0.2ng/mL vs. 0.6±0.1ng/mL (p<0.05) • Lactate 11.3±2.9mM vs. 11.3±3.3mM at 30min

					(p>0.05), 5.5±3.3mM vs. 3.2±2.2mM at 4hr (p>0.05)
		10 (IV)		vs. 100±7% of baseline (p<0.05)	• TnI 1.2±0.2ng/mL vs. 0.7±0.2ng/mL (p<0.05) • Lactate 11.3±2.9mM vs. 11.7±4.3mM at 30min (p>0.05), 5.5±3.3mM vs. 3.1±1.0mM at 4hr (p>0.05)
		25 (IV)		vs. 85±11% of baseline (p<0.05)	• TnI 1.2±0.2ng/mL vs. 1.2±0.2ng/mL (p>0.05) • Lactate 11.3±2.9mM vs. 11.8±1.8mM at 30min (p>0.05), 5.5±3.3mM vs. 2.6±0.6mM at 4hr (p>0.05)
Gill *et al.* (2013)	pig (mixed, NR, 1-4d, 8/8)	10 (IV)	120 (ventilation with FiO_2 0.10-0.15)	NR	• TnI 1.2±0.2ng/mL vs. 0.6±0.2ng/mL (p<0.05)
Cardiac transplantation					
Laudi *et al.* (2006)*	rat (Lewis, ♂, NR, 7/7/7/7)	12.5 x3 (PO)	NR	NR	• 28d survival 75% vs. 100% if administered 3d prior vs. 33% if administer day of transplant vs. 78% if administered 3d post-transplant (p=0.041)

***** conference abstract; † results presented with standard deviation; ‡ p value not reported; CI=cardiac index, CO=cardiac output, CsA=cyclosporine A, EM=electron microscopy, FiO_2 = fraction of inspired oxygen, IV=intravenous, NR=not reported, ns=not significant, PO=per os, TnI=cardiac troponin I, U/O=urine output

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CHAPTER 2 – CYCLOSPORINE A FOR THE PREVENTION OF ISCHEMIA REPERFUSION INJURY

PART 2 – PROTECTIVE EFFECTS OF CYCLOSPORINE AND ITS ANALOG NIM-811 IN A MURINE MODEL OF HEPATIC ISCHEMIA/REPERFUSION INJURY

2.2 – Protective Effects of Cyclosporine & its Analogue NIM-811 in a Murine Model of Hepatic Ischemia/Reperfusion Injury

A version of this section is currently under review in the *Liver Research*.

Title: Protective Effects of Cyclosporine and its Analogue NIM-811 in a Murine Model of Hepatic Ischemia/Reperfusion Injury

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2.2.1 – Preface

Background

The liver is susceptible to ischemia reperfusion injury (IRI) during hepatic surgery, when vessels are compressed to control bleeding, or liver transplantation, where there is an obligate period of ischemia. A hallmark of IRI is mitochondrial dysfunction resulting in generation of reactive oxygen species, and cell death through necrosis or apoptosis. Cyclosporine (CsA), well known as an immunosuppressive agent through inhibition of calcineurin, has the additional effect of inhibiting the mitochondrial permeability transition pore (mPTP), preventing mitochondrial swelling and injury. Its non-immunosuppressive analog, NIM-811, has a similar effect on the mPTP. In this study, we tested the effect of both agents on mitigating warm hepatic IRI in a murine model.

Methods

Before ischemic insult, mice were treated with control (normal saline), CsA 2.5, 10 or 25mg/kg, or with 10mg/kg of the non-immunosuppressive CsA analog NIM-811 by intraperitoneal injection. Mice were then subjected to 60 minutes of partial warm hepatic ischemia by selective pedicle clamping and recovered for 6 hours after reperfusion. Serum alanine transaminase (ALT) was measured, and liver tissue was examined histologically for apoptosis and for levels of inflammatory cytokines.

Results

Mice treated with 10 or 25mg/kg of CsA or NIM-811 showed significantly decreased ALT compared to saline-treated controls ($p=0.001$, 0.010 and 0.033, respectively). Liver tissue also showed reduced histological injury scores at all doses of CsA and NIM-811 ($p=0.041$, <0.001,

0.003, 0.043 for 2.5mg/kg CsA, 10mg/kg CsA, 25mg/kg CsA, and NIM-811, respectively). Significant decrease in apoptosis was also observed at all doses of CsA ($p=0.012$, 0.007, <0.001 for 2.5mg/kg CsA, 10mg/kg CsA, and 25mg/kg CsA, respectively).

Conclusions

Premedication with CsA or NIM-811 mitigates hepatic IRI in mice, as evidenced by decreased ALT and reduced injury on histology. This may have potential implications for mitigating IRI in liver transplantation and hepatic resection, particularly in the setting of normothermic machine perfusion.

2.2.2 – Introduction

Ischemia/reperfusion injury (IRI) results from the transient loss of blood flow to an organ.[1] The ischemic phase is marked by cell death from metabolic stress resulting from lack of oxygen, while the reperfusion phase that occurs after restoration of blood flow is marked by activation of the innate immune system, leading to infiltration with peripheral immune cells.[2] Clinically, hepatic IRI may occur during liver resection, if intermittent compression of arterial and portal venous inflow (Pringle maneuver) is used to control bleeding, or in liver transplantation, where there is an obligate period of ischemia during transport and implantation.[3] Liver IRI during transplantation is mainly addressed through the use of preservation solutions during cold ischemia and, more recently, machine perfusion of grafts.[4] While several pharmacological strategies have demonstrated efficacy in preclinical studies, few have proven effective clinically and none have been adopted universally.

Cyclosporine (CsA) is well known for its immunosuppressive effect, which works by preventing T cell activation through inhibition of calcineurin.[5] A secondary effect of CsA is inhibition of cyclophilin D, a key component of the mitochondrial permeability transition pore (mPTP).[6] Mitochondria feature prominently in the pathophysiology of IRI due to their role in ATP production, ROS generation, and initiation of apoptosis and necrosis. mPTP inhibition has the potential to prevent mitochondrial swelling during oxidative stress, which, if severe enough, leads to irreversible cell death. The CsA analog, NIM-811, similarly inhibits cyclophilin D, but lacks the immunosuppressive effect of CsA.[7]

Numerous preclinical studies have shown CsA to be protective against myocardial IRI.[8] However, the evidence in hepatic IRI is mixed, with some studies showing benefit and others having no effect depending on the dosing strategy and outcome measured.[9-12] Additionally, while

NIM-811 has been shown to be protective against liver injury in models of small-for-size syndrome, it has not been shown to be directly protective against ischemia. This is potentially important if NIM-811 can offer the same protective effect as CsA, without inducing unnecessary immune suppression. In the present study, we tested NIM-811 and escalating doses of CsA in a murine model of warm hepatic IRI.

2.2.3 – Methods

Animal model

A similar model was used and was reported in our previous study.[14] The study protocol (AUP00002033) was approved by the Institutional Animal Care Committee at the University of Alberta, following guidelines set out by the Canadian Council of Animal Care. Male C57BL/6 mice were purchased from Charles River Laboratories (Saint-Constant, QC, Canada), aged 10-12 weeks. Mice were injected intraperitoneally (IP) two hours prior to ischemia with either 100µL of normal saline (NS) or 2.5mg/kg, 10mg/kg or 25mg/kg of CsA (Sandimmune IV, Novartis Pharmaceuticals Canada Inc.; Dorval, QC, Canada) diluted in the same volume of NS. An additional group was treated in the same way with 10mg/kg of NIM-811 (MedChemExpress; Monmouth Junction, NJ, USA).

To induce liver ischemia, mice were anesthetized with isoflurane and a midline laparotomy was performed. Under 10x magnification (MZ6, Leica Microsystems Inc.; Concord, ON, Canada), two atraumatic microvascular clamps (Micro Serrefines, Fine Science Tools Inc.; North Vancouver, BC, Canada) were placed across the arterial and portal venous branches leading to the median and left lobes (approximately 70% of the liver mass). Ischemia was verified visually by immediate blanching of the lobes (**Figure 2.2.1**). Of note, this model used partial hepatic ischemia

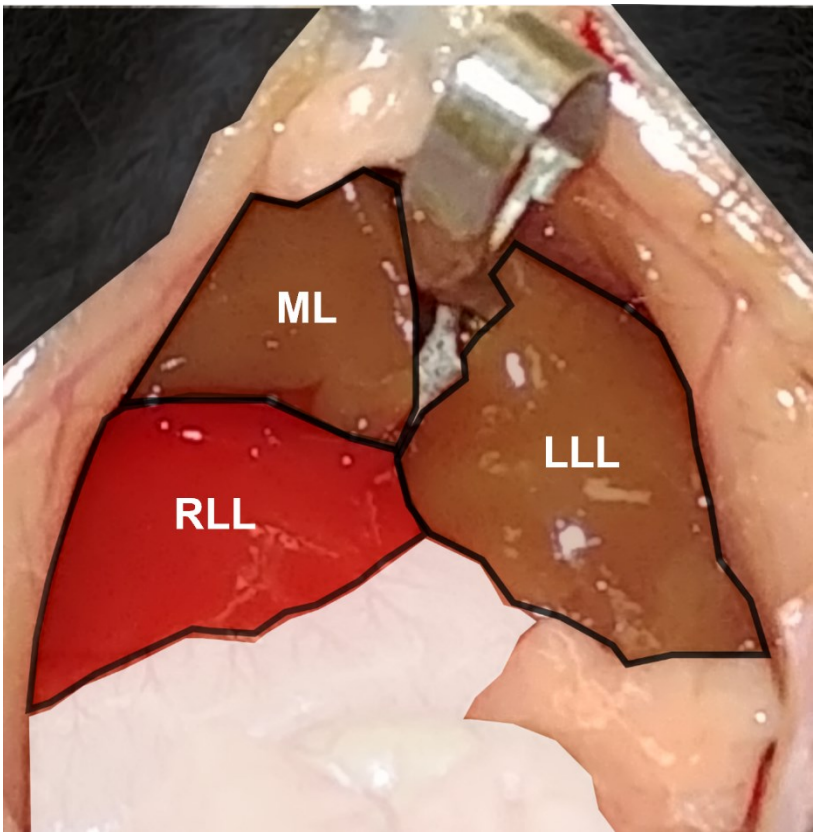

Figure 2.2.1. Expected Intraoperative Findings. Blanching of the median (ML) and left lateral (LLL) lobes with the application of a vascular clamp. The unclamped right lateral lobe (RLL) appears slightly hyperemic.

to avoid venous congestion of the bowel that would result from occlusion of the entire portal vein. 5 IU of heparin (Frensenius Kabi Canada; Toronto, ON, Canada) diluted in 500µL of NS was given IP and the abdominal skin was closed with running 4-0 polypropylene suture (Prolene, Ethicon; Cincinnati, OH, USA) to prevent heat loss. A heating pad was placed beneath the mice (kept at 37ºC) and mice were maintained under inhalational anesthetic (Isoflurane, Frensenius Kabi Canada; Toronto, ON, Canada) for the duration of ischemia.

At the end of one hour, clamps were removed and return of blood flow was observed. A bolus of 500µL NS was given IP before closing fascia with running 4-0 polyglactin suture (Vicryl, Ethicon; Cincinnati, OH, USA). Skin was secured with 9mm wound clips (Autoclip, Becton Dickinson Canada Inc.; Mississauga, ON, Canada). For post-operative pain control, 200µL of local anesthetic (0.25% bupivacaine, Sensorcaine, AstraZeneca Canada Inc.; Mississauga, ON, Canada) was applied topically to the wound and mice were injected with 100µL of 0.015mg/kg buprenorphine subcutaneously (SC; Temgesic, Schering-Plough Canada Inc.; Pointe-Claire, QC, Canada). Mice were recovered under heat lamps and returned to their cage, where they had free access to food and water.

Six hours post-ischemia, mice were sacrificed humanely by exsanguination and thoracotomy under isoflurane anesthesia. A six-hour endpoint was chosen as this was found to be the time of peak serum alanine transaminase (ALT) elevation based on our preliminary tests and reference sources.[14,15] The laparotomy incision was reopened. Blood was removed via cardiac puncture using a 23G needle and collected in capillary blood collection tubes (Microvette 500, Sarstedt AG & Co.; Nümbrecht, Germany) for subsequent centrifugation at 10,000g for 5 minutes. The resultant serum was frozen at -80ºC. Hepatectomy was also performed. A portion of the ischemic and non-ischemic control lobes were immediately frozen at -80ºC, while a second portion

was fixed in 10% formalin. A schematic of the model is shown in **Figure 2.2.2**. Five sham surgical animals were included, who underwent laparotomy and hepatic manipulation, but were not subject to hepatic ischemia.

Determination of serum ALT

Serum ALT was determined using a VetTest Chemistry Analyzer (IDEXX Laboratories Canada Corp.; Markham, ON, Canada). NS was used to dilute samples as required.

Histology

Formalin fixed tissue, collected six hours post-ischemia, was embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Slides were reviewed by a trained pathologist (AT), who was blinded to the treatment groups. Scores were assigned based on presence of necrosis (0 absent, 1 pericentral, 2 zonal, 3 panlobular), hemorrhage (0 absent, 1 pericentral, 2 zonal, 3 panlobular), cholestasis (0 absent, 1 present), and sinusoidal dilatation (0 none, 1 mild, 2 moderate, 3 severe), in concordance with a previously published scoring system.[16]

Proinflammatory cytokines

Frozen tissue samples were thawed and a portion (~0.5mg) was placed in lysis buffer (50nM HEPES, 1mM EDTA, 150nM NaCl, 1% Triton-X) and homogenized. A pro-inflammatory panel (mouse) Kit (Meso Scale Discovery) was used to measure tissue cytokines – interleukin (IL) 1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, keratinocyte chemoattractant (KC)/human growth-regulated oncogene (GRO), tissue necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ) – using a Meso Sector S 600 plate reader (Meso Scale Diagnostics LLC; Rockville, MD, USA).

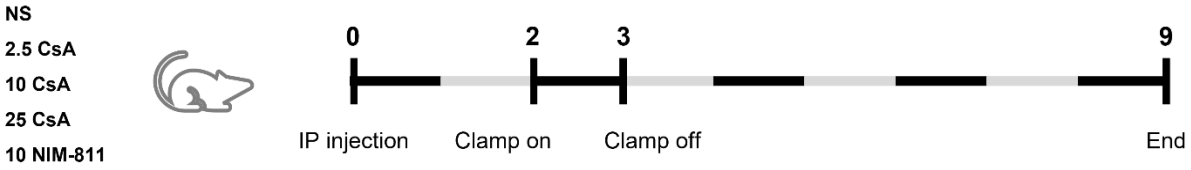

Figure 2.2.2. Model Schematic. Timeline (in hours) of cyclosporine A treatment and induction of hepatic ischemia/reperfusion injury.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

Deadend™ fluorometric TUNEL system (Promega North America; Madison, WI, USA) was used to identify apoptosis in paraffin-embedded tissue. Briefly, slides were washed in xylene, followed by rehydration with ethanol. Tissue on the slides was fixed using 4% paraformaldehyde and permeabilized with proteinase K. A nucleotide mixture containing fluorescein-12-dUTP was added to both tissue sections on the slide, while recombinant terminal deoxynucleotidyl transferase was added only to one (the other serving as a negative control). The reaction was stopped after one hour at 37ºC, the slides were washed, and mounted with media containing 4′,6-diamidino-2-phenylindole (DAPI) for counter-staining. Slides were viewed and imaged at 20x (Axio Observer Z1, Carl Zeiss Canada Ltd.; Toronto, ON, Canada). Quantification of the images was performed using Fiji software (General Public License).

Statistical analysis

Statistical analysis was performed using SPSS (IBM Canada; Markham, ON, Canada). Data was tested for normality using the Shapiro-Wilk test and histogram of distribution. Non-normal continuous data were transformed for normality using a two-step approach, as previously described.[17] Student's t-test were used for comparison between two groups and ordinary one-way or ANOVA tests were used for multi-group comparisons. Data are presented as medians and interquartile range. A p-value of 0.05 was used as the cut-off for statistical significance.

2.2.4 – Results

CsA reduces serum ALT following warm IRI

Serum ALT, measured at 6 hours post-ischemia, was reduced in mice treated with 10 or 25mg/kg of CsA compared to mice treated with NS only (1793.5 [1603-2157] U/L and 1823 [1668-2932] U/L vs. 3300.5 [2481-4911] U/L; $p<0.001$ and 0.007, respectively). No significant difference was seen in serum ALT of mice treated with 2.5mg/kg of CsA compared to control mice (4041 [2327-4871] U/L; $p=0.845$). Serum ALT of sham mice was significantly lower than all treatment groups (383 [229-562] U/L; $p<0.001$). Mice treated with 10mg/kg NIM-811 also had significantly lower serum ALT compared to controls (2375 [1963-2919] U/L; $p=0.031$). **Figure 2.2.3** illustrates the differences in serum ALT seen between groups.

Serum CsA levels 2 hours post-injection were 414.5 (412.75-416.25) µg/L, 1145 (1102.5-1187.5) µg/L, and >2500µg/L for the 2.5, 10, and 25mg/kg doses, respectively. All levels were within or above the range required for chronic immunosuppression.[18] Treating mice with CsA only (25mg/kg) had no effect of serum ALT at the same time point (67 [61-87] U/L; normal range 28-132U/L).

CsA decreases necrosis upon histological evaluation

Mice treated with CsA had significantly lower total scores of histological injury compared to controls at all doses ($p=0.042$, $p<0.001$ and $p=0.011$ for 2.5mg/kg, 10mg/kg and 25mg/kg doses respectively). This was driven mainly by lower sub-scores of necrosis, which were seen across doses in CsA-treated mice ($p=0.008$, $p<0.001$ and 0.002 for 2.5mg/kg, 10mg/kg and 25mg/kg doses respectively). Mice treated with CsA also had lower sub-scores for sinusoidal dilatation ($p=0.021$, $p<0.001$ and $p=0.003$ for 2.5mg/kg, 10mg/kg and 25mg/kg doses respectively), though

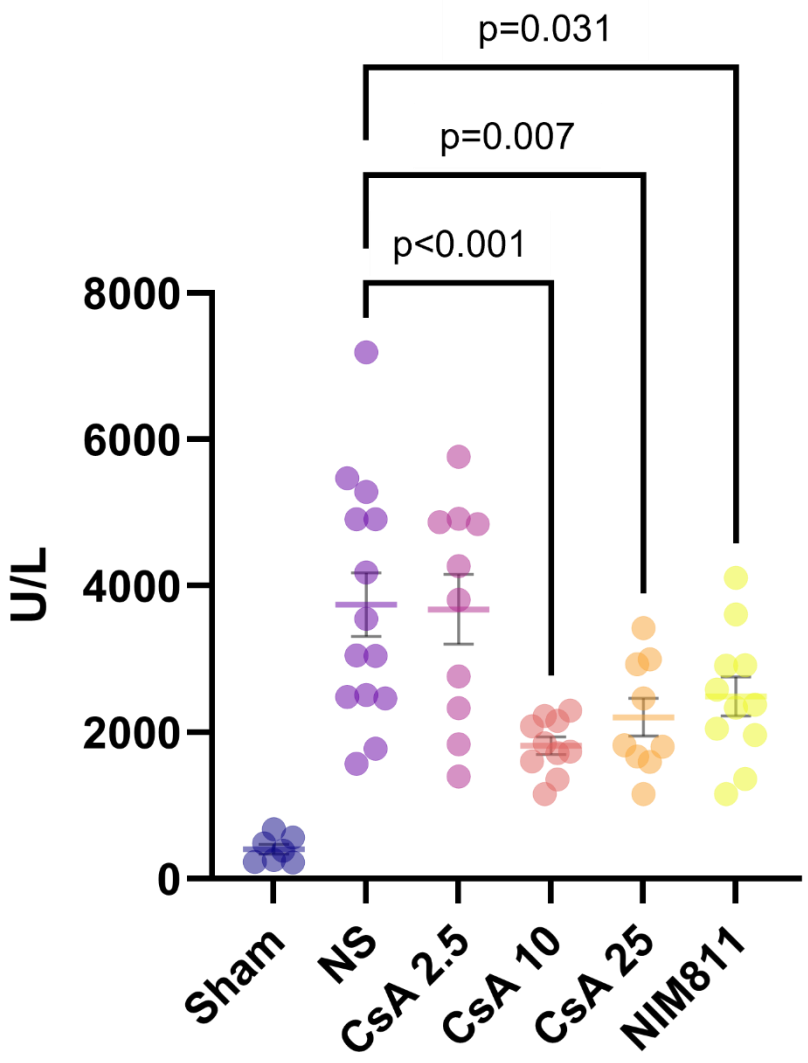

Figure 2.2.3. Effect of Cyclosporine A on Serum Transaminases. Serum alanine transferase (ALT) collected 6 hours after partial liver ischemia in mice treated with normal saline (NS), 2.5mg/kg, 10mg/kg or 25mg/kg of cyclosporine A (CsA 2.5, 10, 25) or 10mg/kg of NIM-811 (NIM811).

only pericentral dilatation was seen in NS-treated mice. Significantly lower histological scores of injury were also noticed in mice treated with NIM-811 ($p=0.043$). Sub-scores of necrosis were not different between NIM-811 treated mice and controls ($p=0.158$); however, these mice had significantly lower scores of sinusoidal dilation ($p<0.001$). **Figure 2.2.4** illustrates the differences in histological scores between treatment groups.

Apoptosis from IRI is decreased in mice treated with CsA

The percentage of apoptotic cells detected by TUNEL staining was significantly decreased compared to controls at all doses of CsA groups compared to the NS-treated controls ($p=0.012$, $p=0.003$ and $p<0.001$ for 2.5mg/kg, 10mg/kg and 25mg/kg doses respectively; **Figure 2.2.5A**). Representative images of the TUNEL-stained tissue sections from the NS and 10mg/kg CsA-treated groups are shown in **Figure 2.2.5B** (20x magnification). Bright green nuclei (seen most clearly in the magnified inset) are indicative of positive TUNEL staining.

Proinflammatory cytokine profiles at 6 hours post-ischemia in mice treated with CsA

Proinflammatory cytokines were measured in liver tissue at 6 hours post-ischemia in the CsA group compared with NS-treated controls. Tissue levels of IL-1β, IL-2, IL-4, IL-10 and KC/GRO were decreased with the highest dose of CsA (25mg/kg; $p=0.044$, $p=0.021$, $p=0.045$, $p=0.038$, $p=0.046$, respectively) compared to NS-control. Proinflammatory cytokine levels for each group are shown in **Figure 2.2.6**.

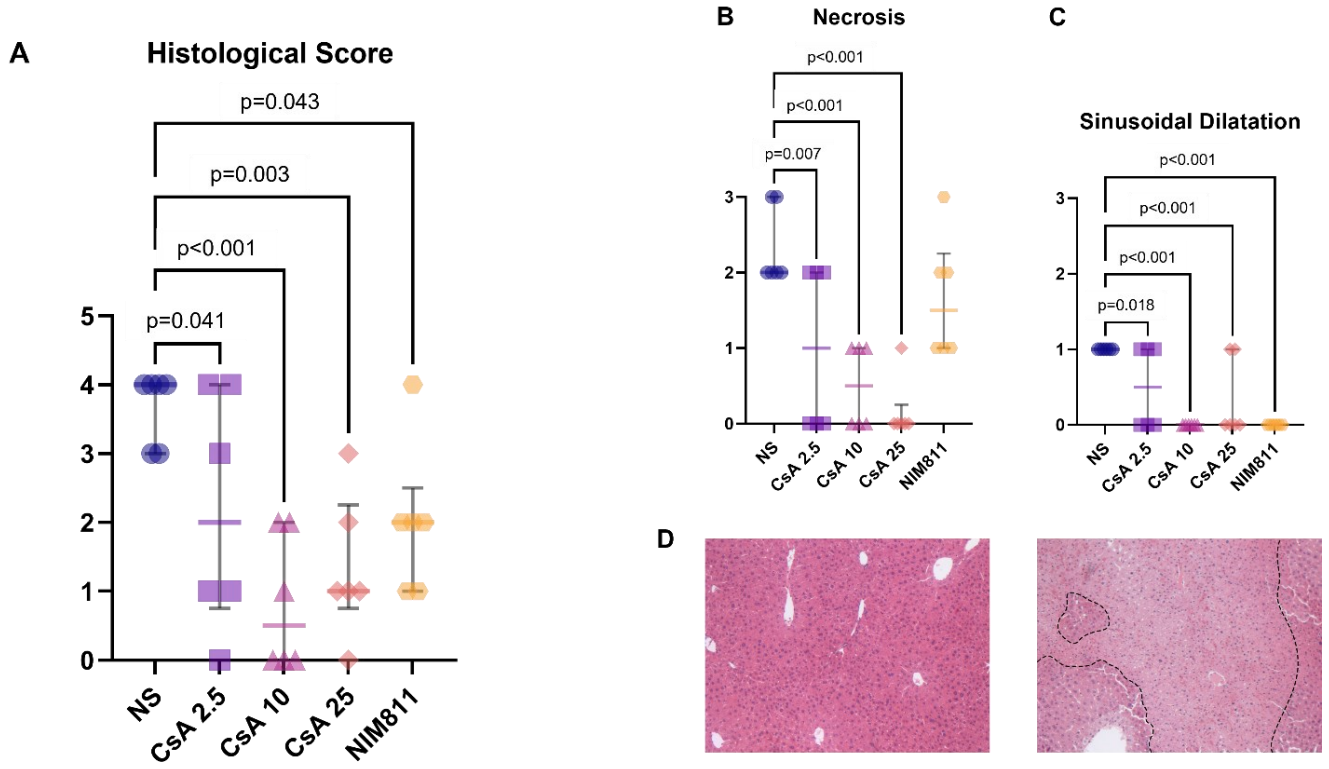

Figure 2.2.4. Effect of Cyclosporine A on Tissue Injury. Total histological score (A) of ischemic lobes taken after 6 hours of partial liver ischemia in mice treated with normal saline (NS), 2.5mg/kg, 10mg/kg or 25mg/kg of cyclosporine A (CsA 2.5, 10, 25) or 10mg/kg of NIM-811 (NIM811). Sub-scores of necrosis (B) and sinusoidal dilatation (C) for the same tissue samples. Representative histology showing absent (left) and pan-lobular (right; outlined with dashed line) necrosis (D).

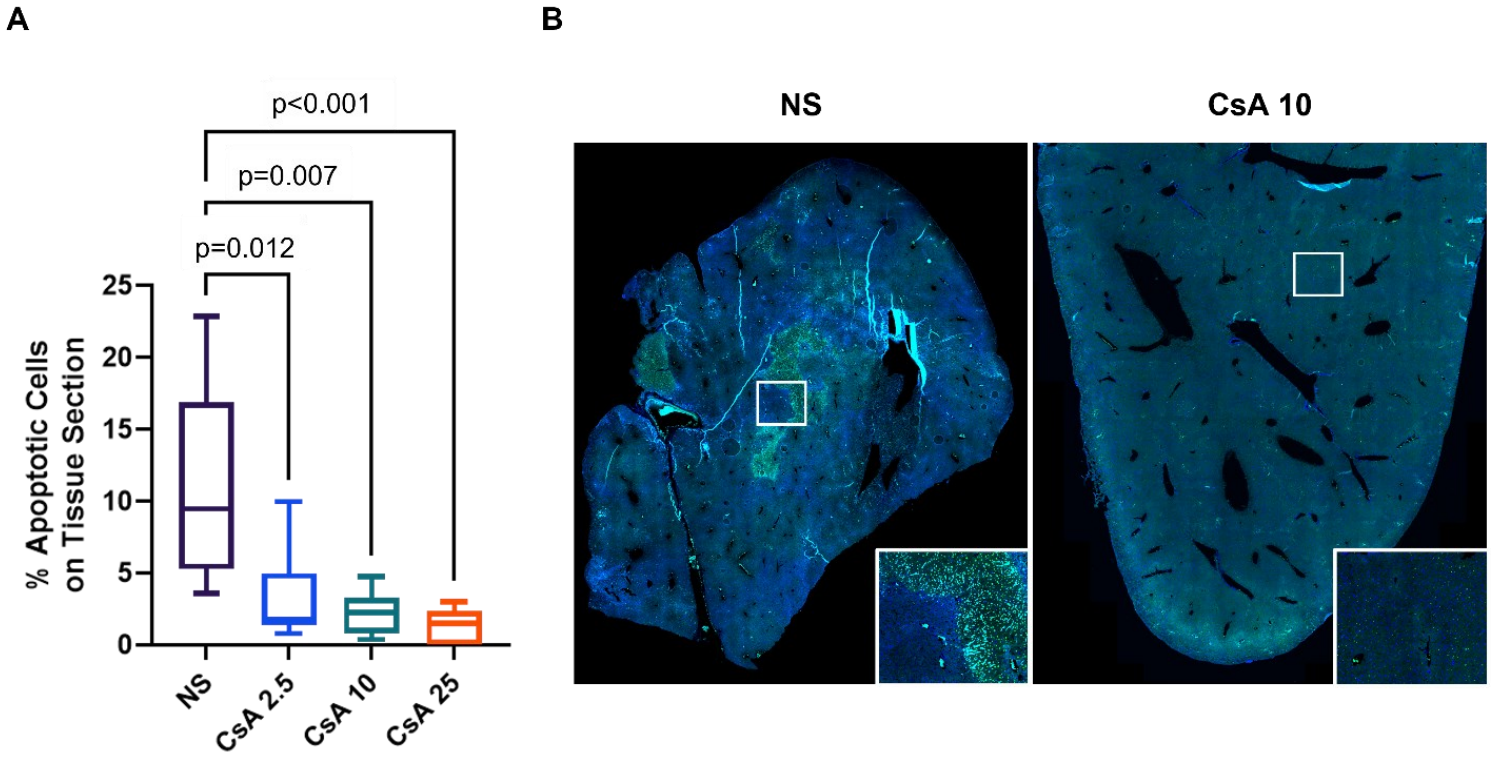

Figure 2.2.5. Effect of Cyclosporine A on Cell Death from Apoptosis. TdT-mediated dUTP nick-end label (TUNEL) staining for the detection of apoptotic cells. Quantification of percentage of apoptotic cells after 6 hours of partial liver ischemia in mice treated with normal saline (NS), 2.5mg/kg, 10mg/kg or 25mg/kg of cyclosporine A (CsA 2.5, 10, 25) (A). Representative images from control (NS) and 10mg/kg CsA-treated (CsA 10) groups at 20x magnification with DAPI counterstain (B).

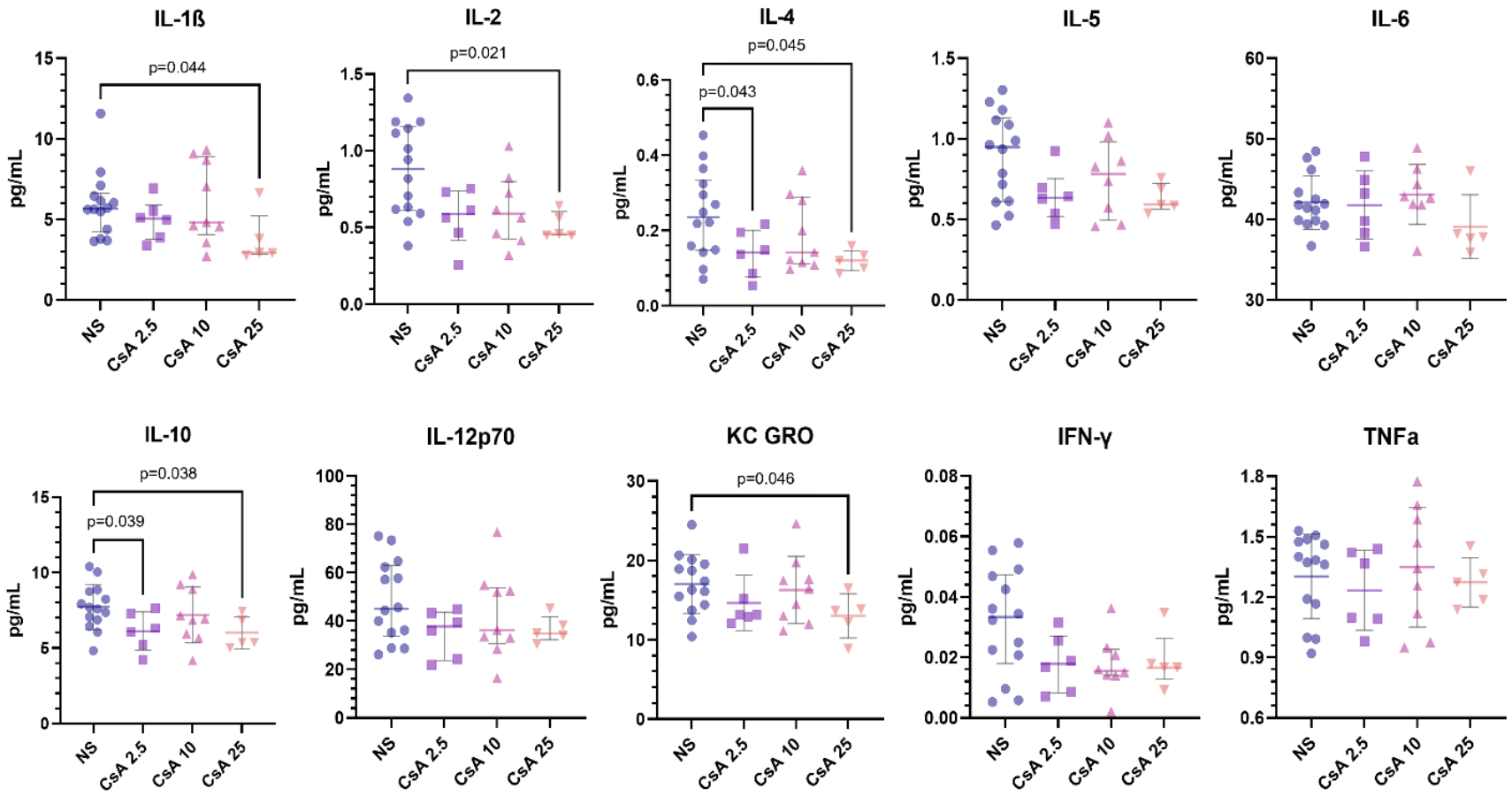

Figure 2.2.6. Effect of Cyclosporine A on Tissue Cytokines. Proinflammatory cytokine levels measured in liver tissue 6 hours post-ischemia. IL=interleukin, KCGRO=keratinocyte chemoattractant/human growth-regulated oncogene, IFNγ=interferon gamma, TNFα=tissue necrosis factor alpha

2.2.5 – Discussion

Our study provides evidence that pre-administration of escalating doses of CsA prior to warm liver IRI is beneficial in reducing hepatic injury. We found a greater than 50% reduction in serum ALT in mice treated with 10 or 25mg/kg CsA compared to mice treated only with NS. These findings correlated with histological scores showing a lower degree of necrosis. We also observed that CsA administration decreased levels of apoptosis following warm liver IRI. NIM-811, a CsA analog that lacks immunosuppressive properties, showed similar reductions in serum ALT and histologic liver injury, suggesting that the protective effect of CsA is unrelated to its immunosuppressive effect.

CsA has previously been shown to mitigate IRI in preclinical studies and has been extensively explored, particularly in relation to myocardial IRI.[19] However, these findings have not reliably translated into clinical practice, perhaps best exemplified by the CIRCUS trial.[20] Difficulties in translating findings of preclinical studies of myocardial and cerebral infarction models, in particular, reflect the fact that these events occur unexpectedly and patients present after the onset of ischemia. In these settings, CsA cannot be given before ischemia and the duration of ischemia cannot be controlled. Both of these variables have been reported to impact the efficacy of CsA in preclinical studies, which seems to work best if given prior to ischemia and has diminished efficacy beyond a critical window of ischemia.[8,21,22] In contrast, hepatic ischemia can often be anticipated, particularly in the setting of liver transplantation, where it is kept within certain limits. In addition, being able to achieve the same effect with NIM-811 means that unnecessary immunosuppression can potentially be avoided.

The observation in our study that CsA prevents both necrotic and apoptotic cell death is likely due to its action at the mitochondrial level. This is supported by our finding that the non-

immunosuppressive NIM-811 similarly reduced hepatic injury. In instances of severe insult, mitochondrial swelling and rupture can precipitate necrotic cell death.[23] Less severe damage might induce an apoptotic response. The effect of CsA on other programmed cell death responses, such as necroptosis and ferroptosis, is less clear and was not investigated in this study. It is worth noting that, our study found some benefit in terms of reduced histological injury and apoptosis with even the lowest tested dose of CsA (2.5mg/kg), though this was not reflected in serum ALT. CsA doses of 10 and 25mg/kg had similar benefits, suggesting that increasing the dose beyond 10mg/kg will not lead to improved protection. This is important considering the impacts that side effects of CsA (most notably renal dysfunction) could have in this population.[24]

The role of proinflammatory cytokines in inducing IRI through the recruitment of immune cells is well documented.[25] In this study, we found small, but significant differences in levels of proinflammatory cytokines. Some that achieved statistical significance (IL-2 and IL-4) are associated with T cell activation, which tends to happen later in the course of IRI when the adaptive immune system becomes involved.[26] This could also be a reflection of calcineurin inhibition by CsA.[5] The absence of significant differences in other proinflammatory cytokine levels in our study could be due to the later timepoint (chosen to detect maximal tissue damage). However, it also reflects the fact that immunosuppression is not thought to be the main mechanism of protection from IRI by CsA. If this were the case, we would expect to see more pronounced differences in tissue cytokines earlier in the course of IRI. Other studies have implicated a variety of different cytokines in liver IRI, including TNFα, IL-1β, and IL-6.[27] Of these, only IL- IL-1β was found to be slightly decreased with the highest dose of CsA at 6 hours. While liver enzymes were significantly different at the 6-hour timepoint we examined in this study, further examination of

multiple timepoints could potentially identify peak levels, which may differ between different cytokines.

There are challenges with modeling liver IRI in mice that have been well described in the literature, particularly with respect to variability in the level of injury between animals, which can be difficult to control even within the same study.[28] It is known that differences in sex, strain, and even sub-strain of mice can affect outcomes. Our choice of male mice is in line with the majority of other studies, as female sex is thought to be protective against liver injury.[29] In our experience, we found several factors that decreased variation in serum transaminases in the model. Applying the clamp under magnification was important, as it allowed consistency in application and verification that the clamp crossed the whole portal venous branch and captured the smaller arterial branches. We also observed that application of a second clamp reduced variability. It is sometimes the case that the portal branches to the upper lobes are abnormally short or that branches to the right lobe occur higher than usual, resulting in ischemia to the right lobe with clamping. This often resulted in venous congestion of the bowels, precipitating demise of the animal.

Heparin was added to prevent the formation of blood clots, which could lead to additional hepatic injury.[15] Effects of fasting state, body temperature, and diurnal variations in serum transaminases were not examined in this study, though, we did maintain consistency with respect to the timing of experiments and use of warming devices during and after surgery. Whereas fasting is employed in models of toxic liver injury, such as with acetaminophen, as a means of reducing glutathione levels and increasing susceptibility to injury, fasting in IRI liver models is not routinely used and prolonged fasting has even been found to be protective against liver IRI.[30]

The utility of a murine model such as this provides a relatively straightforward means of screening novel compounds with the potential to protect against liver IRI. However, there are

several important differences from clinical scenarios that limit its direct translation. Liver transplantation is the most common clinical setting in which liver IRI occurs. Features of IRI in liver transplantation lacking from this model include complete vascular isolation (i.e., no possibility for hepatic venous backflow), a period of cold ischemia, increasing use of normothermic machine or regional oxygenated perfusion technologies, and the use of immunosuppression to prevent tissue rejection. This model fits more closely with IRI encountered during hepatic resection. However, clinically, this type of ischemia usually occurs for a much shorter period (ideally <15 minutes) or consists of multiple brief periods of ischemia interspersed with reperfusion, which may be less injurious to the liver. Only rarely is an extended duration of vascular inflow occlusion required to control bleeding. It may be that the effect of CsA or NIM-811 would be less pronounced in the clinical setting.

2.2.6 – Conclusions

Overall, our findings provide evidence that CsA can reduce hepatic warm IRI in a murine model. Similar findings using its non-immunosuppressive analog, NIM-811, suggest that this effect is not mediated through immunosuppressive pathways. This has potential implications for liver transplantation and hepatic resection. It would be beneficial to test CsA or NIM-811 in a large animal model of hepatic IRI, preferably one of liver transplantation, prior to the initiation of a clinical trial potentially with application during normothermic machine perfusion.

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CHAPTER 2 – CYCLOSPORINE A FOR THE PREVENTION OF ISCHEMIA REPERFUSION INJURY

PART 3 – CYCLOSPORINE A DOES NOT MITIGATE LIVER ISCHEMIA/REPERFUSION INJURY IN AN *EX VIVO* PORCINE MODEL OF DONATION AFTER CIRCULATORY DEATH

2.3 – Cyclosporine A Does Not Mitigate Liver Ischemia/Reperfusion Injury in an *Ex Vivo* Porcine Model of Donation After Cardiac Death

A version of this section is currently under review in *Annals of Transplantation*.

Title: Cyclosporine A Does Not Mitigate Liver Ischemia/Reperfusion Injury in an *Ex Vivo* Porcine Model of Donation After Circulatory Death

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References: 19

2.3.1 – Preface

Background: Ischemia/reperfusion injury (IRI) is an inherent problem in organ transplantation, owing to the obligate period of ischemia that organs must endure. Cyclosporine A (CsA), though better known as an immunosuppressant, has been shown to mitigate warm IRI in a variety of organ types, including the liver. However, there is little evidence for CsA in preventing hepatic IRI in the transplant setting.

Material & Methods: In the present study, we tested the effect of CsA on hepatic IRI in a large animal *ex vivo* model of donation after circulatory death (DCD). Porcine donors were pre-treated with either normal saline control or 20mg/kg of CsA. Animals were subject to either 45 or 60 minutes of warm ischemia before hepatectomy, followed by 2 or 4 hours of cold storage prior to reperfusion on an *ex vivo* circuit. Over the course of a 12-hour perfusion, perfusion parameters were recorded and perfusate samples and biopsies were taken at regular intervals.

Results: Peak perfusate lactate dehydrogenase was significantly decreased in the lower ischemia group treated with CsA compared to the untreated group (4,220U/L [3,515-5,815] vs. 11,305 [10,100-11,674]; p=0.023). However, no difference was seen between controls and CsA-treated groups on other parameters in perfusate alanine or asparagine aminotransferase (p=0.912, 0.455, respectively). Correspondingly, we found no difference on midpoint histological injury score (p=0.271).

Conclusion: Taken together, we found minimal evidence that CsA is protective against hepatic IRI in our DCD model.

2.3.2 – Background

Ischemia/reperfusion injury (IRI) occurs following the return of blood flow to previously ischemic tissue.[1] Ischemia itself is characterized by metabolic derangements, including depletion of the adenosine triphosphate (ATP) reserve with concomitant increase in lactic acid. Prolonged ischemia results in cell death by mechanisms such as the loss of cellular membrane potential. Cell death is exacerbated upon the return of blood flow by the intense generation of reactive oxygen species (ROS) and the initiation of a proinflammatory cascade, including the recruitment of immune cells and activation of the complement system.[2]

IRI is relevant to several clinical scenarios, in which organs are partially or wholly deprived of blood flow for a period of time. Organ transplantation is a notable example, as there is an obligate period of ischemia from when the organ is explanted from the donor to when it is implanted into the recipient. For the most part this occurs in the context of cold storage, where the organ is kept at 4ºC to minimize its metabolic demands.[3] However, due to rising demand, organs are increasing being used following circulatory death, where there is an additional period of warm ischemia before the organs can be procured.[4] Organs donated after circulatory death (DCD) are at more susceptible to IRI, which, for the liver, means higher risk of primary non-function and decreased graft survival.[5]

Cyclosporine A (CsA) is one of the early immunosuppressant drugs that enabled the success of solid organ transplantation. Its immunosuppressive function is achieved through the inhibition of calcineurin, which prevents T cell activation.[7] An additional effect of CsA is its inhibition of the mitochondria permeability transition pore (mPTP) via its binding to cyclophilin d.[8] This secondary function has been explored to prevent mitochondrial swelling and dysfunction in the setting of IRI.

CsA has been demonstrated to reduce warm hepatic IRI in several preclinical models, including a porcine model of hepatic artery clamping.[9-11] However, the evidence in transplantation-related models combining warm and cold ischemia is less robust and does not include any large animals studies.[12,13] In the present study, we explore the effect of CsA in a more clinically relevant large animal model of hepatic IRI following donation after circulatory death.

2.3.3 – Materials & Methods

The following study was done in accordance with the Canadian Council on Animal Care Guidelines and Policies. All experiments were conducted with approval from the Animal Care and Use Committee (Health Sciences) for the University of Alberta (AUP00001036; approved 04/07/2014). This study used female domestic swine weighing 40-50kg. Animals were supplied by the Swine Research and Technology Centre at the University of Alberta.

Study Design

This study consists of an *ex vivo* model of liver DCD, in which livers enduring a period of warm, followed by cold ischemia were subject to whole-blood reperfusion using an *ex vivo* perfusion device. Four groups of five animals each were included in the study, testing two levels of ischemic injury with or without CsA (**Figure 2.3.1**). At the lower degree of ischemic injury, livers were subject to 45 minutes of warm ischemia, followed by 2 hours of cold ischemia (at 4ºC), whereas the higher degree of injury consisted of 60 minutes of warm ischemia, followed by 4 hours of cold ischemia. A dose of either normal saline (control) or 20mg/kg CsA (treatment) was administered prior to the onset of warm ischemia.

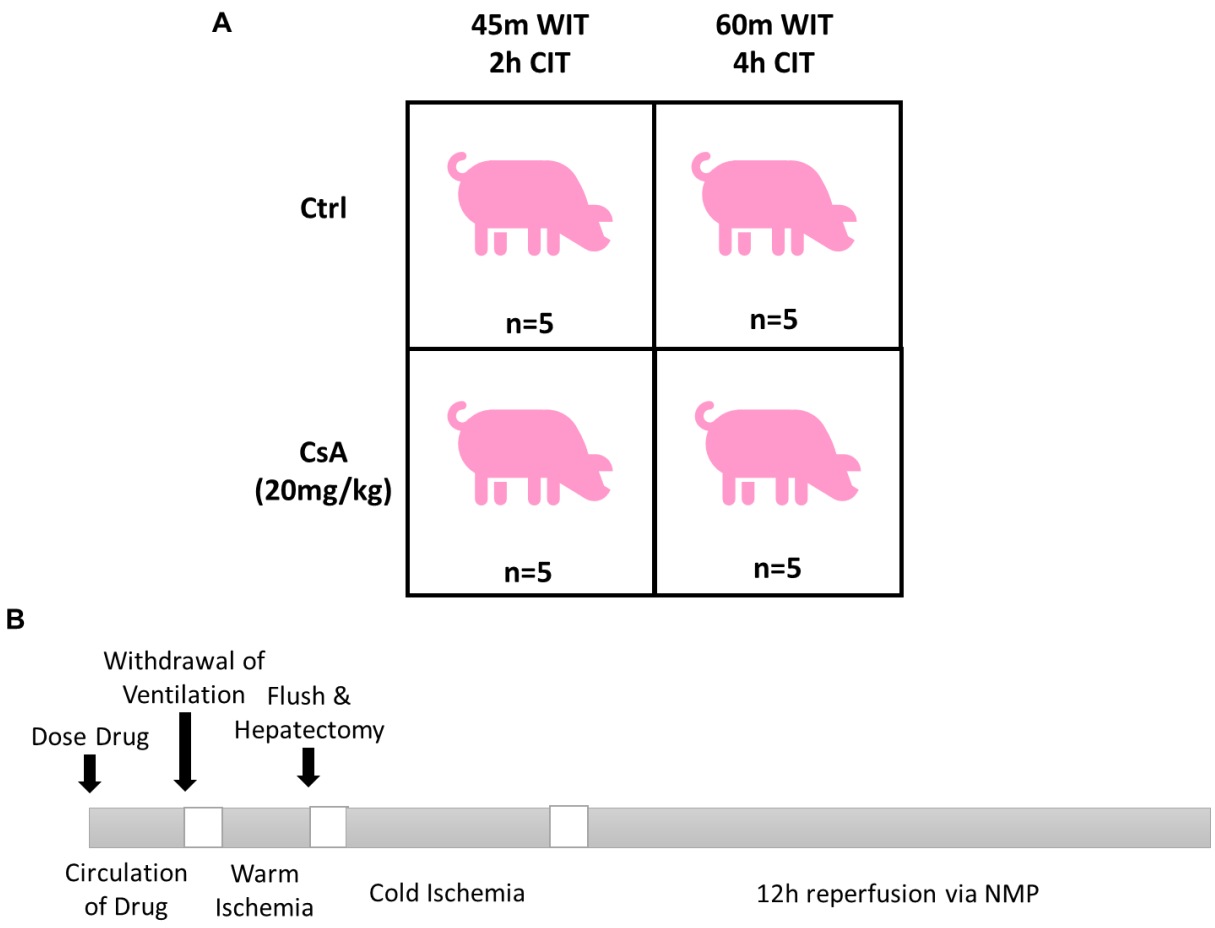

Figure 2.3.1. Study groups treated with normal saline (Ctrl) or cyclosporine A (CsA) and subject to either 45 minutes of warm ischemia (WIT), followed by 2 hours of cold ischemia (CIT) or 60 minutes of WIT followed by 4 hours of CIT (A). Outline of experimental procedure (B).

Liver Procurement

For liver procurement, pigs were sedated with 20mg/kg of ketamine (Ketaset; Zoetis Canada Inc., Kirkland, QC, Canada) and 0.05 mg/kg of atropine (Atro-SA; Rafter 8 Products Inc., Calgary, AB, Canada). They then underwent endotracheal intubation and were maintained on inhalational anesthesia (3-4% isoflurane; Frensenius Kabi Canada, Toronto, ON, Canada). An orogastric tube was inserted to decompress the stomach and a 20G venous cannula was inserted into an ear vein for fluid administration. A cut-down to the right carotid artery was performed to insert an arterial pressure monitoring line.

Either control saline or drug were infused via the ear vein. A 30 minute period was allowed for the treatment to circulate prior to proceeding with abdominal dissection. A midline laparotomy was performed extending from the xyphoid process to pubic synthesis. Abdominal contents were retracted to expose the infra-renal aorta. Overlying peritoneum was incised and a segment of aorta was dissected and encircled with 0 silk ties (Perma Hand; Ethicon Inc., Bridgewater, NJ, USA) for ease of future cannulation. Next, the gallbladder was removed from the surface of the liver using a combination of electrocautery and blunt dissection, ligating the cystic duct and artery together. The main portal vein and supraceliac aorta were then dissected in turn and encircled with a 2-0 silk for later identification. A thoracotomy was performed to facilitate blood collection via the right atrium, which was achieved by inserting a two-staged venous canula. Blood was collected into a sterile container to the point of complete exsanguination (1.5-2L). Warm ischemic time was measured from the point at which systolic blood pressure dropped below 50mmHg.

Following the period of warm ischemia, the supraceliac aorta was clamped and a cannula was inserted into the infra-renal aorta through which 2L of cold University of Wisconsin (UW) solution (Belzar UW® Cold Storage Solution; Bridge to Life Ltd., Colombia, SC, USA) was

infused. Ice was then packed into the abdomen and the suprahepatic inferior vena cava (IVC) was incised to vent the flush. Upon completion of the intra-abdominal flush, hepatectomy was performed. The liver was weighed on the back table, flushed via the portal vein with an additional 0.5L of UW solution and placed on ice slush in an organ retrieval bag for the duration of the cold ischemic time.

NMP Perfusion

Upon completion of the cold ischemic period, livers were perfused for 12 hours according to our previously established protocol.[13,14] As illustrated in **Supplementary Figure 2.3.1**, perfusate is circulated via two BPX-80 Bi-Medicus centrifugal pumps (Medtronic of Canada Ltd., Brampton, ON, Canada). One pump directs perfusate through an EOS ECMO oxygenator (Sorin Group Canada Inc., Burnaby, BC, Canada) before entering the hepatic artery, while the second pump directs perfusate through a MYOtherm XP heat exchanger (Medtronic of Canada Ltd., Brampton, ON, Canada) before it enters the portal vein. Perfusate drains from the liver's IVC into a filtered CARD EVO cardiotomy reservoir (Sorin Group Canada Inc., Burnaby, BC, Canada) before returning to the pumps. Perfusate is heated as it passes through the heat exchanger and oxygenator by CW-05G water bath (Lab Companion, Jeio Tech Inc., Billerica, MA, USA). Transonic Clamp-on Tubing Flowsensors (6PXL and 7PXL; Transonic Systems Inc., Ithaca, NY, USA) measure arterial and portal venous flows, while pressures are measured using TruWave pressure transducers (Edwards Life Sciences Canada Inc., Mississauga, ON, Canada). The speed of the centrifugal pumps is controlled by computer to maintain the desired hepatic artery pressure and portal venous flow. Inflows of oxygen and carbon dioxide are titrated to maintain a partial

pressure of arterial oxygen between 100 and 120mmHg and a partial pressure of carbon dioxide between 35 and 45mmHg.

Prior to commencing perfusion, the circuit is primed with a 1:1 ratio of whole blood to modified Krebs-Henseleit solution (glucose [5mM], sodium chloride [85mM], potassium chloride [5mM], calcium chloride [1mM], magnesium chloride [1mM], sodium bicarbonate [25mM], sodium phosphate monobasic [1mM], sodium pyruvate [5mM], and 8% bovine serum albumin). Piperacillin/tazobactam (3.375g; Sandoz Canada, Boucherville, QC, Canada), methylprednisolone (500mg; Solu-Medrol, Pfizer Canada Inc., Kirkland, QC, Canada), and sodium heparin (5000U; Fresenius Kabi Canada, Toronto, ON, Canada) are added initially as boluses. During perfusion, the organ also receives infusions of regular insulin (2U/h; Humulin R; Eli Lilly & Company, Toronto, ON, Canada) and sodium heparin (1000U/h), as well as boluses of trisaminomethane (THAM, 3mol/L; Sigma-Aldrich Canada, Oakville, ON, Canada) and glucose (250mg/mL; Sigma-Aldrich Canada, Oakville, ON, Canada) to maintain perfusate pH and glucose within physiological range (7.35–7.45 and 6–10 mmol/L, respectively).

Each liver is flushed with 2L of normal saline prior to canulation. An 8 Fr arterial cannula (Medtronic of Canada Ltd., Brampton, ON, Canada) and 1/4’ polycarbonate tubing connector are used to attach the hepatic artery and portal vein to the circuit, respectively. The common bile duct is cannulated with a thin piece of tubing draining to a separate reservoir. Arterial and portal venous flows are gradually increased to physiological values, as the organ reaches its target temperature of 37ºC.

Perfusate Biochemistry

Perfusate hemoglobin, pH, electrolytes, lactate, glucose, and partial pressures of oxygen and carbon dioxide were measured using a point-of-care blood gas machine (ABL Flex Analyzer; Radiometer Canada, Mississauga, ON, Canada) sampling perfusate from the arterial and portal venous inflows and IVC outflow every 2 hours. Perfusate sampled was also samples from the IVC outflow for measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and total and conjugated bilirubin using a Beckman Coulter Unicel Dxc800 Syncron (Beckman Coulter Canada LP, Mississauga, ON, Canada).

Histology

Incisional biopsies were taken from the right lateral lobe after 2, 6 and 12 hours of perfusion. A portion of the tissue was fixed in 10% formalin, while the remainder was flash frozen in liquid nitrogen for tissue assays. Formalin fixed tissue was then paraffin embedded, cut and stained with hematoxylin and eosin (H&E). H&E slides were examined by a trained pathologist (AT) and scored based on the presence of necrosis, vacuolization, cholestasis, and congestion, as previously reported.[15]

Glutathione & Cytokine Assays

Tissue glutathione was measured using a colorimetric assay kit (Invitrogen EIAGSHC, Fisher Scientific Co., Edmonton, AB, Canada), which was run according to the manufacturer's instructions. Standards and samples were added in duplicate on a 96-well plate and, following completion of the reaction, absorbance at 405nm was measured on a spectrophotometer (Multiskan SkyHigh; Fisher Scientific Co., Edmonton, AB, Canada). Levels of 11 different cytokines were

measured in perfusate at 3 time points (0, 6 and 12 hours) using a porcine-specific multiplex assay (Discovery Assay® Array PD13; Eve Technologies Corp., Calgary, AB, Canada), run according to manufacturer's instructions.

Statistical Analysis

Data are reported as medians (interquartile range). Normality was tested using the Shapiro-Wilk test and histogram distribution. Non-normal data were transformed using a process previously described.[16] Student's t-test was used for comparison between two groups. Ordinary one-way ANOVA tests were used for multiple group comparison, with Fisher's least significant difference procedure applied for pairwise comparison in post-hoc analysis. A p-value of 0.05 was taken as significant. SPSS Software version 26 (IBM Canada; Markham, ON, Canada) was used for statistical analysis.

2.3.4 – Results

CsA Treatment has No Effect on Liver Perfusion Parameters or Bile Production

Perfusion parameters, including flow rate, pressure, and resistance are measured as a routine part of organ monitoring. Perfusion parameters were largely similar between groups (**Supplementary Figure 2.3.2**). Peak HA flow was lower in the higher ischemia group treated with CsA compared to the lower ischemia control (330.3mL/min [327.0-363.9] vs. 433.2mL/min [425.1-446.9]; p=0.016). However, minimum HA resistance was no different between groups (p=0.681). In contrast, peak PV flow was no different between groups (p=0.709), while minimum PV resistance was significantly higher in the higher ischemia group treated with CsA compared to

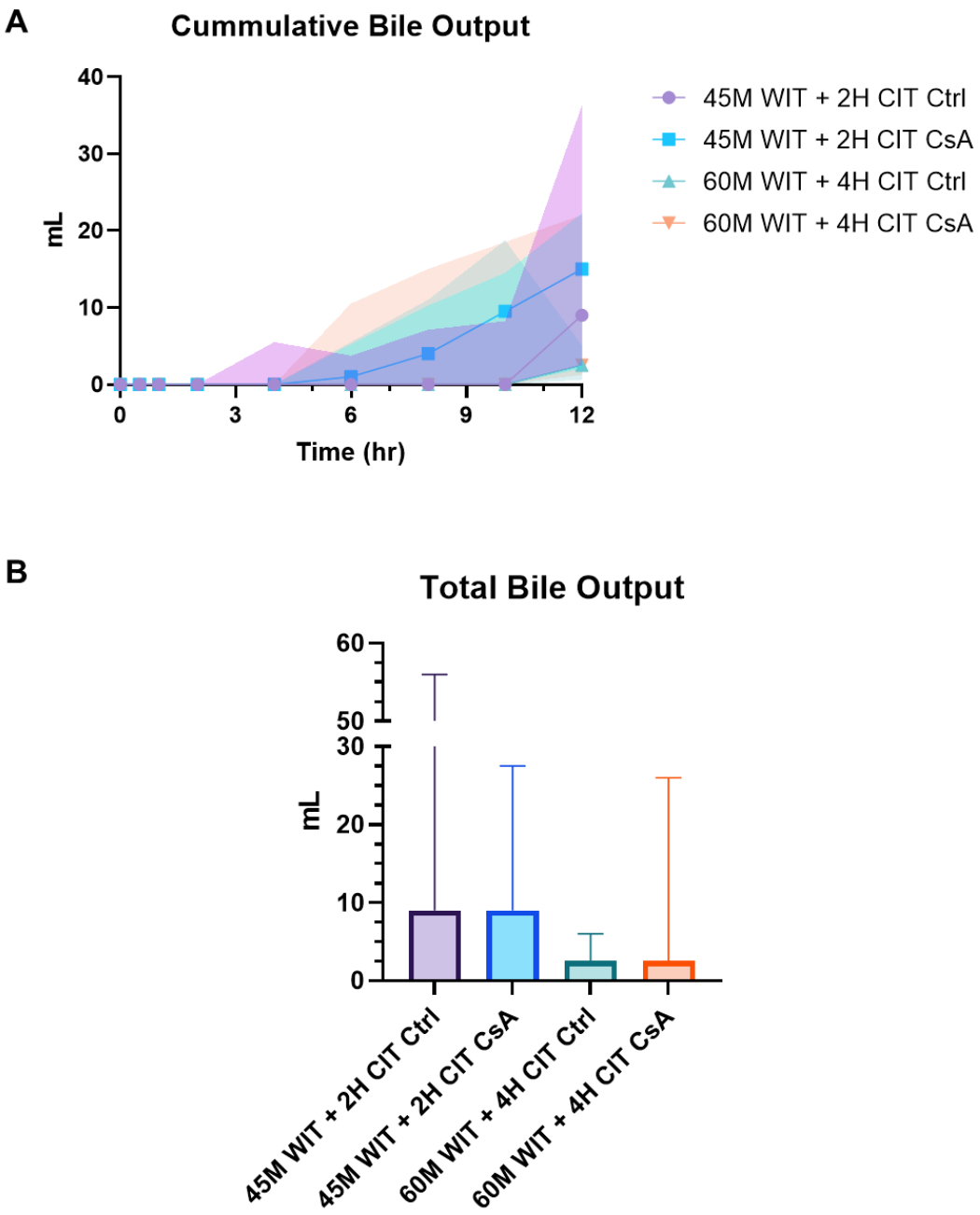

Figure 2.3.2. Cumulative (A) and total (B) bile output over 12 hours reperfusion for control (Ctrl) and treated (CsA) livers subject to 45 or 60 minutes of warm ischemia (45M WIT, 60M WIT) and 2 or 4 hours of cold ischemia (2H CIT, 4H CIT). Total bile output was not significantly different (p=0.642). CIT=cold ischemia time; WIT=warm ischemia time

the lower ischemia control (1.0mmHg/min/mL [0.4-1.2] vs. 0.3mmHg/min/mL [0.1-0.4]; p=0.009). Median cumulative bile production was higher for livers in the lower ischemia group (12.5mL [2.5-17.0] vs. 2.5mL [2.0-18.0]), but this did not achieve statistical significance (p=0.277; **Figure 2.3.2**). No significant difference in bile production was seen between CsA-treated livers and controls at either degree of ischemia (p=0.987, 0.465 comparing control and treated livers in the lower and higher ischemia levels, respectively).

Peak LDH is Decreased in Livers Treated with CsA After 45min of Warm Ischemia & 2h of Cold Ischemia

Perfusate ALT, AST, and LDH were measured as markers of cellular damage. Peak LDH was significantly lower in the lower ischemia treatment group (4,220U/L [3515-5810]) compared to the other groups (11,305U/L [10,100-11,674], 7939U/L [6,692-8,760], 7,992U/L [7,692-10,971]; p=0.023, 0.035, 0.019 for the lower ischemia control and the higher ischemia control and treated, respectively). However, peak values of perfusate ALT (which is more specific to the liver) for the lower ischemia group treated with CsA (302U/L [191-314]) were no different from those of the other groups (334U/L [254-334], 306U/L [295-335], 313U/L [217-331]; p=0.519, 0.584, 0.747 for the lower ischemia control and the higher ischemia control and treated, respectively; **Figure 2.3.3**). Peak values of perfusate AST were similarly no different in the lower ischemia treatment group (8,265U/L [4,176-8,660], 11,305U/L [10,100-11,674], 7,939U/L [6,692-8,760], 7,992U/L [7,692-10,971]; p=0.120, 0.382, 0.357 for the lower ischemia control and the higher ischemia control and treated, respectively).

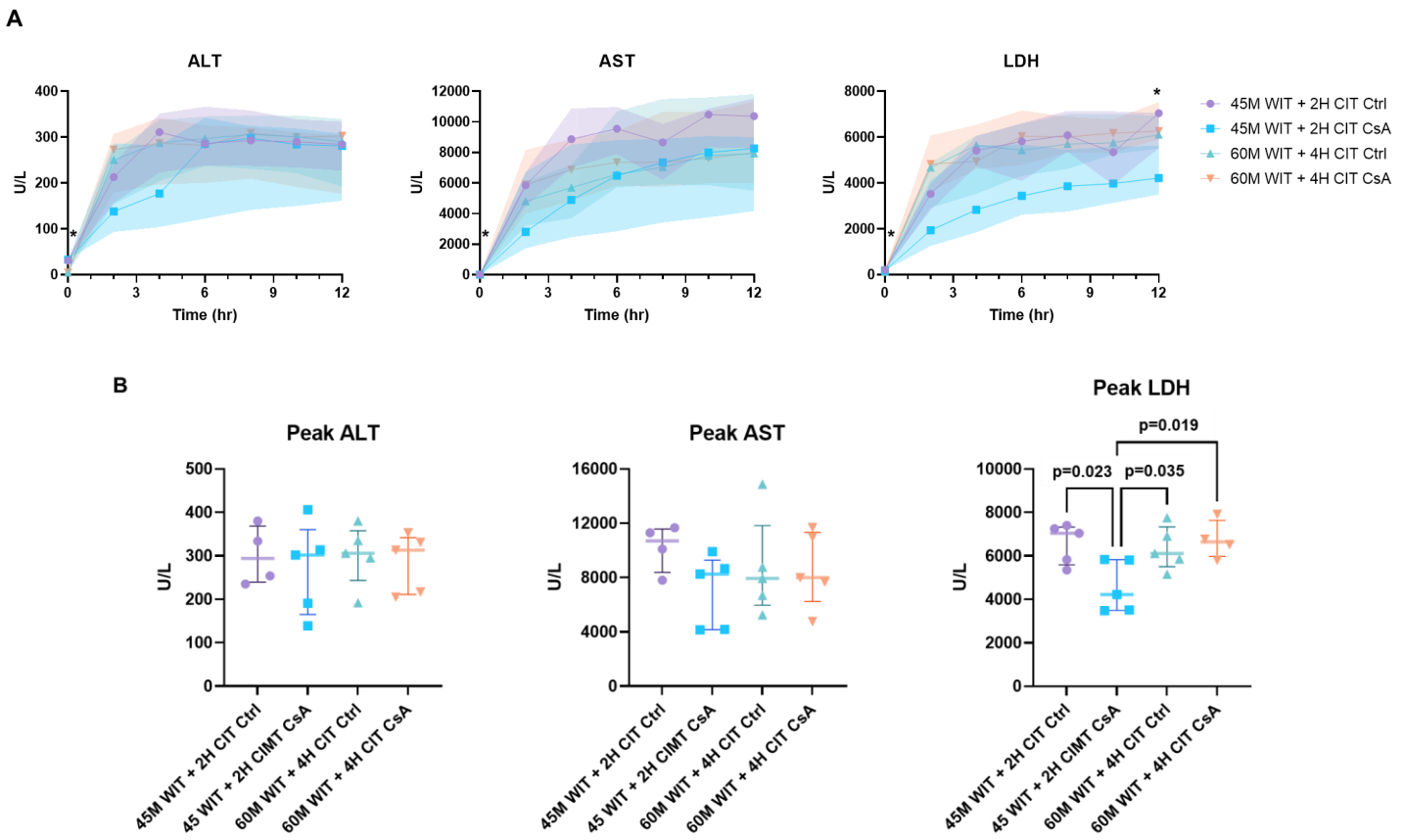

Figure 2.3.3. Perfusate levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) over the course of reperfusion (A) and at their peak (B) for control (Ctrl) and treated (CsA) livers subject to 45 or 60 minutes of warm ischemia (45M WIT, 60M WIT) and 2 or 4 hours of cold ischemia (2H CIT, 4H CIT). Peak ALT and AST were not significantly different between groups (p=0.912 and 0.455, respectively). CIT=cold ischemia time; WIT=warm ischemia time

End pH is Decreased in Livers with Less Ischemic Injury, but is not Affected by CsA Treatment

The liver plays a vital role in maintaining serum pH and regulating glycolysis, including metabolism of its principle by-product, lactate.[17] As such, acidosis and rising perfusate lactate are indicative of poor liver function. We found pH at the end of perfusion was significantly decreased in the higher ischemia groups compared to the lower ischemia groups (7.346 [7.324-7.361] vs. 7.407 [7.384-7.461]; p=0.001), despite having no difference in the amount of THAM required to maintain pH (42mL [35-48] vs. 45mL [33-50] for the lower and higher ischemia groups, respectively; p=0.605; **Figure 2.3.4**). However, there was no difference in end pH between control and treated groups at either level of ischemia (p=0.752, 0.517 for the lower and higher ischemia groups, respectively). Perfusate lactate levels did not differ significantly over the course of the perfusion, nor were peak lactate levels different between groups (2.8mmol/L [2.7-4.2], 3.3mmol/L [2.1-5.1], 3.5mmol/L [2.7-9.8], 4.1mmol/L [2.8-6.8] for control and treated livers in the lower ischemia group and control and treated livers in the higher ischemia group, respectively; **Figure 2.3.5**).

Peak O_2 Consumption is Increased in Livers with Less Ischemic Injury, but is not Affected by CsA

Oxygen consumption is indicative of the metabolic activity of an organ. We found that peak oxygen consumption was also higher for livers in the lower ischemia groups compared to those in the higher ischemia groups (**Figure 2.3.6**). Peak oxygen consumption was 0.011mL/min/g tissue (0.011-0.012) for the control and 0.014mL/min/g tissue (0.009-0.016) for the treated groups at the higher ischemia level compared to 0.017mL/min/g tissue (0.015-0.018) for the control (p=0.029, 0.063 respectively) and 0.020mL/min/g tissue (0.016-0.021) for the treated (p=0.007, 0.016 respectively) groups at the lower ischemia level. There was no difference, however, between

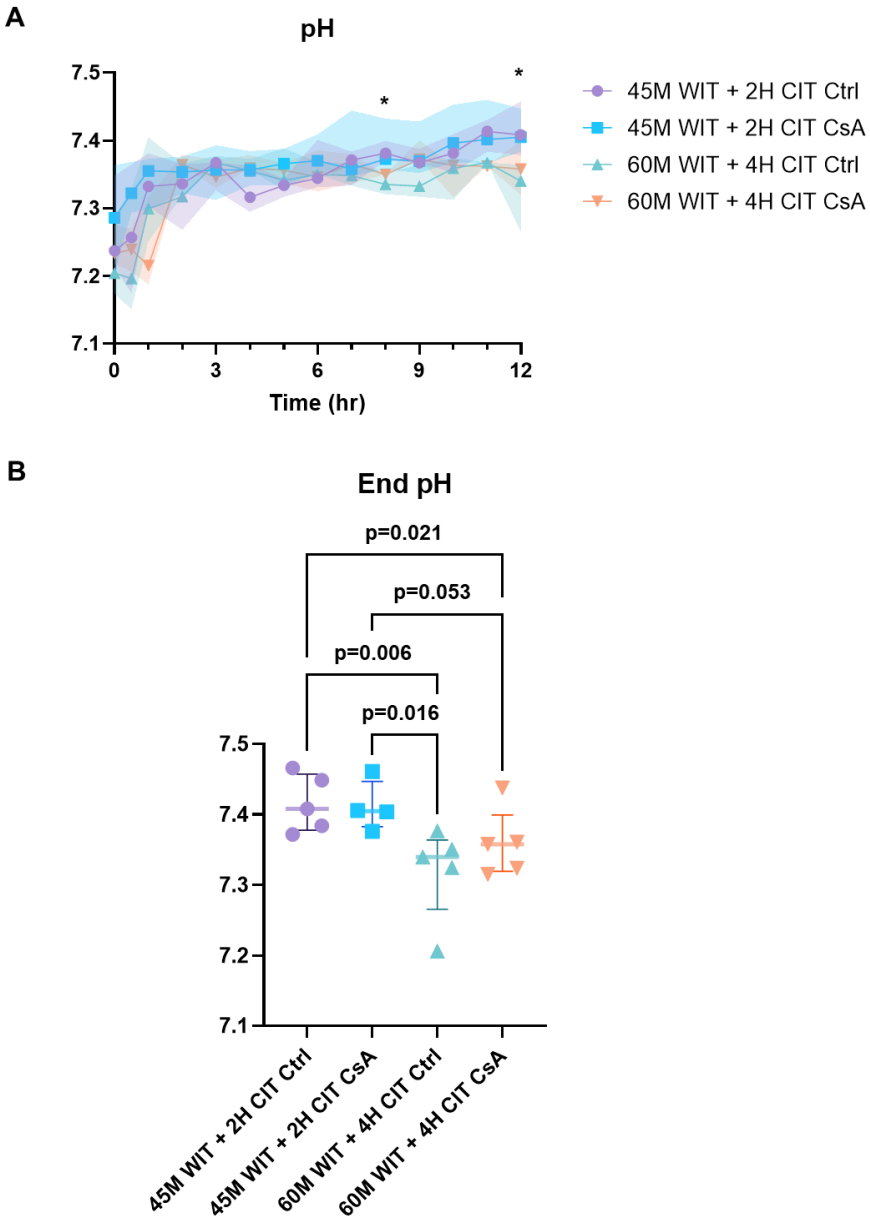

Figure 2.3.4. Perfusate pH over the course of reperfusion (A) and at the end (B) for control (Ctrl) and treated (CsA) livers subject to 45 or 60 minutes of warm ischemia (45M WIT, 60M WIT) and 2 or 4 hours of cold ischemia (2H CIT, 4H CIT). CIT=cold ischemia time; WIT=warm ischemia time

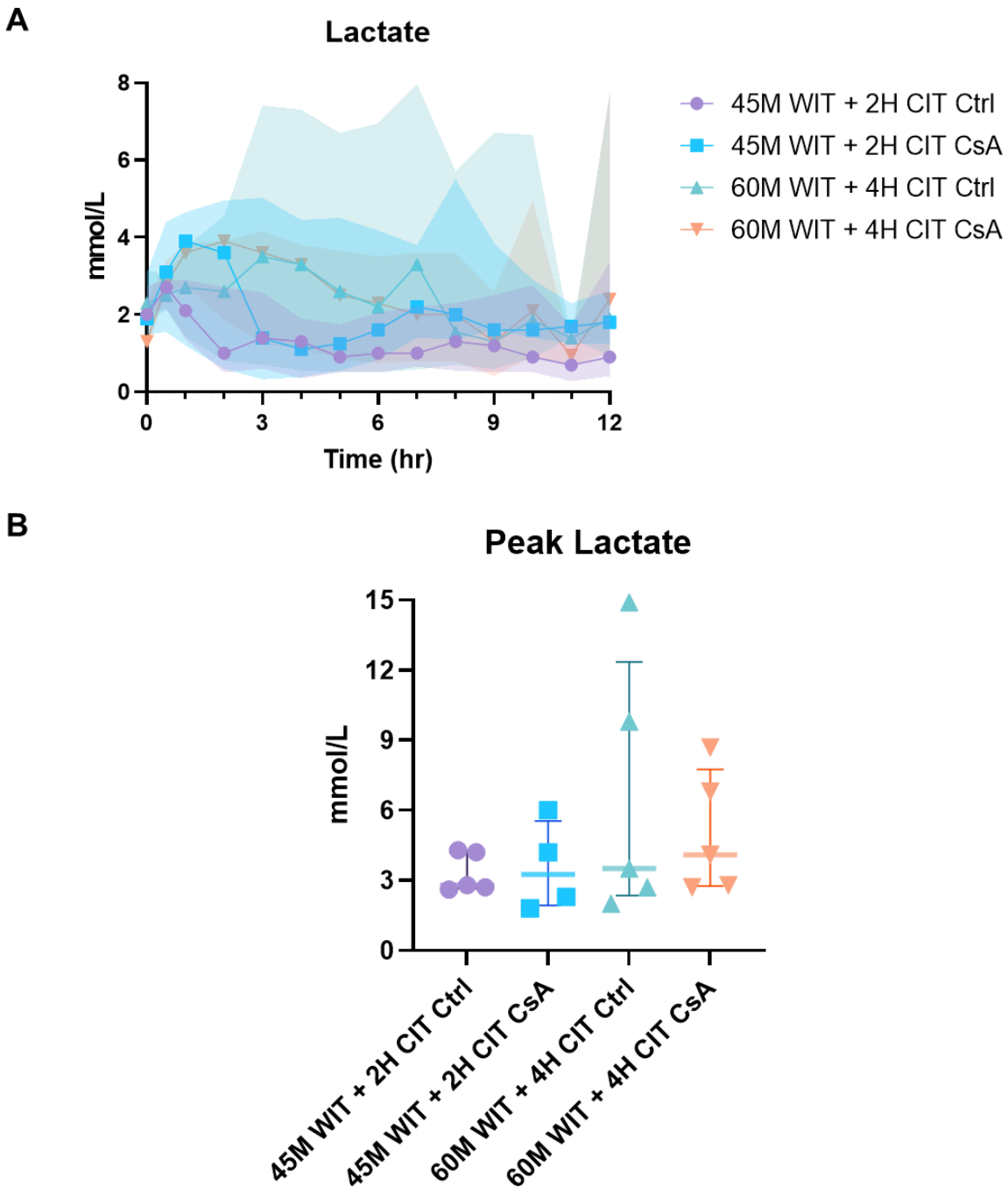

Figure 2.3.5. Perfusate lactate over the course of reperfusion (A) and at peak levels (B) for control (Ctrl) and treated (CsA) livers subject to 45 or 60 minutes of warm ischemia (45M WIT, 60M WIT) and 2 or 4 hours of cold ischemia (2H CIT, 4H CIT). Peak lactate was not significantly different between groups (p=0.656). CIT=cold ischemia time, WIT=warm ischemia time

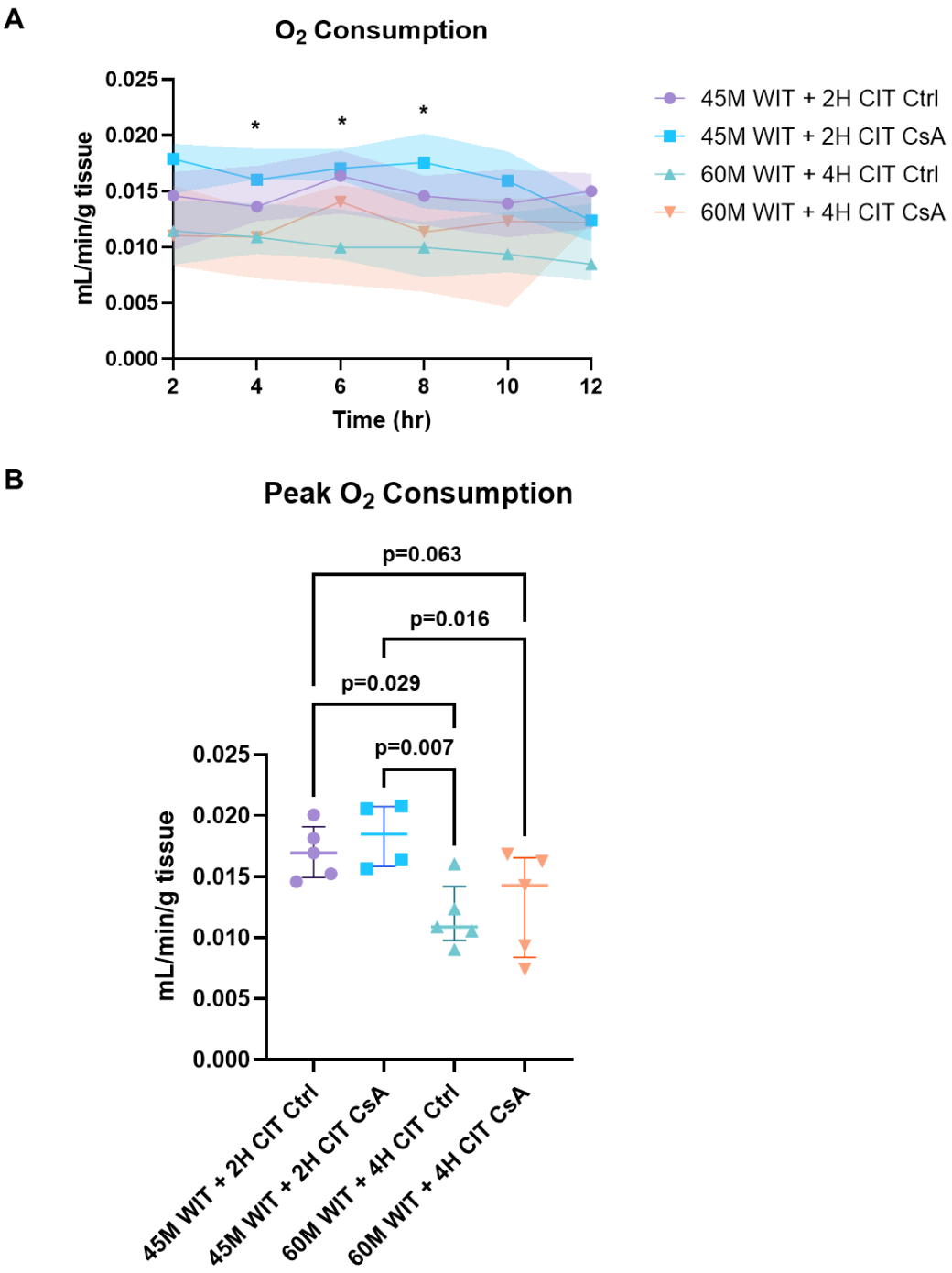

Figure 2.3.6. Oxygen (O_2) consumption over the over the course of reperfusion (A) and at peak levels (B) for control (Ctrl) and treated (CsA) livers subject to 45 or 60 minutes of warm ischemia control and CsA-treated groups with the same degree of ischemia (p=0.417, 0.689 for the lower

and higher levels of ischemia, respectively). (45M WIT, 60M WIT) and 2 or 4 hours of cold ischemia (2H CIT, 4H CIT). CIT=cold ischemia time; WIT=warm ischemia time

Histological Injury Score is Decreased at the End of Perfusion for Livers with Less Ischemic Injury

Total histological injury score was significantly lower for the lower ischemia groups after 12 hours of perfusion compared to the higher ischemia control (p=0.011, 0.027 for the lower injury control and treated groups, respectively). Histological sub-scores of sinusoidal congestion and necrosis were no different between groups at any of the time points. However, the vacuolization sub-score was significantly lower at 6 and 12 hours in the lower ischemia groups compared to the higher ischemia groups (p=0.019, <0.001 for 6 and 12 hours, respectively). Despite this, no difference in histological injury score was seen between control and CsA-treated groups at any of the time points for either degree of ischemia (**Figure 2.3.7**).

IL-10 is Increased in the Perfusate of Livers Treated with CsA After 45min of Warm Ischemia & 2h of Cold Ischemia

The anti-inflammatory cytokine IL-10 was significantly increased in the lower ischemia CsA-treated group (1608.92pg/mL [1228.61-2497.26]) compared to the lower ischemia control group and both of the higher ischemia groups (451.61pg/mL [386.26-548.88], 419.01pg/mL [364.35-484.14], 391.72pg/mL [270.34-527.34]; p=0.043, 0.042, 0.018 for lower ischemia control and higher ischemia control and treated, respectively). No difference was seen between groups in other inflammatory cytokines measured in the perfusate (**Figure 2.3.8**). Tissue glutathione,

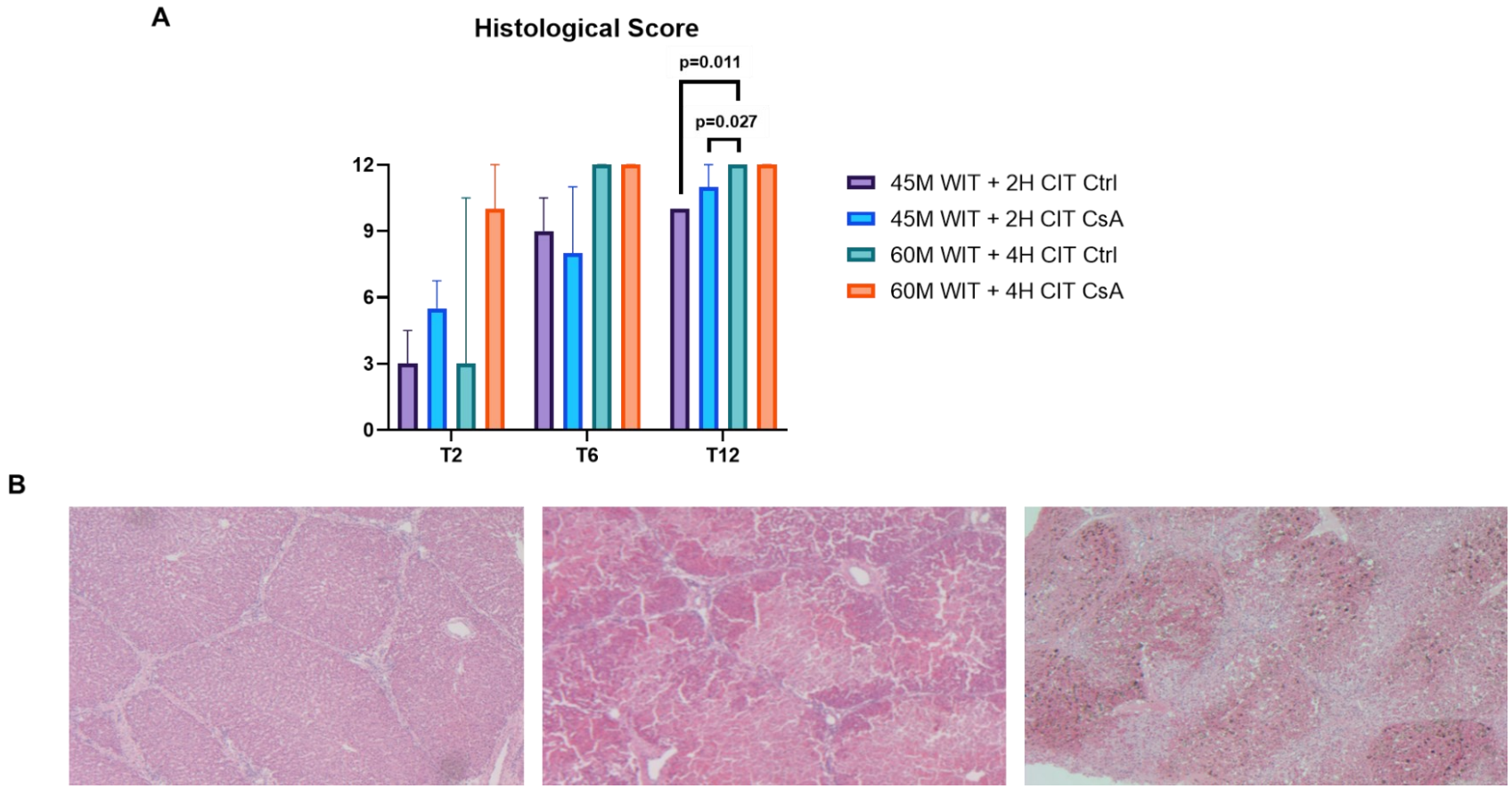

Figure 2.3.7. Histological injury scores for biopsies taken at 2 (T2), 6 (T6), and 12 (T12) hours (A) for control (Ctrl) and treated (CsA) livers subject to 45 or 60 minutes of warm ischemia (45M WIT, 60M WIT) and 2 or 4 hours of cold ischemia (2H CIT, 4H CIT). Histological injury scores were not significantly different at 2 or 6 hours (p=0.054 and 0.271, respectively). Representative images of minimal, moderate, and severe injury taken at 50x magnification (left to right, B). CIT=cold ischemia time; WIT=warm ischemia time

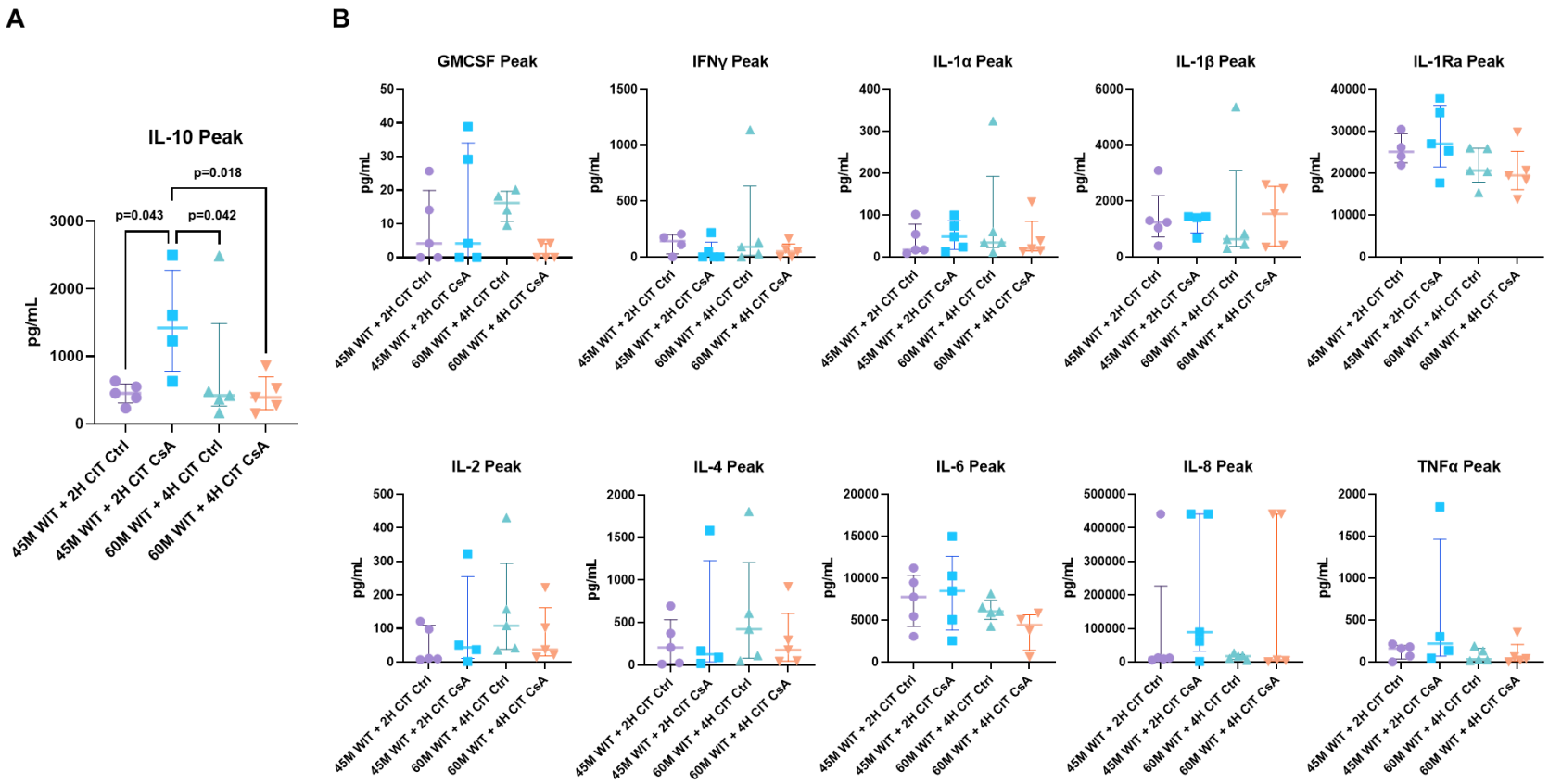

Figure 2.3.8. Peak levels of perfusate cytokines for control (Ctrl) and treated (CsA) livers subject to 45 or 60 minutes of warm ischemia (45M WIT, 60M WIT) and 2 or 4 hours of cold ischemia (2H CIT, 4H CIT). CIT=cold ischemia time; GMCSF=granulocyte macrophage colony stimulating factor; IFNγ=interferon gamma; IL=interleukin; TNFα=tissue necrosis factor alpha; WIT=warm ischemia time

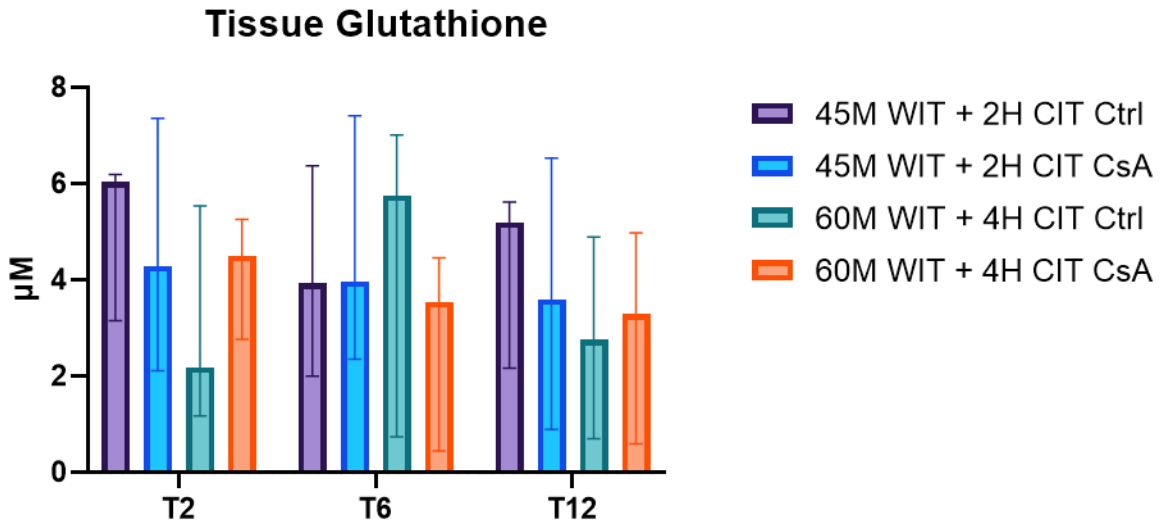

Figure 2.3.9. Tissue glutathione concentrations for liver biopsies taken at 2 (T2), 6 (T6), and 12 (T12) hours after reperfusion for control (Ctrl) and treated (CsA) livers subject to 45 or 60 minutes of warm ischemia (45M WIT, 60M WIT) and 2 or 4 hours of cold ischemia (2H CIT, 4H CIT). Glutathione concentration was not significantly different at any timepoint (p=0.709, 0.618, 0.486 at 2, 6 and 12 hours, respectively). CIT=cold ischemia time; WIT=warm ischemia time

measured as a marker of oxidative stress, showed no difference was seen between groups (**Figure 2.3.9**).

2.3.5 – Discussion

In the present study, we tested the effect of CsA on hepatic IRI in a novel porcine DCD model using *ex vivo* perfusion. We found minimal effect of CsA on reducing hepatic IRI at two different severities of ischemic injury. End pH and peak oxygen consumption were higher in the lower ischemia livers and histological score at 12 hours was lower in the lower ischemia livers compared to the higher ischemia control. However, these parameters were no different between control and treated livers within the same injury level. Only the peak LDH was lower in the CsA-treated group with the lower ischemia, while the anti-inflammatory cytokine IL-10 was higher, compared to the other groups.

CsA has shown promise as an agent to mitigate IRI in multiple preclinical studies, particularly in relation to cardiac ischemia.[18] Several preclinical studies of warm hepatic IRI have shown that pre-treatment with CsA can decrease serum transaminases several hours post-reperfusion.[8,9] Indeed, in our own studies, we found that mice treated with doses of CsA greater than 10mg/kg had decreased serum ALT, as well as decreased histological evidence of injury after 6 hours of reperfusion (manuscript submitted for publication). We additionally found a similar effect with the CsA analog, NIM-811, suggesting that the mechanism is unrelated to its immunosuppressive effect. Studies of the effect of CsA on warm hepatic IRI in porcine models have shown improved survival with pre-administration.[10] Less is known about the effect of CsA on liver IRI in the setting of combined warm and cold ischemia. The few rat liver transplantation

studies that exist suggest that CsA may improve survival if given to the recipient prior to transplant following up to 12 hours of SCS, but may be less effective if given to the donor only.[11,12]

There are several potential reasons why CsA was not effective at reducing IRI in our model. The dose of 20mg/kg was chosen based on a review of literature, including both hepatic and non-hepatic models.[18] The most common dose administered was 10mg/kg. A higher dose was chosen, as we observed that studies including porcine and/or female animals tended to reported weaker effects.[18] Indeed, the serum level of CsA at the time of hepatectomy was above the limit of detection at our clinical lab (5,000ng/mL) for all animals treated with CsA, so it is unlikely that dose was the issue. It may be that CsA is less effective in pigs compared to rats or less effective in female animals. Pigs were used in our model owing to their physiologic similarity to humans and, for reasons of size and husbandry, female pigs are more easily obtained in our setting (male animals are typically neutered by this age and size to limit their aggressiveness).[19]

It is possible that the duration of ischemia in our model was too long and that CsA would be effective with shorter ischemic times (either warm or cold ischemia). We chose warm ischemic times of 45 and 60 minutes to reflect the upper limits of what would be tolerated clinically (also considering that these are young, healthy animals). Knaak *et al.* published a similar DCD model employing 45 minutes of warm ischemia and 4 hours of SCS.[20] Our use of a whole blood-base perfusate in a normothermic setting (37ºC), rather than diluted filtered red blood cells at subnormothermic temperatures (33ºC) may explain why we observed a more severe injury with similar ischemic times. Perhaps still, CsA may be more effective in reducing injury from warm ischemia, rather than cold ischemia (or both as is contained in our model). A study by Tarrab *et al.* using a model of rat liver transplantation found that CsA given to the recipient was not

protective against IRI following prolonged SCS (24 hours).[12] This would certainly limits the utility of CsA in the setting of organ transplantation, given the obligate period of cold ischemia.

An additional limitation to our study includes that the liver was not transplanted into a live animal for reperfusion. Reperfusion in the *ex vivo* setting allows for a potentially longer reperfusion phase (particularly in an injury model) and is less labour-intensive (as transplantation of a large animal requires invasive monitoring and may not be recoverable). However, *ex vivo* reperfusion lacks the interaction with other organ systems that can also play a role in IRI.[21,22]

2.3.6 – Conclusions

Overall, our study found minimal benefit for pre-treatment with CsA to prevent IRI in a large animal DCD model. Despite some studies showing CsA to be protective in warm hepatic IRI, we did not find it to be beneficial preventing injury following combined warm and cold ischemia within the limitations of this model. However, our model is novel in its use of whole blood-based *ex vivo* perfusion to induce IRI in an isolated liver and may serve as a basis for testing other compounds that may be beneficial in preventing IRI in the setting of liver transplantation.

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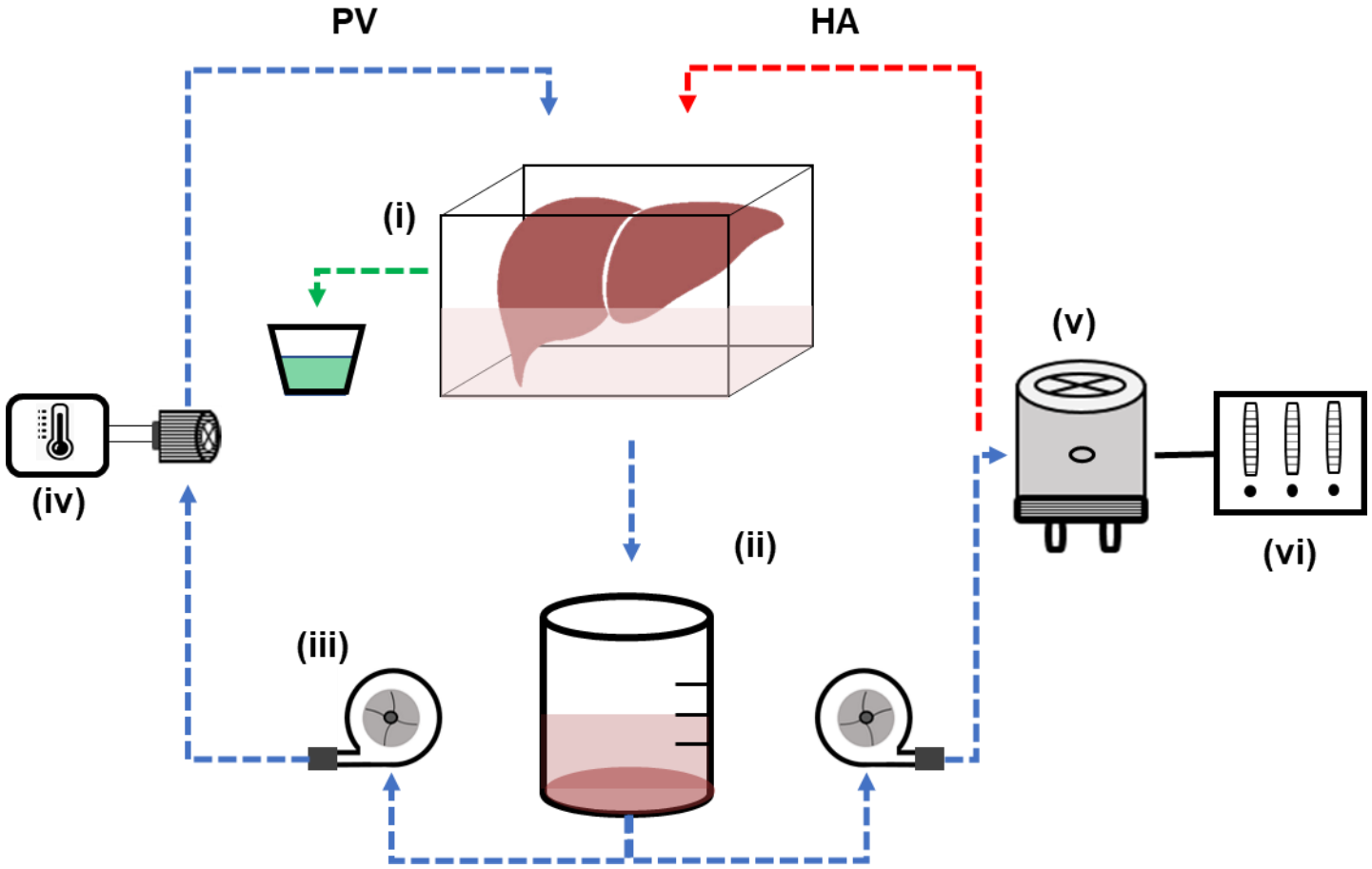
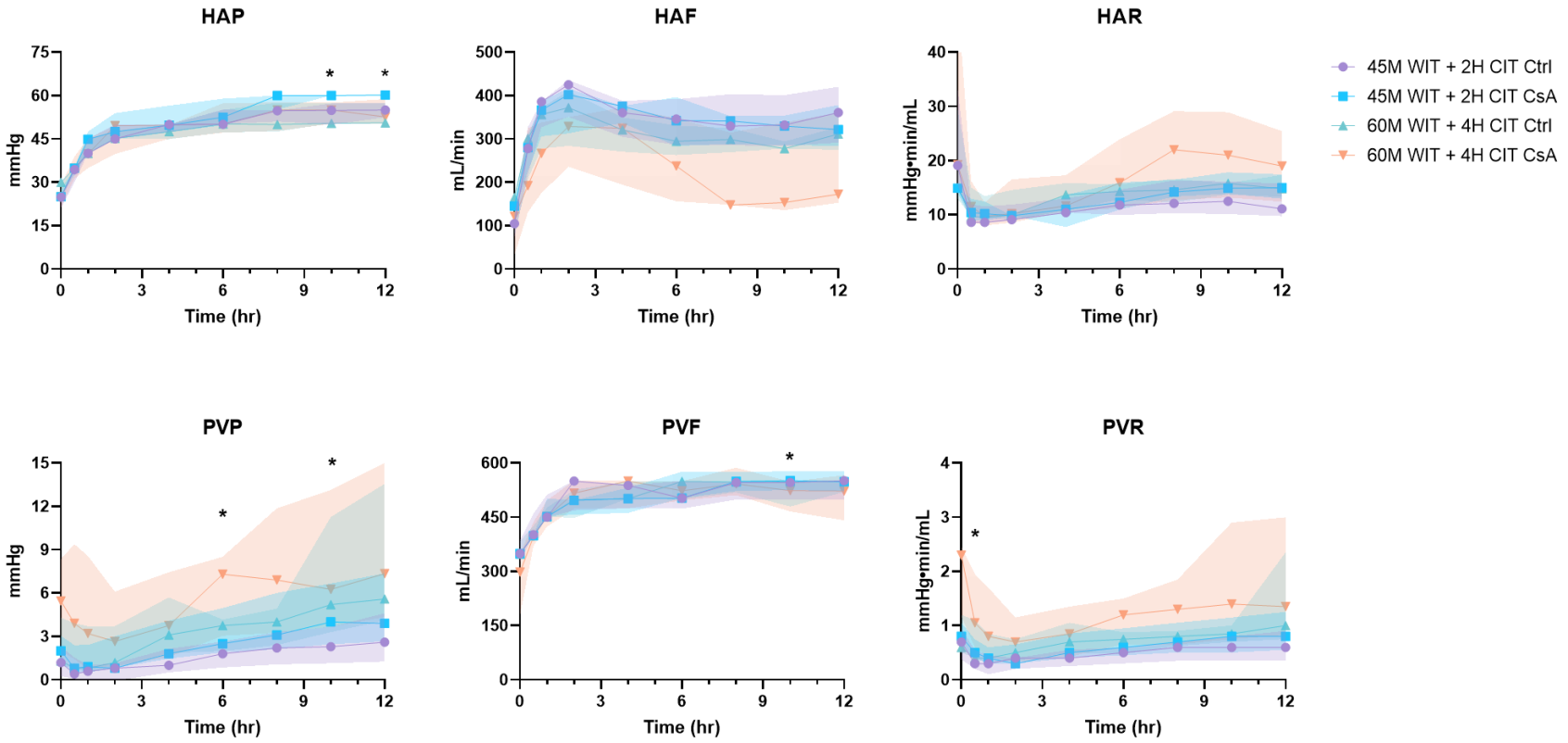
2.3.8 – Supplementary Material



Supplementary Figure 2.3.1. Liver *ex vivo* normothermic machine perfusion schematic. Reprinted with permission from Hefler *et al.* Biomedicines (2022) Oct 6;10(10):2496.



Supplementary Figure 2.3.2. Perfusion parameters for control (Ctrl) and treated (CsA) livers subject to 45 or 60 minutes of warm ischemia (45M WIT, 60M WIT) and 2 or 4 hours of cold ischemia (2H CIT, 4H CIT). HAP=hepatic artery pressure; HAF=hepatic artery flow; HAR=hepatic artery resistance; PVP=portal venous pressure; PVF=portal venous flow; PVR=portal venous resistance

CHAPTER 3 – USING NORMOTHERMIC MACHINE PERFUSION TO MODEL ACUTE LIVER FAILURE

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PART 1 - PRECLINICAL MODELS OF ACUTE LIVER FAILURE: A COMPREHENSIVE REVIEW

3.1 – Preclinical Models of Acute Liver Failure: A Comprehensive Review

Preclinical models of acute liver failure: a comprehensive review

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ABSTRACT

Acute liver failure is marked by the rapid deterioration of liver function in a previously well patient over period of days to weeks. Though relatively rare, it is associated with high morbidity and mortality. This makes it a challenging disease to study clinically, necessitating reliance on preclinical models as means to explore pathophysiology and novel therapies. Preclinical models of acute liver failure are artificial by nature, and generally fall into one of three categories: surgical, pharmacologic or immunogenic. This article reviews preclinical models of acute liver failure and considers their relevance in modeling clinical disease.

Subjects Biochemistry, Gastroenterology and Hepatology
Keywords Acute liver failure, Preclinical models, Surgical models, Toxicity models

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Additional Information and Declarations can be found on page 26

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INTRODUCTION

Acute liver failure (ALF) refers to the rapid deterioration of liver function in an individual without pre-existing liver disease within 26 weeks (*O'Grady, Schalm & Williams, 1993*). ALF is a challenging disease to study clinically due to its relative rarity and high mortality. Annual cases in the US are estimated to be in the range of 2,000 to 3,000 (*Stravitz & Lee, 2019*). A report of 1,147 cases by the US ALF study group found an overall mortality of 30% (*Lee et al., 2008*). Animal models are important for the study of such rare diseases, both for understanding pathophysiology and for development of potential treatments, as the lack of access to patients and severity of their illness makes clinical studies challenging.

The main considerations with using laboratory animals to model human disease are their appropriateness and generalizability. Animals that are closer to humans evolutionarily are considered more generalizable. However, rodent models, though less comparable to humans than some large animal models (*e.g.*, pigs and non-human primates) are more prevalent for economic, ethical and logistic reasons.

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Figure count: 3

References: 219

3.1.1 – Preface

Acute liver failure is marked by the rapid deterioration of liver function in a previously well patient over period of days to weeks. Though relatively rare, it is associated with high morbidity and mortality. This makes it a challenging disease to study clinically, necessitating reliance on preclinical models as means to explore pathophysiology and novel therapies. Preclinical models of acute liver failure are artificial by nature, and generally fall into one of three categories: surgical, pharmacologic or immunogenic. This article reviews preclinical models of acute liver failure and considers their relevance in modeling clinical disease.

3.1.2 – Introduction

Acute liver failure (ALF) refers to the rapid deterioration of liver function in an individual without pre-existing liver disease within 26 weeks (O'Grady, Schlam & Williams, 1993). ALF is a challenging disease to study clinically due to its relative rarity and high mortality. Annual cases in the US are estimated to be in the range of 2,000 to 3,000 (Stravitz & Lee, 2019). A report of 1,147 cases by the US ALF study group found an overall mortality of 30% (Lee et al., 2008). Animal models are important for the study of such rare diseases, both for understanding pathophysiology and for development of potential treatments, as the lack of access to patients and severity of their illness makes clinical studies challenging.

The main considerations with using laboratory animals to model human disease are their appropriateness and generalizability. Animals that are closer to humans evolutionarily are considered more generalizable. However, rodent models, though less comparable to humans than some large animal models (e.g., pigs and non-human primates) are more prevalent for economic, ethical and logistic reasons.

Broadly, artificially-induced preclinical ALF models can be grouped into surgical, pharmacological or immunogenic approaches (Table 3.1.1). Common techniques used in surgical models include total hepatectomy, major liver resection, temporary hepatic ischemia, and a combination of portacaval shunting with hepatic artery ligation (Argibay et al., 1996; de Groot et al., 1987; Makino et al., 2005; Yamaguchi et al., 1989). A range of pharmacological models have also been studied. Among the most widely used are acetaminophen (APAP), d-galactosamine (d-Gal), and carbon tetrachloride (CCl_4) (Apte, 2014; Frank et al., 2020; Mossanen & Tacke, 2015) (Table 3.1.2). Immunogenic models refer to models of viral hepatitis, as well as means of inducing hepatitis by activating cell death pathways, such as with anti-Fas antibodies (Bajt et al., 2000; Belz,

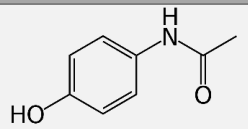
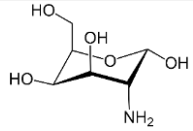
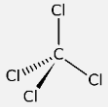
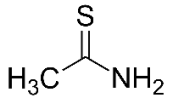
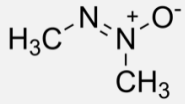
Table 3.1.1. Commonly used preclinical models of acute liver failure.

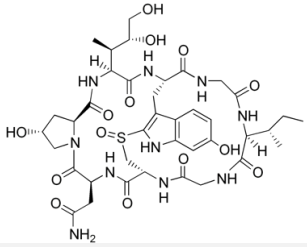
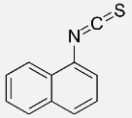
		Features	*Advantages*	*Disadvantages*
Surgical	Anhepatic	• Complete removal of liver • Single or multi-stage procedure	• Highly reproducible • Useful for testing liver support devices	• Irreversible • Lacks inflammatory response • Technically challenging in small animals
	Partial hepatectomy	• Resection of 70-97% of liver mass	• Direct clinical correlate • Useful for studies of hepatic regeneration	• Does not typically produce HE • Specific to post-hepatectomy liver failure
	Hepatic artery ligation & portocaval anastomosis	• One- or two-stage procedure	• Reliably produces progressive HE & coma	• Irreversible • No direct clinical correlate
Pharmacological	Acetaminophen	• Injury result of toxic metabolite (i.e. NAPQI)	• Direct clinical correlate • Dose dependent effect	• May require cytochrome P450 induction or glutathione depletion • Ineffective in rats • Complicated by methemoglobinemia in large animal models
	D-galactosamine	• Depletes uridine, interfering with RNA synthesis • Given with or without lipopolysaccharide	• Demonstrated efficacy in variety of small & large animal models	• No direct clinical correlate • Different histological pattern of injury from most hepatotoxins
	Carbon tetrachloride	• Produces highly reactive carbon-chloride radicals • Also used in chronic liver injury models	• Representative of generalized hepatotoxic injury	• Specific chemical not encountered clinically • Poor model for HE • Between species variability
Immunogenic	Concanavalin A	• Immunogenic lectin derived from jack bean	• Representative of T cell mediated hepatitis, relevant to autoimmune hepatitis clinically	• Batch-to-batch variability • Species & strain differences in susceptibility

	Fas antibody	• Binds & activates Fas cell death receptor	• Specifically induces cell death by apoptosis, an important mechanism in a variety of causes of ALF	• Also affects extra-hepatic tissues (e.g. spleen, thymus) • Limited experience outside of murine models • Strain specific differences in mice
	Viral	• Includes endemic, species-specific viruses & genetically modified viruses &/or viruses applied to genetically modified hosts	• Important cause of ALF clinically • Use of endemic murine or leporine viruses limits risk of transmission to research personnel	• Difficult to reliably induce ALF using human viruses
Other	Long-Evans cinnamon rat	• Defect in gene encoding copper-transporting P-type ATPase, homologous to defect seen in Wilson's disease	• Closely representative of Wilson's disease • ALF occurs spontaneously	• Not relevant to other causes of ALF

ALF: acute liver failure, HE: hepatic encephalopathy, NAPQI: N-acetyl-p-benzoquinone imine, RNA: ribonucleic acid

Table 3.1.2. Pharmacological models of acute liver failure.

	Structure	*Mechanism*	*Route of Administration*	*Animal Models*	*Dosage*
Acetaminophen		Alternative metabolism by cytochrome P450 leads to production of toxic metabolite NAPQI	PO, SC, IP, IV	Mouse, rat*, rabbit, dog, pig	200-900mg/kg (mouse)†
d-Galactosamine		Depletion of intracellular uridine stores necessary for RNA synthesis	IP, IV	Mouse, rat, rabbit, dog, pig, primate	400-1000mg/kg or 300-700mg/kg with 0.1mg/kg LPS
Carbon Tetrachloride		Cytochrome P450 metabolism produces highly reactive trichloromethyl & trichloromethylperoxy radicals	PO, IP, IV	Mouse, rat, rabbit, pig	0.5-2.5mL/kg
Thioacetamide		Metabolism by cytochrome P450 or FAD-containing monooxygenase into hepatotoxins TASO & $TASO_2$, which forms acetylimidolysine causing their denaturation	IP‡	Mouse, rat	200-1600mg/kg (bolus) or 200-600mg/kg daily x2-4d
Azoxymethane		Causes DNA alkylation leading to tumour formation in models of colorectal cancer, but mechanism not well characterized in hepatotoxic models	IP	Mouse	50 or 100mg/kg

A-Amanitin		Inhibitor of RNA polymerase II, disproportionately affecting cells with high metabolism	IP	Mouse, pig, primate	0.6mg/kg or 0.1mg/kg with 0.1µg/kg LPS
A- Naphthyl Isothiocyanate		Cholestatic injury caused by accumulation in cholangiocytes leads to impaired bile flow & eventually hepatocyte necrosis	PO	Mouse, rat	25-100mg/kg

PO: *per os*, IP: intraperitoneally, SC: subcutaneously, IV: intravenously, LPS: lipopolysaccharide

*relatively resistant, effective only at high doses

†repeated or continuous administration often required for large animal models

‡can be administered PO, but not typically done with acute models

2004). There are additional models for specific etiologies of ALF that fall outside of these categories, such as the Long Evans Cinnamon rat, which models ALF in the context of abnormal copper metabolism, similar to Wilson's disease seen in humans (Siaj et al., 2012).

This review provides an overview and summary of animal models of ALF reported in the literature to date, including a discussion of technical aspects. Our aim is to bring together these divergent approaches and expertise into a single resource. We hope that it will be useful to researchers new to the field, as well as those more experienced seeking to explore alternative approaches.

3.1.3 – Survey Methodology

A literature search was conducted of PubMed, MedLine and Web of Science using the search terms "acute liver failure" and "fulminant liver failure". Clinical studies were excluded from the search results. There was no restriction based on language. Models categorized by their method of inducing liver failure and representative articles were chosen for inclusion. Older literature reviews on the same topic were consulted to ensure key topics were not missed (Rahman & Hodgson, 2000; Filiponi & Mosca, 2001; Bélanger & Butterworth, 2005).

3.1.4 – Defining ALF

ALF is defined clinically as severe liver injury (evidenced by dramatically increased liver transaminases) with acute onset of hepatic encephalopathy (HE) and synthetic liver dysfunction, marked by elevated international normalized ratio (INR; typically $\geq$1.5), in a previously well individual without pre-existing liver disease (Stravitz & Lee, 2019). The timeline for the development of ALF is less than 26 weeks (Stravitz & Lee, 2019). However, ALF may be

subdivided into hyperacute (<7 days), acute (7 to 21 days), and subacute (21 days to 26 weeks) (Stravitz & Lee, 2019).

Definitions of ALF in animal models are less precise (Fig. 3.1.1). The main criterion is often simply elevation of transaminases. Coagulopathy is not often assessed, especially in small animals, where blood volume is prohibitive and other pathophysiological aspects are usually being investigated concomitantly. There are scoring systems for HE, even in small animals, but these are often only reported when HE is the focus of the study (Butterworth et al., 2009). In contrast to the clinical presentation, where tissue biopsies are not routinely taken, histology takes greater emphasis in animal studies, as it can demonstrate the expected findings of hepatic necrosis (Mitchell et al., 1973a). Lethality is also often reported as evidence of sufficient injury, though current animal ethical guidelines counsel strongly against survivability or death as a primary study endpoint (Wang et al., 2000).

The timeline for ALF in animal models is shorter than in humans. Generally, in studies reporting models of ALF, liver failure develops over hours to days after a single inciting event. This can be justified as the inciting event is known, whereas clinically (except in cases of acute overdose) patients may present with progressive worsening over days and weeks. Laboratory animals also have shorter lifespans. For instance, the median lifespan of laboratory mice is 28 months, so applying the clinical definition of 26 weeks would represent over 20% of the animal's life. Additionally, most aspects of rodent biology, including metabolic rate, and RNA and protein turnover, are several times that of humans (Agoston, 2017). This is especially true for more complex biological processes. For instance, in rodent models of chronic liver injury, fibrosis develops in as little as 4 weeks, whereas cirrhosis in humans takes years (Schuppan & Afdhal, 2008; Yanguas et al., 2016).

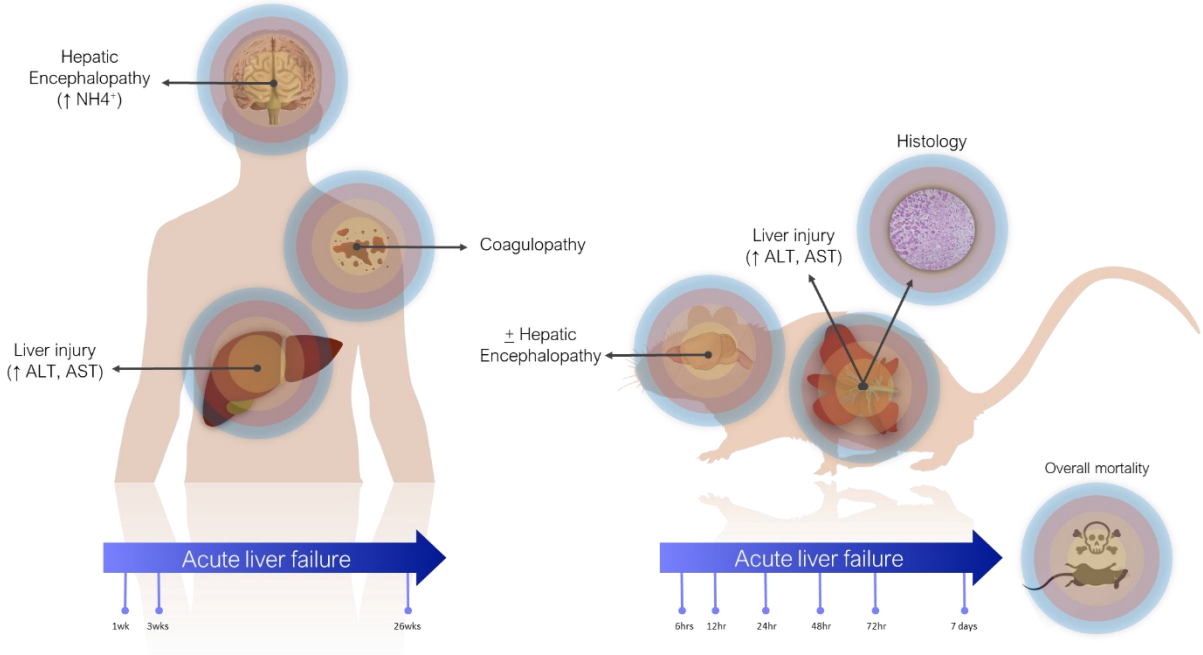


Figure 3.1.1. Differences between clinical and preclinical models of acute liver failure.

In actuality, the majority of animal models purported to represent ALF may correspond more closely with acute liver injury (ALI) (Koch et al., 2017). Clinically, ALI is a less precisely defined term, generally referring to elevated transaminases, possibly with synthetic dysfunction, but without acute HE. Still, both exist on the same spectrum of injury, with ALF at the more severe end, and it is reasonable to expect that treatments effective against ALI would also work against ALF by the same cause (though perhaps to a lesser degree).

3.1.5 – Animal Selection

Animal models of ALF are uniformly mammalian. Though other liver diseases have been studied in lower vertebrates, they are less suitable to model complex clinical syndromes, such as ALF. The most common animals used are mice and rats, which have obvious economic and logistic advantages. However, rodents can differ from animals with closer evolutionary relationships to humans, particularly with regards to inflammatory and metabolic responses (Seok et al., 2013). For instance, hepatic metabolism by cytochrome P450 (CYP450) in humans has been shown to more closely resembles dogs than rats (Nishimuta et al., 2013). This concern is not unique to animal models of ALF. There are many examples of therapies that work well in rodents, but failed to be translatable to humans. A systematic review including 76 highly-cited (>500 citations) animal studies found that only 37% were replicated in clinical trials (Hackam & Redelmeier, 2006).

Pigs have emerged as the large animal model of choice for liver disease. They share anatomical and physiologic similarities to humans and are a more socially acceptable alternative to dogs or non-human primates, as well as being more accessible than primates. Rabbits have a specific role for the study of viral hepatitis, as there exists a viral disease specific to rabbits (i.e.,

rabbit haemorrhagic disease) that is characterized by acute necrotizing hepatitis and is typically fatal within 72 hours (Abrantes et al., 2012). Small primate models, such as mouse lemurs, are being considered by some as a way to maximize the ratio between fidelity and cost, but have not yet been adapted to the study of ALF (Ezran et al., 2017).

Humanized models, in which immunodeficient mice are engrafted with human cells or tissues, are being used increasingly, particularly in pharmacologic and immunologic studies (Allen et al., 2019). In the case of mice with humanized liver, human hepatocytes are transplanted into immunodeficient mice, resulting in the replacement of over 70% of the native mouse liver (Strom, Davila & Grompe, 2010). There are reports of these mice being used to study ALF. Torres et al. (2019) described a model of APAP-induced ALF in mice with humanized livers, but found that the mice were less susceptible to hepatotoxicity than their wildtype counterparts. This may reflect the importance of other factors, such as an appropriate immune response, in producing the full syndrome of ALF.

In addition to species considerations, several criteria have been proposed for ideal animal models of ALF. The first and most recognized were put forth by Terblanche and Hickman (1991). Among these, they emphasized the importance of reversibility (i.e., that a treatment intervention could lead to survival), reproducibility (often judged by the following criteria), consequent death (from liver failure and not secondary causes), and a therapeutic window for intervention (necessitated by the first criteria). Consciousness has also been suggested to be important for assessment of HE, but may not be possible for ethical reasons (Bélanger & Butterworth, 2005).

3.1.6 - Surgical Models of ALF

Some surgical models, such as portacaval anastomosis (PCA) with hepatic artery ligation and partial hepatectomy with ischemia or portacaval shunt, seek to create a generalized model of ALF, whereas other surgical models are aimed at specific causes of ALF (e.g., post-hepatectomy or ischemia).

Anhepatic Models

Anhepatic models have been described in rats, rabbits, dogs, and pigs (Joyeux et al., 1977; Yamaguchi et al., 1989; Nyberg et al., 1993; Frühauf et al. 2004). Both single and multi-stage procedures have been reported. With the former, it is necessary to reconstitute flow between the infrahepatic inferior vena cava (IVC) and the portal vein, caudally, and the suprahepatic IVC cranially. This can be done using a simple shunt or anastomosis with a prosthetic graft (e.g., polyethylene terephthalate or polytetrafluoroethylene) (Arkadopoulos et al., 1998; Filipponi et al., 1999; Frühauf et al. 2004). Multi-stage procedures were developed specifically for use in rats. A two-stage procedure was described by Rozga et al. (1992). In the first stage, atrophy of the posterior lobes (right and caudate lobes) is induced by ligation of posterior portal branches. After two weeks, in the second stage, the hypertrophied anterior lobes (median and left lobes) are resected, a portacaval anastomosis is created, and the hepatic artery is ligated (fully devascularizing the atrophied posterior lobes). The atrophy of the posterior lobes, which are normally adherent to the IVC, obviates the need for vena cavo-caval anastomosis. This reduces technical complexity and associated mortality, which can be significant in small animal models. Three-stage techniques have also been described earlier in the literature, in which ligation of the IVC above the renal veins is carried out in the first stage (Holmin, Alinder & Herlin, 1982). Two

months later, after adequate collateral circulation has developed, a portacaval anastomosis and total hepatectomy are carried out in separate operations.

While anhepatic models produce the purest form of liver failure in some sense, they lack a direct clinical correlate. The only time a patient is without a liver is in the anhepatic phase between hepatectomy and graft implantation during a liver transplantation, which typically lasts for little over an hour (IJtsma et al., 2009). While absence in the liver's synthetic function is readily apparent in such models, they lack features of systemic dysfunction (e.g., acidemia, elevated lactate) that accompany clinical cases of ALF, caused by hepatocyte death.

Anhepatic models also lack reversibility. They have been used in metabolic studies, as well as studies of HE. They are unsuitable for most therapeutic studies, though may provide an advantage in studies of extracorporeal liver support (e.g., bioartificial livers [BALs]) due to the consistency of absolute hepatic insufficiency. Because of the lack of systemic dysfunction, they do have a longer survival time. In a direct comparison between models, Frühauf et al. (2004) found an average survival time of 17.1 hours in an anhepatic porcine model, compared to 9.8 hours in a devascularized model.

Partial Hepatectomy

An alternative to total hepatectomy are major liver resection models, which generally range from 70% to 95% resection of the liver mass. The lower end corresponds to the 20-30% recommended to avoid post-operative liver failure after hepatic resection of an otherwise healthy liver (Guglielmi et al., 2012). This has been reported in mice, rats, rabbits, pigs, and baboons.

The rodent liver has four lobes: right, median, left, and caudate (Rogers & Dintzis, 2018). Nomenclature is expectedly imprecise, particularly in older literature. Both the right and caudate

lobes have two distinct segments, which are nearly bisected by a transverse septum. The two segments of the right lobe may be labeled superior and inferior or anterior and posterior. The caudate lobe has also been referred to as the omental lobe. In some studies, the inferior segment of the right lobe is referred to as the caudate lobe, while what is otherwise known as the caudate lobe is referred to as the quadrate lobe and papillary process (Longo et al., 2005; Abe et al., 2009). The median lobe of the rat is reliably bilobed and may be referred to separately as right and left median lobes (usually with the descriptor 'lateral' added to the right and left lobes) (Rogers & Dintzis, 2018). Notable differences between the mouse and rat livers are the relatively larger size of the left lobe in the former and the lack of a gallbladder in the latter (Fig. 3.1.2).

Livers of other commonly used experimental animals are similarly lobulated (Vons et al., 2009; Nykonenko, Vávra & Zonča, 2017; Stan, 2018). Nomenclature used is also similar to rodents. The livers of rabbits, dogs, and pigs are generally described as having six lobes (Budras et al., 2007; Swindle, 2016; Stan, 2018). In these animals, the equivalent of the median lobe is divided into distinct right and left lobes and a small quadrate lobe is found inferior to them, to which the gallbladder is partially attached. Right and left lateral and caudate lobes appear in a similar position as rodents. Studies on the surgical anatomy of the liver in non-human primates is limited. A detailed study in the cynomolgus monkey (*Macaca fasciularis*) described four lobes as in rodents (Vons et al., 2009). Despite differences in external appearance, the same internal segmentation is consistent between species (Kogure et al., 1999; Nykonenko, Vávra & Zonča, 2017).

In both the mouse and rat, resection of the median and left lobes is generally referred to as 70% hepatectomy (sometimes 2/3), while 90% hepatectomy adds resection of the right lobe (Bustos et al., 2003; Makino et al., 2005). Some studies report more precise percentages based on

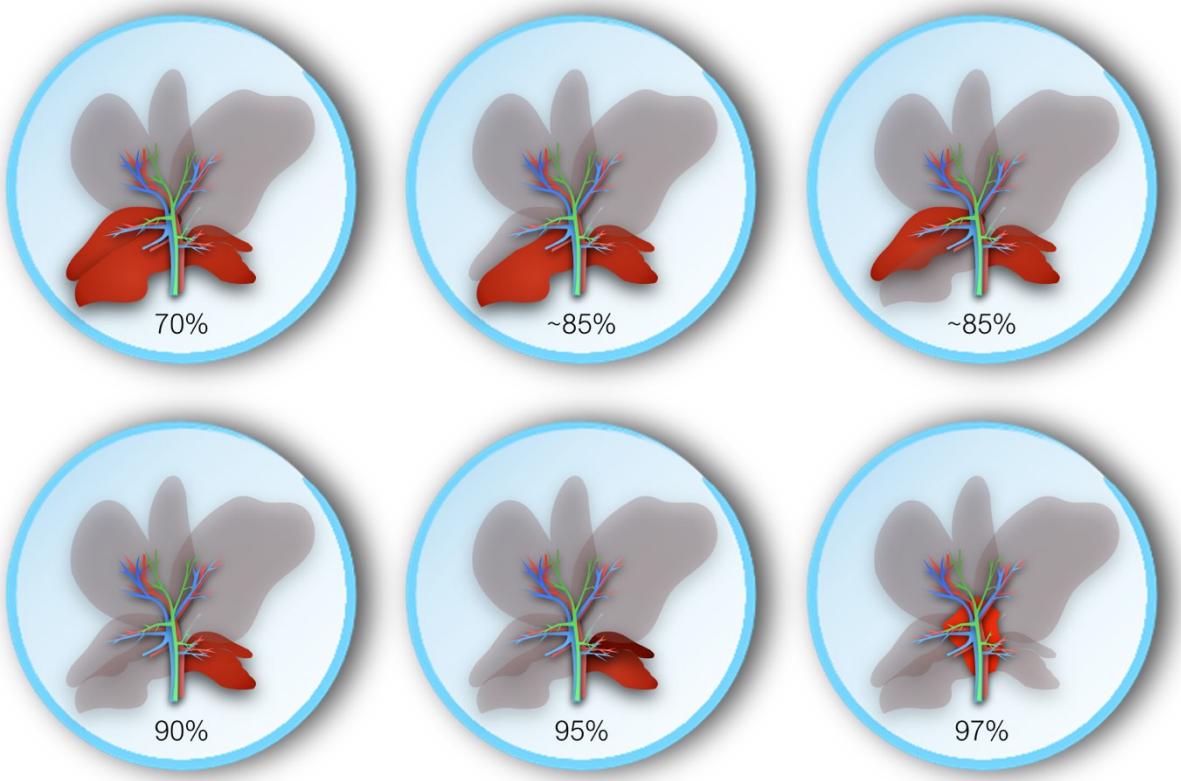


Figure 3.1.2. Lobes resected in different murine models of post-hepatectomy liver failure.

measured volumes, which may vary slightly between species and strains (Inderbitzin et al., 2006; Lehmann et al., 2012). Alternative approaches describe sparing only the superior or inferior segment of the right lobe (variably reported as 80-87% hepatectomy) (Ogata et al., 2008; Ohashi et al., 2013). A 95% hepatectomy has also been described, which includes resection of all lobes except the inferior segment of the caudate, and even a 97% hepatectomy where all lobes are resected and the only pericaval hepatic tissue (at the base of the resected lobes) remains (Madrahimov et al., 2006). Resection in rodents is best achieved by ligating portal structures separately, rather than together with hepatic parenchyma, as this avoids venous outflow obstruction (Makino et al., 2005). Figure 3.1.3 illustrates the different degrees of hepatic resection employed in models of ALF following partial hepatectomy.

Though 70% hepatectomy has been described by some studies as a model of ALF, it may be more suitable for regenerative studies of partial hepatectomy. A study in mice found 100% survival at one week following 70% hepatectomy, whereas 90% hepatectomized mice all died within 24 hours (Makino et al., 2005). Evidence of regeneration was also seen by 10 hours in the 70% group, but not in the 90% group. A similar study in rats likewise found improved hepatocyte regeneration in rats with 70% compared to 90% hepatectomy (Meier et al., 2016). 90% hepatectomized rats also showed evidence of coagulopathy and higher elevation in transaminases more consistent with ALF.

Pagano et al. (2012) found that more extensive hepatic resection was required to produce liver failure in pigs. Even with resection of left lateral, left and right median, and half of the right lateral lobe (~80% hepatectomy), only 65% of the animals in their study developed liver failure, whereas none of the animals with lesser resections did. 90% hepatectomy models have also been

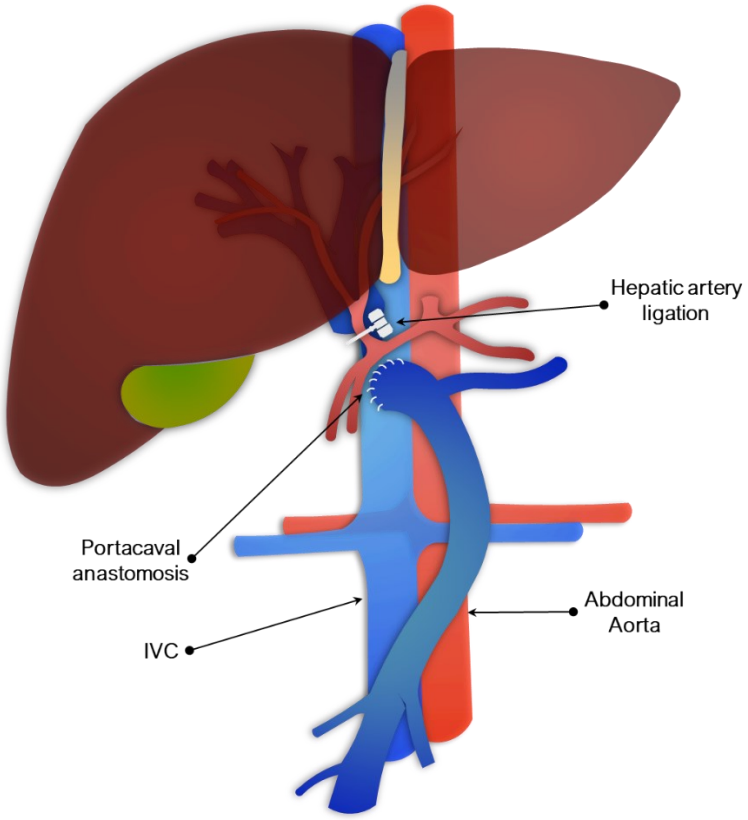


Figure 3.1.3. Portocaval anastomosis and hepatic artery ligation as a model of acute liver failure.

reported in rabbits, pigs and baboons (Berlinger et al., 1987; Asencio et al., 2017; Navarro-Alvarez et al, 2018). As in rodents, these models are based on resection of all lobes except for the caudate. Variations of major liver resection models have also been described. These can be used to emphasize a particular aspect of ALF that may be lacking or at least less apparent in models of partial hepatectomy alone. A model of 70% hepatectomy plus ischemia to remnant or right lobe only has been described in rats, rabbits, pigs, and baboons (Eguchi et al., 1996; Hung et al., 2007; Arkadopoulos et al., 2011; Machaidze et al., 2017). The addition of ischemia induces liver necrosis in the remnant lobes, which is otherwise lacking from models of partial hepatectomy alone. This adds a systemic inflammatory response that more closely parallels many clinical cases of ALF. The addition of common bile duct (CBD) ligation to partial hepatectomy has been also described as a means of reliably inducing jaundice, though this is obstructive and not hepatocellular in nature (Moharib et al., 2014). Likewise, PCA has been added to reliably induce HE, which may be absent or at least not readily apparent in animal models of partial hepatectomy, especially given the briefer course of disease in comparison to clinical cases (Filipponi et al., 1991). A less commonly used model, reported by Baimakhanov et al. (2016), employed a combination of partial hepatectomy and radiation-induced liver damage (50Gy) to the remaining lobes. The addition of radiation inhibits proliferation of remaining hepatocytes.

Portocaval Anastomosis & Hepatic Artery Ligation

Another commonly used surgical model involved PCA plus hepatic artery ligation. This has been described in rats, rabbits, dogs, and pigs, though not in mice, presumably because it is made more impracticable by their size (Fick, Schalm & de Vlieger, 1987; Bonato et al., 1989; Ash et al., 1993; Argibay et al., 1996). PCA may be done at the same time as arterial ligation or separately.

The two-stage procedure is used to reduce mortality and typically consists of the portacaval anastomosis, followed by hepatic artery ligation 48 to 72 hours later. The second stage may alternatively be done by placing slings around the hepatic artery and bile duct at the first operation, which are then exteriorized and later tied off to induce global liver ischemia (Khalili et al., 2001). Portacaval shunting alone (sometimes referred to as an Eck fistula) has been used to study HE, but is insufficient to fully model ALF (Starzl, Porter & Francavilla, 1983). As with total hepatectomy models, PCA and hepatic artery ligation result in irreversible loss of entire liver function. However, leaving the liver *in situ* adds physiological effect of hepatic necrosis seen clinically. As with total hepatectomy models, these models of complete devascularization are not reversible, but have utility in the study of HE or assessment of BALs.

To add reversibility, models of temporary ischemia have been described in dogs, and pigs (Horák, Horký & Ruzbarský, 1980; de Groot et al., 1987). In pigs, temporary ischemia of 4 to 14 hours in duration by occlusion of the hepatic artery has been reported, with or without occlusion of the CBD, following PCA. De Groot et al. (1987) reported 50% mortality and 15-67% hepatic necrosis with 4-hour occlusion compared to 85% mortality and 49-75% necrosis with 6 hours of ischemia. Fourneau et al. (2000) tested increasing ischemic times from 6 to 14 hours and found 100% mortality in 5 animals subjected to 10 hours of ischemia, with occlusion of the CBD. Animals without CBD occlusion, however, were able to survive up to 14 hours of ischemia.

3.1.7 – Pharmacological Models of ALF

Acetaminophen

Acetaminophen (APAP) is a commonly used pharmacological model. This reflects the fact that it is the most prevalent cause of ALF in North America clinically and is readily available as

an over-the-counter medication (Rubin et al., 2018). The mechanism of APAP toxicity has been well studied. Briefly, toxicity occurs when excessive doses of APAP cause metabolism through a secondary pathway mediated by the CYP450 family of enzymes. This pathway produces the hepatotoxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI), which is thought to induce toxicity chiefly through formation of mitochondrial protein adducts and generation of reactive oxygen species (Ramachandran & Jaeschke, 2019). Glutathione (GSH) is an antioxidant molecule found in nearly all eukaryote cells, which is capable of detoxifying NAPQI. This is the principle upon which treatment with N-acetylcysteine (NAC) is based. NAC is hydrolyzed to cysteine in cells, providing the rate-limiting substrate necessary for GSH synthesis (Heard, 2008).

Reduction of GSH stores by approximately 80% is necessary for hepatotoxicity to occur (Mitchell et al., 1973a). Sensitizing agents have been employed to this end. One of the most commonly employed is phenobarbital, which works by inducing CYP450 (Miller et al., 1976). Another CYP450 inducer, 20-methylcholanthrene (20-MC), has also been reported to be effective (Rahman, Selden & Hodgson, 2002). An alternative strategy is inhibition of GSH synthase, such as with buthionine sulfoximine (BSO), which has been shown to decrease GSH stores to 15-20% when given at 2mg/kg two hours prior to APAP administration in mice (Kelly et al., 1992).

APAP toxicity models have been described in mice, rabbits, dogs, and pigs (Francavilla et al., 1989; Rahman, Selden & Hodgson, 2002; Newsome et al., 2010; Mossanen & Tacke, 2015). It can be administered intravenously (IV), intraperitoneally (IP), subcutaneously (SC), or orally (PO). IP is preferred for mice, whereas IV or SC is more commonly used for large animal models. PO reflects what is seen clinically, but has the disadvantage in animal models of slower onset and more variable absorption. Though an IV formulation exists for human use, the aqueous solubility of APAP is suitable for animal experiments, where prolonged storage is not required, with most

protocols using normal saline (NS) or phosphate buffered saline (PBS) as the diluent prior to administration (Chiam et al., 2018). Less commonly, the prodrug of APAP, propacetamol, can be used at twice the dose (Tsai et al., 2015). The animal is typically fasted overnight prior to administration, regardless of whether anesthesia is used, as this is reasoned to ensure comparable levels of GSH between animals (Vogt & Richie, 1993). Because of the depletion in GSH that results, fasting also serves to exacerbate hepatotoxicity caused by APAP (Walker et al., 1982).

The relative resistance of rats to APAP-induced hepatotoxicity has been described in early literature (Mitchell et al., 1973b). McGill et al. (2012) compared mechanisms of APAP-toxicity directly between mice and rats. They found less depletion of GSH in rats, despite higher doses (1g/kg vs. 300mg/kg IP), and concluded that this was the result of lower rates of mitochondrial protein binding. Despite this, several studies report the use of rats as an APAP-toxicity model with doses ranging from 400mg/kg IP to 5g/kg PO (Panatto et al., 2011; Mahmoud et al., 2016). The lower toxicity of APAP in rats may be offset by combining it with other forms of acute (partial hepatectomy) or chronic (non-alcoholic steatohepatitis) hepatic injury (Kučera et al., 2012; Sahay et al., 2019).

Mice are the most common animal model used for APAP-induced ALF. Doses range from 200-900mg/kg IP, given as a single bolus. PO is less commonly used, though dosing is similar, as APAP bioavailability of enteral APAP is close to 90%, with several studies using 300mg/kg (McGill et al., 2012). Most studies using higher doses (≥500mg/kg) report mortality >90% within 48 to 72 hours. Lv et al. (2019) found 95% mortality at 48 hours using a dose of 900mg/kg.

Several studies have directly compared different doses. Barman et al. (2016) found all mice (C57 BL/6 ♂, aged 8-10 weeks) survived to 5 days with a dose of 200mg/kg, whereas only 75% survived with the 350mg/kg dose, and all mice given 400mg/kg died within 80 hours. Serum

transaminases and histology were consistent with ALF at the highest dose, with levels of alanine aminotransferase (ALT) greater than 5,000 U/L and close to 50% necrosis at 12 hours. Ham et al. (2015) using essentially the same strain of mice (C57 BL/6 ♂, aged 9-10 weeks) found 100% survival at 3 days with 400mg/kg, 70% survival with 500mg/kg, and 100% mortality with 600mg/kg. Transaminase levels were reported at 48 hours, so were considerably lower (just under 600 U/L). Shen et al. (2018) compared 3 doses of APAP (again using male C57 BL/6 mice) and found similar levels of serum transaminases at 24 hours with 300 and 500mg/kg doses (>5,000 U/L). However, 180mg/kg produced only minor increases. Monitoring levels at various time points, they found that serum transaminases peaked at 6 hours post injection and remained elevated at similar levels up to 24 hours. By 48 hours, levels had returned to normal.

Another important factor in these models is the use of the same sex. Studies tend to use male mice, as females have been found to be less susceptible to APAP-induced hepatotoxicity (Du et al., 2014). Pharmaceutical CYP450 induction or GSH depletion may be used, but is not required in mice and appears less frequently in the literature compared to other animal models. GSH depletion by overnight fasting is frequently employed.

Rabbits are less widely used compared to mice. Rahman et al. (2002) developed a model consisting of five doses of 500mg/kg SC over 24 hours. Both GSH depletion with BSO and CYP450 induction with 20-MC were found to be necessary to achieve hepatic necrosis**.** However, they were not successful using phenobarbital with BSO or BSO alone with these doses of APAP.

While mouse models are not typically anesthetized, modern protocols using large animal models maintain sedation for the duration of the experiment to avoid undue suffering to the animal and allow for invasive monitoring. An early study by Henne-Bruns et al. (1988) described the challenges of developing a porcine model. They reported that at lower doses (500-1,000mg/kg),

though hepatocyte necrosis was seen on histology, all animals recovered, whereas higher doses (1,000-2,000mg/kg) resulted in rapid death due to methemoglobinemia. Gazzard et al. (1975) found a similar problem in canines. Methemoglobin levels exceeded 40% in animals given 0.75 and 1g/kg IP. Given PO at doses of 1g/kg all animals died, but only two-thirds showed elevated transaminases and only one had evidence of hepatic necrosis on autopsy.

Treatment of methemoglobinemia, such as with methylene blue, has not been used to address this problem. Instead, most large animal models have moved to using continuous or multiple doses of APAP. Francavilla et al. (1989) succeeded in developing a reproducible canine model, using 750mg/kg SC, followed by two doses of 200mg/kg at 9 and 24 hours. However, it has not been replicated in recent literature, as dogs have largely fallen out of favor as a large animal model.

Two porcine models of APAP toxicity have emerged in the current literature**.** Newsome et al. (2010) describe a model in which APAP is administered IV. Animals were pretreated with 20mg of phenobarbital for 5 days prior to the experiment. On the day of study, pigs were bolused with 0.1875g/kg of APAP, followed by a 12-hour infusion of 1.8mg/kg/min, adjusted to achieve a blood APAP concentration between 200 and 300mg/L. Experiments lasted up to 28 hours, with 2 of the 9 treated animals surviving, 5 dying of multiorgan failure or sepsis, and 2 succumbing to respiratory failure induced by methemoglobinemia. Aspartate aminotransferase (AST) levels reached the several hundreds, and factors V and VII showed a significant decrease, though increased intracranial pressure (ICP) was not seen. Animals developed varying degrees of necrosis, with 6 showing at least moderately severe liver injury and 3 having only mild injury. Of note, the point of care (POC) assay used in this study to maintain the APAP levels has been shown to be unreliable and is no longer commercially available (Egleston et al., 1997). We were unable to find any quantitative POC tests for APAP on the market today.

A second porcine model of APAP-induced ALF has been described by Thiel et al. (2011). In their study, APAP was administered via nasojejunal tube, first as a 250mg/kg bolus, then 1-3g/hr to maintain APAP plasma levels between 300-450mg/L until ALF was achieved (as judged by impaired coagulation). The method of APAP measurement was not reported. They found 100% mortality by 30 hours (no control group was provided for comparison). AST levels rose into the several hundreds and coagulopathy was demonstrated, with an average INR of 5.7 by the end of the study. They also reported ICP elevated to >30mmHg. Expected findings of centrilobular necrosis were confirmed on histology. Both porcine models required maintenance under anesthesia and continuous monitoring, as well as adjustment of APAP levels, which makes these models expensive and challenging to reproduce.

Yu et al. (2015) attempted to develop a primate model of APAP toxicity in *M. fasicularis*, but found the animal to be resistant to hepatotoxicity up to doses of 900mg/kg per day for 14 days. Only minor, sporadic increases in liver transaminases were seen in the macaques, despite achieving a blood concentration 3.5 times that associated with liver toxicity in humans.

D-Galactosamine

D-galactosamine (d-Gal) is another commonly used pharmacological agent for inducing ALF in animal models. D-Gal is an amino sugar derived from galactose, otherwise found as a component of cellular glycoproteins and glycoprotein hormones, such as luteinizing hormone and follicle stimulating hormone (Apte, 2014). It causes toxicity by depleting intracellular stores of uridine – a necessary component of RNA synthesis – through metabolism in the Leloir pathway, which results in the accumulation of UDP-galactosamine. This also deprives the cell of uridine derivatives, UDP-glucose and UDP-galactose, necessary for glycogen synthesis. It has further

been shown to activate Kupffer cells, leading to the release of proinflammatory cytokines and neutrophil infiltration (Yang et al., 2016). D-Gal characteristically results in diffuse rather than zonal necrosis seen with most hepatotoxic drugs.

D-Gal may be given with or without lipopolysaccharide (LPS; sometimes referred to as endotoxin), a component of the outer membrane of Gram-negative bacteria, which exacerbates the immune-mediated liver injury (Hamesch et al., 2015). Recognized as a pathogen-associated molecular pattern, LPS triggers a massive release of inflammatory cytokines. In mice, pre-treatment with LPS has been shown to ameliorate this effect, though it is not clear that this would be the case in humans, as our susceptibility to LPS is several magnitudes higher than rodents (Zhang et al., 2014). Common sources of LPS include *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella enterica*. Biological activity can vary based on bacterial source and supplier, so preliminary testing may be necessary prior to proceeding with the full model (Hamesch et al., 2015).

LPS has been used on its own to induce ALF in rodents, but requires bacterial inoculation, commonly with *Cutibacterium acnes* (formerly *Propionibacterium acnes*), to achieve full effect (Brodsky et al., 2009). It has been observed that LPS enhances acute and chronic infections in mice, particularly resulting in bacterial infiltration into the liver (Hamesch et al., 2015). LPS has also been combined with partial hepatectomy in rats to produce a surgical model of ALF with an inflammatory component (Skawran et al., 2003).

D-Gal dosed at 400-1,000mg/kg when given alone or at 300-700mg/kg when given with 0.1mg/kg of LPS has been shown to consistently induce ALF in rodents (Apte, 2014). Higher doses, up to 3g/kg, have been used in some studies, particularly in those investigating HE, as doses in this range will render rats comatose within 36 to 48 hours and cause >90% mortality within 72

hours (Birraux et al., 1998). Slower onset HE can be achieved using multiple small doses over a period of weeks. For instance, Ganai and Husain (2018) administered 250mg/kg via IP injection biweekly to achieve HE in 30 days.

Galun et al. (2000) tested escalating doses in male Fischer rats from 100 to 500mg/kg, looking for a sub-lethal model of ALF. They found that doses greater that 300mg/kg resulted in significant elevation in ALT (>1,500U/L), as well as a sharp decline in the activity of coagulation factors V and VII. In female rats, doses greater than 300mg/kg uniformly resulted in death, whereas in male rats, doses of at least 1g/kg were required to ensure 100% mortality by 5 days. In a study using male Wistar rats, Cauli et al. (2008) found that a dose of 2.5g/kg IP resulted in 100% mortality in an average time of 24.5 hours, whereas a 1.5g/kg dose had only 77% mortality at 7 days, with an average time to death of 48 hours.

The majority of rodent studies use both d-Gal and LPS, particularly in mice. The dose of LPS varied from study to study, ranging from 1-500µg/kg (Zhang et al., 2011; Liu et al., 2019). It is difficult to judge from existing studies whether LPS has a dose-dependent effect when administered with d-Gal or whether a certain minimal amount is required, as this has not been investigated directly. Separate studies using different doses of LPS report comparable levels of liver injury. For instance, Chen et al. (2012) reported serum AST and ALT levels close to 2000U/L and 80% mortality within 12 hours in an ALF model administering 800mg/kg d-Gal and 20µg/kg LPS to BALB/c mice. Takamura et al. (2007) similarly reported serum ALT of nearly 2,000U/L and AST of 1,500U/L in BALB/c mice, as well as 80% mortality within 12 hours using the same does of d-Gal, but with 100µg/kg of LPS. The LPS used in these studies was purchased from the same manufacturer, but the bacterial species of origin is not reported, highlighting an additional challenge in interpreting these studies in relation to LPS. Tumour necrosis factor α (TNFα) has

also been used in combination with d-Gal, instead of LPS. Wang et al. (2018) found similar peak transaminases (>7,500U/L) in mice comparing d-Gal administered with either 5μg/kg of LPS or 10μg/kg of TNFα, though the peak occurred later (6h vs. 9h) in mice receiving TNFα.

D-Gal has also been used in other animal models of ALF, including rabbits, dogs, pigs, and monkeys (Ferenci et al., 1984; Sielaff et al., 1995; Kalpana et al., 1999). Most of the studies using rabbits reported doses in millimoles, ranging from 3.25-5.1mmol/kg (equivalent to 582-914mg/kg) (Ferenci et al., 1984; Jauregui et al., 1995). Not all of the parameters of ALF were reported in these studies, but they were successful at inducing hepatic coma at these doses. Wang et al. (2000) administered doses as high as 1.2g/kg to rabbits for the purpose of testing a bioartificial liver (BAL), at which they demonstrated uniform lethality, elevated transaminases into the several thousands, and extensive hepatic necrosis.

Canine studies have used doses of d-Gal alone ranging from 0.5-2g/kg to induce ALF (Sielaff et al., 1995; Nyberg, Cerra & Gruetter, 1998). Though, some studies have reported that doses greater than 1.5g/kg are necessary to induce sufficient hepatic injury. For instance, Nyberg, Cerra & Gruetter (1998) found that doses of 1.7 and 2g/kg resulted in dramatic elevations in AST (>4,000U/L), INR (>10), and intracranial pressure (>3x baseline), whereas the lower dose of 1g/kg produced comparatively mild liver injury, with AST of 287U/L and INR of 1.5. Using a dose of 0.5g/kg in beagles, Zhang et al. (2012) reported a rise in serum transaminases to nearly 1,000U/L, tripling of prothrombin time (PT), and doubling serum ammonia after 24 hours, consistent with the clinical definition. Insufficient detail was provided in these studies to assess whether these differences could be due to breed, age or other factors.

Similar doses have been reported in porcine studies, ranging from 0.3 to 1.5g/kg (Cao et al., 2012; Sang et al., 2016). Ho et al. (2002) compared 0.5 and 1g/kg doses and found that both

resulted in manifestations of ALF (including elevated transaminases, coagulopathy, and severe hepatic necrosis), though the animals receiving the lower dose survived longer. Several studies using the higher dose of 1.5g/kg reported similar mean survival times, ranging from 3 to 4 days, and elevations in AST of several thousand (Cao et al., 2012; Li et al., 2012; Shi et al., 2017). Li et al. (2012) additionally reported significant coagulopathy, with a PT more than 6x normal, and hyperammonemia, close to 150μmol/L by the third day.

Feng et al. (2017) compared several doses of d-Gal in *M. fascicularis* – 0.3g/kg, 0.25g/kg, and 0.2g/kg. The lowest dose did not result in biochemical or physiologic changes consistent with ALF, though animals did experience elevated transaminases into the several hundreds. Both of the higher doses produced similar elevations in transaminases (>1,000U/L), PT, and ICP, among other measures. The animals receiving the highest dose, however, had a shorter survival time and more extensive necrosis on pathology.

Carbon Tetrachloride

Though more commonly used in chronic models of hepatic fibrosis, carbon tetrachloride (CCl_4) can be given in a single large dose to induce acute injury. Hepatic fibrosis is achieved by administering multiple small doses. Particularly in small animals (e.g., rodents), this can be achieved on a timescale (2 to 4 weeks) that would fit into the clinical definition of ALF in humans. However, it is not clear that this represents the same disease process and is likely more reflective of a chronic injury, especially given shorter animal lifespans (as discussed above).

CCl_4 is activated by one of several members of CYP450 enzymes to produce a trichloromethyl radical (CCl_3^*), which binds with a variety of macromolecules (nucleic acids, proteins, lipids), disrupting vital intracellular processes (Weber, Boll & Stampfl, 2003). CCl_3^* can

be further oxidized, creating a highly reactive trichloromethylperoxy radical (CCl_3OO^*). This molecule is particularly reactive with phospholipids and triglycerides (Boll et al., 2001). Lipid peroxidation of the membrane lipids destroys the integrity of cell and organelle membranes, resulting in permeabilization and loss of ion gradients. Reactive aldehydes produced from oxidative degradation of fatty acids bind to functional groups of proteins, inhibiting enzymatic function. It is unclear whether any single injury pattern predominates. More likely, it is the cascade of multiple insults unleashed by CCl_4 that culminates in cell death.

CCl_4 has been used to create models of ALF in rodents, as well as rabbits and pigs (Choi & Burm, 2005; Nardo et al., 2008; Frank et al., 2020). Rodent studies typically use doses ranging from 0.5 to 2.5ml/kg administered IP or PO (via gavage). Similar doses are used for mice and rats. Gastrointestinal absorption is known to be rapid, though this is somewhat impaired after dilution in oil (e.g., corn, olive), a common vehicle used for either route (Kim et al., 1990). Frank et al. (2020) tested doses of 0.5, 1.25, and 2.5ml/kg (absolute CCl_4 volume) administered orally to male Sprague Dawley rats. All doses produced elevation of serum transaminases into the several thousands after 24 hours and high grade tissue injury on histology, though the effect was greater with higher doses. Mark et al. (2010) found a similar dose dependant effect on survival of female C57BL/6 mice after administering 1, 1.5, 2, and 2.25ml/kg. The two highest doses resulted in 100% mortality by 5 days, compared to 60% and 80% mortality for the lowest.

A rabbit model of CCl_4-induced ALF has been reported by some studies, though is not well characterized**.** A representative study by Choi and Burm (2005) administered escalating SC doses of CCl_4 to achieve varying degrees of hepatic injury in white male New Zealand rabbits. The lowest tested dose, 0.5ml/kg, resulted in only mild increases in serum transaminases. Doubling the dose to 1ml/kg resulted in increased transaminases to the several hundreds. Only the highest tested

dose, 2ml/kg, produced transaminases in a range consistent with ALF, with an ALT of 552U/L and an AST of 1,476U/L. The absence of additional parameters (e.g., INR, serum ammonia) and survival data make it difficult to judge if this model is truly representative of ALF.

Several studies using a porcine model of acute CCl_4-induced hepatotoxicity have also been described. Nardo et al. (2008) administered CCl_4 at a dose of 0.45mg/kg IP, which resulted in elevated transaminases (>1,000U/L), and decreased PT to 34% of normal at 24 hours. These animals had 100% mortality at 48 hours.

Some porcine models have combined surgery and CCl_4 administration. An early model described by Alp and Hickman (1987) involved occlusion of the hepatic artery for 2 hours prior to IP injection of 0.5mg/kg. The development of ALF was evidenced by serum biochemistry, including an AST in the several thousand, elevated serum ammonia more than 8x normal, and prothrombin index to 32% of normal. Yuasa et al. (2008) described a laparoscopic approach, involving ligation of all hepatic arterial branches, and direct intra-portal injection of CCl_4, followed by portal vein occlusion for 30 minutes. They tested three doses – 5, 7.5, and 10mL – in 25kg pigs and concluded the middle dose caused injury suitable for an ALF model, whereas the lower dose led to eventual recovery and the higher dose precipitated rapid demise.

Thioacetamide

Thioacetamide is an organosulfur compound, which, like CCl_4, can be used in both acute and chronic models of liver injury. In the liver, it undergoes a two-step activation, first to thioacetamide-S-oxide (TASO), then to thioacetamide-S,S-dioxide ($TASO_2$), which is mediated by either CYP450 enzymes or flavin adenine dinucleotide-containing monooxygenase (Hajovsky et al., 2012). Both metabolites have cytotoxic effects. TASO has been shown to inhibit

mitochondrial activity and alter cell membrane permeability, leading to nuclear enlargement and increased intracellular Ca^{2+} concentration (Akhtar & Sheikh, 2013). $TASO_2$ binds and forms acetylimidolysine with multiple proteins, leading to their denaturation. This causes dysregulation of multiple cellular processes, including mitochondrial respiration, the endoplasmic reticular transport system, and heat shock proteins (Akhtar & Sheikh, 2013).

The majority of ALF models using thioacetamide have been reported in rodents. Thioacetamide is typically administered by IP injection in acute models, as opposed to chronic models, where it may alternatively be given PO**.** For acute models, thioacetamide is typically dissolved in NS or PBS and may be given as a single dose (200-1,600mg/kg) or as multiple daily doses (200-600mg/kg/d) for two to four days (Wallace et al., 2015). It has been particularly well characterized as a rodent model of HE. Strain-specific differences in the development of hepatic fibrosis in chronic models using thioacetamide has been noted, though this has not been well studied in acute models (Wallace et al., 2015).

Miranda et al. (2010) reported a study testing single doses of 200, 600, and 1200mg/kg in male C57 BL/6 mice. They found no significant difference in neurological assessment between the 200mg/kg dose and controls. Though they did not report additional measures for this dose, at 600mg/kg serum ALT was elevated over 1,500U/L at 24 hours and histology showed evidence of periacinar hemorrhagic necrosis. At the highest dose, mortality was significantly increased, reaching 75% at 50 hours, compared to 33% with 600mg/kg. Similarly, Koblihová et al. (2014) tested single doses of 175, 262.5, and 350mg/kg in male rats, both Lewis and Wistar stains. They found the Wistar rats to be more susceptible to death compared to the Lewis rats, with even the smallest dose resulting in a steady drop in survival after 48 hours, increasing in a dose-dependent fashion. In contrast, Wistar rats had higher peak ALT (~700-1,000U/L), with little appreciable

difference between doses for both strains. Peak plasma ammonia, though elevated from baseline, appeared to decrease with increasing thioacetamide, though this was not compared directly. Histology of rats administered the 350mg/kg dose of both strains confirmed expected findings of hemorrhagic necrosis involving mainly perivenular zones.

Studies comparing single versus multiple doses tend to be somewhat confounded by the differential timing of measurements, but nonetheless show increased level of injury (in both serum biochemistry and histology) and more profound HE, as would be expected with repeat dosing (Mladenović et al., 2012; El Khiat et al., 2019). A unique finding that has been reported in several studies is the susceptibility of streptozosin-treated diabetic rats to thioacetamide, such that one-tenth of a dose produces the same degree of injury in diabetic as non-diabetic rats (30 vs. 300mg/kg) (Sawant et al., 2006).

Azoxymethane

Azoxymethane is a compound that was originally isolated from cycad palm nuts (*Cycas circinalis*) and has been widely used in animal models of colorectal cancer, where it induces tumour formation via DNA alkylation (Laqueur et al., 1963; Fiala et al., 1991). While hepatic metabolism of azoxymethane into methylazoxymethanol is key to its carcinogenicity, the specific mechanism of its hepatoxicity has not been clearly elucidated. It has been shown to cause oxidative stress, at least in colonic epithelial cells, by decreasing activity of antioxidant enzymes and depleting intracellular GSH (Waly et al., 2014). It is also known to cause lipid peroxidation, which can disrupt membrane integrity (Waly et al., 2016).

With respect to ALF, azoxymethane has predominantly been studied in mouse models. As with thioacetamide, azoxymethane is often employed for the study of HE**.** Commonly used doses are 50 or 100mg/kg, diluted in NS or PBS and administered via IP injection.

The use of azoxymethane to induce ALF in mice was first described by Matkowskyj et al. (1999) in a study of male C57 BL/6s. They reported that a 100mg/kg resulted in decline in activity within 6 hours, followed by rapid progression through all stages of HE, ending with hepatic coma and death within 41 hours. This was associated with a dramatic elevation in serum ALT, peaking at 12,231U/L by 36 hours. Histology showed hemorrhagic centrilobular necrosis with eventual obliteration of hepatic veins.

Many rodent models of ALF neglect to demonstrate coagulopathy, which is a key component of the clinical diagnosis. However, this has been explicitly studied in azoxymethane-induced liver failure. Doering et al. (2002) reported (♂ C57 BL/6 mice) reduction of factor V and factor VII activity to 2.4% and 10.1%, respectively, by 48 hours after 30mg/kg of azoxymethane. 50mg/kg caused even more dramatic reduction to less than 2% by 36 hours. In this study, all mice receiving 30 or 50mg/kg eventually progressed to hepatic coma and death, in an average of 45 and 33 hours respectively, whereas mice receiving a lower dose of 15mg/kg failed to develop HE and survived to 72 hours.

Other Pharmacological Agents for Inducing ALF in Animal Models

Toxic mushrooms belonging to the genus *Amanita* have been well documented to cause ALF in humans, though estimated to be responsible for <1% of cases (Karvellas et al., 2016). The specific toxin, α-amanitin, is a cyclic peptide, 8 amino acids in length (Santi et al., 2012). A-amanitin is an inhibitor of RNA polymerase II, and so disproportionately affects organs with high

rates of protein synthesis, such as the liver (Karvellas et al., 2016). It is readily absorbed by hepatocytes on first pass metabolism, and though ~60% secreted in bile, it is returned to liver via enterohepatic circulation. It has also been shown to cause damage to kidneys, pancreas, adrenals and testes.

A-amanitin has been used as a means to induce ALF in mouse, pig, and rhesus macaque models (Takada et al., 2001; Zhou et al., 2012; Jedicke et al., 2014). However, experience with α-amanitin-induced ALF in mice is limited. The available studies have used doses of 0.6mg/kg and show 100% mortality within 72 hours and histologic evidence of necrosis (Jedicke et al., 2014; Ferriero et al., 2018).

Large animal models have been successful in achieving ALF using a combination of α-amanitin and LPS. Two porcine studies describe 0.1mg/kg of α-amanitin and 1.0μg/kg of LPS, administered directly into the portal circulation (Takada et al., 2001; Ishiguro et al., 2003). In comparison to animals treated with α-amanitin alone, Takada et al. (2001) found that co-administration with LPS resulted in all animals succumbing to hepatic coma and death within 5 days, whereas in the other group three of the four animals survived to 7 days and experienced normalization of ICP. Likewise, AST in the α-amanitin only group peaked at less than 1,500U/L, compared to >9,000U/L with the addition of LPS. Histology also showed severe centrilobular hemorrhagic necrosis with combined treatment.

Two studies have been published describing ALF in a *M. mulatta* model, induced via α-amanitin and LPS (Zhou et al., 2012; Li et al., 2018). Both used 0.1mg/kg of α-amanitin and 1.0μg/kg of LPS IP and both reported similar results, consistent with ALF. Serum ALT and AST rose to over 4,000 and 8,000U/L, respectively, PT peaked at 300s, and all untreated animals

progressed rapidly to hepatic coma and death within 72 hours. Histology correspondingly showed extensive hemorrhagic necrosis.

A-naphthyl isothiocyanate (ANIT) has also been used in models of ALF. As opposed to the other drugs mentioned, ANIT is characterized by cholestatic injury (Dahm, Ganey & Roth, 2010). ANIT forms conjugates with GSH, which are transported into the biliary system by multidrug resistance-associated protein 2 (MRP2). Subsequent dissociation from GSH leads to high concentrations of ANIT in cholangiocytes. Injury to cholangiocytes results in impaired bile flow and intrahepatic accumulation of bile acids, progressing to hepatocyte necrosis.

Shen et al. (2020) use 100mg/kg PO to induce ALF in male Sprague-Dawley rats. This resulted in elevations of ALT and AST in the range of 600-750U/L and 750-1,050U/L respectively. Other studies in rats have reported similar findings, including the development of focal areas of necrosis on histology (Yang et al., 2017). Though ANIT has yet to be fully characterized as a model of ALF, it has potential for further development based on its unique mechanism of acute cholestatic liver injury.

Cocaine toxicity is a rare cause of ALF in humans, reported in literature only as individual cases (Kanel et al., 1990). The mechanism has been suggested to be related to metabolites of norcorcaine, itself a secondary metabolite of cocaine, which results from N-demethylation by CYP450 in ~10% of the drug (Kanel et al., 1990). Specifically, the norcocaine nitrosonium ion has been shown to be highly reactive with glutathione and cause lipid peroxidation of cell membranes.

Cocaine has been used to induce ALF in mice, though not commonly. Hayase et al. (2000) administered cocaine at 65mg/kg IP to male ICR mice. This resulted in serum ALT >7,000U/L at 23 hours and significant neurological depression, though this normalized by 72 hours. Because of

its other systemic effects, as well as its illicit status in most countries, it is likely to remain of interest for its specific toxicology, rather than as a generalized ALF model.

Several other compounds for inducing acute hepatotoxicity, including allyl alcohol, bromobenzene, diclofenac, furosemide, and N-nitrosodimethylamine, have been reported less commonly in the literature (Brodie et al., 1971; Belinsky et al., 1985; Ilic et al., 2011; Loukopoulos et al., 2014; McGill et al., 2015). However, because synthetic function and features of HE are not routinely reported especially in rodent models, it is not clear whether these constitute reasonable models for ALF on the basis of serum transaminases and histology. Sasaki et al. (2016) were able to induce ALF in a rodent model using carbamazepine. Specific considerations in this model include to use of F344 rats, daily dosing of carbamazepine for 5 days, and the co-administration of BSO (a GSH-depleting agent) on the final day. These rats demonstrated significant elevations in serum transaminases, with ALT peaking at 16,603U/L 24 hours after the last dose, as well as centrilobular hepatocyte necrosis on histology. This study provides an example of a model of idiosyncratic, drug-induced causes of liver failure.

There are a few specific approaches to idiosyncratic drug-induced liver injury (DILI). One consists of co-administration of the drug with LPS, which is thought to add an inflammatory stimulus to precipitate DILI. This approach has been shown to enhance hepatotoxicity in animal models using amiodarone, chlorpromazine, diclofenac, halothane, ranitidine, and trovafloxacin (Buchweitz et al., 2002; Luyendyk et al., 2003; Deng et al., 2006; Shaw et al., 2007; Dugan et al., 2010; Lu et al., 2012). However, the role of infection or inflammation as a causative factor in human DILI has not been clearly demonstrated (McGill & Jaeschke, 2019). An alternative approach attempts to suppress immune tolerance of the liver, such as by knockout of programmed

cell death protein 1 (PD-1), which, though it has been shown to increase susceptibility to ALI, has not yet been shown to induce clear ALF (Metushi, Hayes & Uetrecht, 2015).

In addition to the above-mentioned models of hepatotoxicity, administration of ammonia directly (either PO or IV) has been used for the acute induction of HE in isolation though it does not result in ALF *per se* (Fick, Schalm & de Vlieger, 1989; Butterworth et al., 2009). These artificial models of hyperammonemia may be more appropriate as a control alongside acute or chronic models of liver injury, given the absence other features of ALF and lack of a clinical correlate.

3.1.8 - Immunogenic Models of ALF

The immune response, both the activation of resident immune cells and infiltration of peripheral lymphocytes, features prominently in many of the models previously described. However, we reserve this section for methods of inducing ALF primarily by direct immune-mediated damage. The one exception is LPS, which would otherwise fit in this category, but was described in conjunction with d-Gal because of their close association in ALF models. We will describe the most well-established models, though the addition of knockout mice and genetically modified viruses introduces complexity and variation that cannot fully be covered here.

Concanavalin A

Concanavalin A (ConA) initiates immune-mediated liver injury and has been used specifically to model autoimmune hepatitis (Heymann et al., 2015). ConA is a lectin derived from the jack bean (*Canavalia ensiformis*) that binds to various sugars, including glycoproteins and glycolipids, mainly through interaction with mannose and glucose moieties. Hepatic injury after

ConA administration occurs primarily by the recruitment and activation of T cells and natural killer T cells in the liver. ConA targets the liver specifically, where it is taken up by liver sinusoidal endothelial cells (LSECs). By itself, ConA has been shown to be minimally toxic to isolated hepatocytes. *In vitro* studies have shown that the presence of lymphocytes and macrophages is necessary to stimulate the release of TNF-α and other inflammatory cytokines by LSECs (Gantner et al., 1995).

ConA for the induction of ALF is well described in mice, though not well characterized in other animal models. ConA is dosed between 8-35mg/kg IV, dissolved in sterile PBS or NS (it is unknown whether it can be administered with the same effect IP) (Byk et al., 2016; Tadokoro et al., 2017). Dose finding experiments are recommended prior to beginning experiments, as its efficacy is known to vary by batch. As well, variations in susceptibility by age, sex, and strain have been observed. In particular, female mice have been noted to have higher susceptibility and also greater variability in outcome (Takamoto et al., 2003).

Wang et al. (2017) administered doses of 10mg/kg to male C57BL/6 mice and reported dramatic elevations in ALT (>8,000U/L) as soon as 12h after injection. This corresponded with greater than 50% necrosis on histology. However, at this dose serum transaminases eventually normalized and mice survived to at least 72h. In contrast, a higher dose of 25mg/kg resulted in 80% mortality by 48h.

There may be other lectins that produce a similar effect**.** For instance, Yu et al. (2020) used a lectin purified from the edible mushroom *Agrocybe aegerita* (AAGL) to induce ALF in male C57BL/6 mice. Administered at a dose of 3mg/kg PO, AAGL resulted in elevation in serum ALT >3,000U/L and massive hepatocyte necrosis on histology within 9h. In contrast with ConA, liver injury caused by AAGL was found to be associated with natural killer T cell infiltration, mediated

by IL-1β. Other immunostimulatory macromolecules, such as α-galactosylceramide, have been used to induce an immune-mediated hepatitis, but have not been convincingly shown to model ALF (Biburger & Tiegs, 2005).

Fas Antibody

A specific Fas antibody has been used in animal models to induce ALF. It is a monoclonal antibody (Jo-2 clone) produced by Pharmingen (a subsidiary of BD Sciences) by exposure of Armenian hamster to a mouse lymphoma cell line transformed with recombinant Fas. It binds to and activates Fas receptor (CD95) inducing apoptosis. Though Fas is expressed by other tissues, including thymus, heart, lung, and ovary, this anti-Fas antibody has been used to specifically induce ALF in animal models.

Fas antibody is typically given IV by tail vein injection in doses ranging from 100 to 600µg/kg, but may alternatively be administered IP (Bajt et al., 2000; Mizuguchi et al., 2007). There is a potential theoretical benefit to IP administration, as it would be more likely to enter the portal circulation (Turner et al., 2011). The Fas antibody acts on hepatocytes, as well as nonparenchymal liver cells. Malassagne et al. (2001) reported rapid lethality – 100% mortality within 8h – with a dose of 250µg/kg in female BALB/c mice. When lowering the dose to 150µg/kg, animals survived to at least 12h, but still developed substantial liver injury, with AST rising to >5,000U/L and severe lesions on histology. Sharma et al. (2011), testing a higher dose of 400µg/kg, reported 100% mortality within 20h and elevated ALT >800U/L at 9h. It is possible that the discrepancy is the result of sex differences which were not reported in this study.

Viral Models of ALF

In addition to the variety of models that may be created using recombinant genetic techniques on the virus and/or host, there are several species-specific viruses associated with fatal hepatitis in wild type animals. Mice are susceptible to murine hepatitis virus (MHV) strain 3, a species of coronavirus. Like all coronaviruses, MHV is an enveloped, positive sense, single stranded RNA virus (Roth-Cross, Bender & Weiss, 2008). It affects the brain and/or liver, producing acute or chronic injury depending on the specific strain. MHV strain 3 results in ALF in susceptible mice strains. Studies typically use BALB/c mice. Some strains, such as A/J mice are completely resistant, whereas others, such as C3H mice, tend to develop non-lethal acute hepatitis, progressing to chronic hepatitis. Following viral replication and lysis in Kupffer cells and LSECs at the liver sinusoids, MHV infects hepatocytes, resulting in focal necrosis (Martin et al., 1994).

Studies have been reported dosing 6-8wk old female BALB/c mice with 20 plaque forming units (PFUs) of MHV that resulted in 100% mortality within 3-5 days and serum ALT of 2,000-2,500U/L at 60h. Histology showed focal necrosis and massive inflammatory cell infiltrate (Zhu et al., 2006; Gao et al., 2010). Other studies, using the high doses of 100 PFU in BALB/c mice of the same sex and age, have reported ALT in excess of 10,000U/L at 72h and enlarging focal necrosis, becoming confluent, between 48 to 72h (Wu et al., 2016; Yu et al., 2017).

Other strains of MHV, including MHV-2, MHV-A59, and MHV-JHM, have also been reported to induce significant hepatitis (Garcia et al., 2021). For instance, MHV-A59 has been shown to cause elevated serum AST of 3,700U/L 24h after administration of 16 complement fixation units to female CD mice (Farivar et al., 1976). Histological sections from livers of these mice correspondingly showed greater than 90% necrosis of hepatocytes.

Rabbit hemorrhagic disease virus (RHDV) is a species of calicivirus affecting wild and domestic European rabbits (*Oryctolagus cuniculus*) that causes acute hepatic necrosis, disseminated intravascular coagulopathy and rapid demise (Belz, 2004). RHDV is a positive sense, single stranded RNA spread by airborne and fecal-oral transmission. Studies administering $2x10^4$ hemagglutination units of RHDV via intramuscular (IM) injection to 9wk old New Zealand white rabbits have reported mortality of 90-100% by 60h, with serum ALT reaching between 1,500-3,500IU/L at 48h (San-Miguel et al., 2006; García-Lastra et al., 2010; Tuñon et al., 2011). Histology showed expected changes of extensive hepatocellular necrosis, edema, hemorrhage, and neutrophil infiltration. Sánchez-Campos et al. (2004) reported elevated PT and decreased factor VII at 48h compared to baseline in addition to elevated transaminases, but it is unclear to what degree this is a consequence of liver failure versus coagulopathic effects induced by the virus.

While no species-specific viral hepatitides are known in non-human primates, attempts have been made to induce ALF using human viruses, though with limited success. Leon et al. (2016) co-infected three *M. fasicularis* macaques with parvovirus B19 and hepatitis A virus (HAV), as this is known to worsen the resultant hepatitis. Despite demonstrating appropriate seroconversion, the animals did not develop ALF and findings on liver histology were only slightly worse compared to animals infected with HAV alone. Lashkevich et al. (1996) described the rapid development of encephalopathy and hepatic necrosis in 'monkeys' after administration of echoviruses isolated from severe pediatric cases. However, details on these cases are lacking and viral genotypes were not reported.

Hepatitis B virus (HBV) is known to cause an acute hepatitis in chimpanzees (*Pan troglodytes*). However, the development of ALF has not been reported. Chen et al. (2020) infected chimpanzees with a procure HBV mutant that is known to be associated with ALF in humans.

While the hepatitis induced by the variant was more severe than that caused by the wild type, none of the animals developed ALF and all had completely recovered within 24 weeks. Given that ALF occurs only 1% of cases of acute hepatitis B in human patients, it may not be possible to achieve sufficient consistency for use in primate models (Manka et al., 2016).

Elimination of the type I interferon (IFN) response, either by genetic knockout of the interferon-α/β receptor or antibody blockade, has been used in several studies to reliably induce ALF by a range of viruses that would otherwise not result in hepatitis or only sporadically so. Type I IFNs, including IFNα and β as the most well-known, play an important role in the acute antiviral response by inhibiting viral replication and spread by infected cells, promoting viral antigen presentation, and activating the adaptive immune system (Murira & Lamarre, 2016). Lindquist et al. (2018) reported a model of Crimean-Congo hemorrhagic fever virus (CCHFV) induced-liver injury in C57Bl/6 mice relying on type I IFN blockade by anti-interferon α/β receptor subunit 1 (IFNAR1) antibody. Mice infected with 100 PFUs of CCHFV, followed by IFN-I blockade 24h later, demonstrated elevated serum transaminases into the several hundreds, widespread inflammation and hepatocellular necrosis on histology, and uniform lethality within 5 days.

Other studies have used *IFNAR* knockout mice to achieve ALF with different species of viruses. Borst et al. (2018) reported a model administering $2x10^6$ PFUs of vaccinia virus (VACV) to *IFNAR*$^{-/-}$ mice, which resulted in elevated serum transaminases close to 1,000U/L by day 4 and uniform death within 5 days. A model described by Parker et al. (2016) required the addition of type II IFN blockade (via *STAT1* knockout) to achieve ALF by herpes simplex virus type 1 (HSV-1) infection. Corneal infection with $2x10^6$ PFUs per eye resulted in dramatic elevation of transaminases (>12,000 U/L) and death by 5 days. Histology showed multifocal necrosis, with infiltrating neutrophils and lymphocytes.

Other Models of Immune-Mediated ALF

Other immunogenic models of ALF have employed a combination of strategies to yield ALF with particular features. For instance, Welz et al. (2018) developed a murine model of CD8+ T cell mediated acute viral hepatitis, which is a characteristic feature of liver injury in hepatitis A virus infection (Kim, Chang & Shin, 2014). In their model, mice that had been either immunized against chicken ovalbumin (OVA) and/or treated with OVA-specific CD8 T cells (OT-I cells) were exposed to a recombinant adenovirus coding for OVA 30 days after immunization. A third group was treated with OT-I cells at the same time as being exposed to the recombinant virus. All three groups showed a dramatic rise in serum ALT 3 days post-infection, with the highest elevation (>7,000 U/L) being found in the third group (combined administration of OT-I cells and virus). Histology showed extensive periportal infiltration of T cells, with confluent areas of hepatocyte necrosis.

A model of autoimmune hepatitis (AIH) was described by Kido et al. (2008), in which PD-1 knockout BALB/c mice undergo thymectomy at 3 days old, leading to the spontaneous development of AIH within several weeks. These mice showed onset of AIH between 2-3wks, marked by peak AST >3,000 U/L and fatality of all mice by 4wks. These mice developed anti-nuclear antibodies, a key feature of AIH type 1 in humans, and histology showed severe hepatocyte degeneration, with massive infiltration of CD4+ and CD8+ T cells.

Knockout models such as these, as well as those of IFN pathways, may be less useful in directly investigating novel therapies, compared to models of wild-type mice. However, they play a greater role in understanding the underlying pathophysiology of viral and immune-mediated forms of ALF.

3.1.9 – Other Models

The final model of ALF that will be discussed is the Long Evans Cinnamon (LEC) rat, which is distinct from the other categories of preclinical models and serves as a specific model of Wilson's disease, an autosomal recessive disease of abnormal copper metabolism that can lead to both acute and chronic liver failure in humans (Członkowska et al., 2018). LEC rats have a genetic defect in the gene encoding a copper transporting P-type ATPase, homologous to the *ATP7B* gene that is known to be responsible for Wilson's disease in humans (Terada & Sugiyama, 1999). As in their human counterparts, they demonstrate excessive deposition of copper in the liver, decreased serum copper and its transporter, ceruloplasmin, as well as decreased biliary excretion. Hepatitis and ALF occurs spontaneously in these animals between 80 and 120 days old (Li et al., 1991).

ALF can be more reliably induced in these animals by dietary copper supplementation or acute administration of copper. Siaj et al. (2012) demonstrated that LEC rats receiving water supplemented with 20mg of copper per litre over 3 months resulted in ALF by 80 days, compared to animals receiving reduced copper diets, which could prevent hepatitis and ensure almost disease-free survival. Interestingly, rats transitioned to high copper diet after 5 months on a regular diet had more rapid elevation of serum transaminases and showed decreased mean lifespan compared to rats receiving the high copper diet for 3 months beginning as pups. Still, both groups developed elevated serum transaminases, peaking at ~1,000 U/L, and had histology showed extensive inflammation, enlarged nuclei, and, to a lesser extent, necrosis. IP injection of 3mg/kg of copper daily for 3 days has been shown to rapidly induced ALF in LEC rats, with death occurring in 50% of animals by 48h (Sugawara et al., 1991).

3.1.10 – Concluding Remarks

Acute liver failure in patients presents two distinct opportunities for effective intervention aimed at improving outcomes. The first is at initial presentation to mitigate acute hepatic necrosis. The second is in the days that follow to enhance liver regeneration. Preclinical models of ALF largely represent models of acute hepatocyte death and their role in the study of pathophysiology and potential therapeutic interventions applies mostly to the former aspect. As opposed to clinical cases, the focus is on markers of hepatocyte death, chiefly serum transaminases and histology, rather than liver function. Particularly, with their brief time course and the lack of reversibility of some models, they are best used to investigate factors that play a role in this phase, as well as opportunities for intervention.

As with any animal model of clinical disease, the goal should be to identify essential elements and eliminate extraneous details to enhance reproducibility, both within the same study and for others trying to replicate it. To paraphrase Albert Einstein, animal models should be "as simple as possible, but no simpler" (Robinson, 2018). Some have sought a generalized model of ALF, while others aim to reproduce a specific cause of ALF seen clinically. Still, other models represent a particular type of ALF, whose specific causes are not easily reproduced in the preclinical setting, such as ConA for immune causes of ALF. Generalized models may be appropriate for evaluating treatments meant to be broadly applicable and may prove to be more reproducible in some instances. However, given complexities of ALF and its multiple etiologies, it is difficult to know how well insights gained from any single model will apply to the clinical setting. It is likely that judicious use of a variety of animal models will continue to be required to enhance our understanding of and explore effective treatment options for ALF.

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CHAPTER 3 – USING NORMOTHERMIC MACHINE PERFUSION TO MODEL ACUTE LIVER FAILURE

PART 2 – MODEL OF ACUTE LIVER FAILURE IN AN ISOLATED PERFUSED LIVER – CHALLENGES & LESSONS LEARNED

3.1 Model of Acute Liver Failure in an Isolated Perfused Porcine Liver – Challenges and Lessons Learned

biomedicines

MDPI

Article

Model of Acute Liver Failure in an Isolated Perfused Porcine Liver—Challenges and Lessons Learned

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Abstract: Acute liver failure (ALF) is a rare but devastating disease associated with substantial morbidity and a mortality rate of almost 45%. Medical treatments, apart from supportive care, are limited and liver transplantation may be the only rescue option. Large animal models, which most closely represent human disease, can be logistically and technically cumbersome, expensive and pose ethical challenges. The development of isolated organ perfusion technologies, originally intended for preservation before transplantation, offers a new platform for experimental models of liver disease, such as ALF. In this study, female domestic swine underwent hepatectomy, followed by perfusion of the isolated liver on a normothermic machine perfusion device. Five control livers were perfused for 24 h at 37 °C, while receiving supplemental oxygen and nutrition. Six livers received toxic doses of acetaminophen given over 12 h, titrated to methemoglobin levels. Perfusate was sampled every 4 h for measurement of biochemical markers of injury (e.g., aspartate aminotransferase [AST], alanine aminotransferase [ALT]). Liver biopsies were taken at the beginning, middle, and end of perfusion for histological assessment. Acetaminophen-treated livers received a median dose of 8.93 g (8.21–9.75 g) of acetaminophen, achieving a peak acetaminophen level of 3780 µmol/L (3189–3913 µmol/L). Peak values of ALT (76 vs. 105 U/L; $p = 0.429$) and AST (3576 vs. 4712 U/L; $p = 0.429$) were not significantly different between groups. However, by the end of perfusion, histology scores were significantly worse in the acetaminophen treated group ($p = 0.016$). All acetaminophen treated livers developed significant methemoglobinemia, with a peak methemoglobin level of 19.3%, compared to 2.0% for control livers ($p = 0.004$). The development of a model of ALF in the ex vivo setting was confounded by the development of toxic methemoglobinemia. Further attempts using alternative agents or dosing strategies may be warranted to explore this setting as a model of liver disease.

Keywords: acute liver failure; porcine model; ex situ machine perfusion; acetaminophen; carbon tetrachloride

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3.2.1 – Preface

Acute liver failure (ALF) is a rare but devastating disease associated with substantial morbidity and a mortality rate of almost 45%. Medical treatments, apart from supportive care, are limited and liver transplantation may be the only rescue option. Large animal models, which most closely represent human disease, can be logistically and technically cumbersome, expensive and pose ethical challenges. The development of isolated organ perfusion technologies, originally intended for preservation before transplantation, offers a new platform for experimental models of liver disease, such as ALF. In this study, female domestic swine underwent hepatectomy, followed by perfusion of the isolated liver on a normothermic machine perfusion device. Five control livers were perfused for 24 hours at 37ºC, while receiving supplemental oxygen and nutrition. Six livers received toxic doses of acetaminophen given over 12 hours, titrated to methemoglobin levels. Perfusate was sampled every 4 hours for measurement of biochemical markers of injury (e.g., aspartate aminotransferase [AST], alanine aminotransferase [ALT]). Liver biopsies were taken at the beginning, middle, and end of perfusion for histological assessment. Acetaminophen-treated livers received a median dose of 8.93g (8.21-9.75g) of acetaminophen, achieving a peak acetaminophen level of 3780µmol/L (3189-3913µmol/L). Peak values of ALT (76 vs. 105U/L; p=0.429) and AST (3576 vs. 4712U/L; p=0.429) were not significantly different between groups. However, by the end of perfusion, histology scores were significantly worse in the acetaminophen treated group (p=0.016). All acetaminophen treated livers developed significant methemoglobinemia, with a peak methemoglobin level of 19.3%, compared to 2.0% for control livers (p=0.004). The development of a model of ALF in the *ex vivo* setting was confounded by the development of toxic methemoglobinemia, most likely due to the metabolite, p-aminophenol.

Further attempts using alternative agents or dosing strategies may be warranted to explore this setting as a model of liver disease.

3.2.1 – Introduction

Acetaminophen toxicity is the most common cause of acute liver failure (ALF) in North America [1]. Despite its low occurrence (10 people per million annually), it is associated with high morbidity and a devastating mortality rate of almost 45% [2]. Early presentations (within 24 hours) may respond to treatment with N-acetylcysteine (NAC), which replenishes the liver's glutathione reserve and facilitates the conversion of acetaminophen into non-toxic metabolites [3,4]. NAC can delay progression, but, otherwise, management is largely supportive, allowing the liver time to recover. Liver transplantation may be considered based on the severity of injury, as judged by the King's College criteria, and the patient's pre-morbid status [5]. While survival rate is high after transplantation (75% at 5 years), transplantation itself is associated with its own morbidity and requires lifelong immunosuppression, which is not to mention the limited supply of suitable organs [6,7].

Acetaminophen is a common over-the-counter analgesic. It is primarily metabolized in the liver, where it undergoes either glucuronidation or sulfonation by cyto-chrome P450 enzymes to generate water soluble metabolites that can be readily excreted in the urine [8]. A small proportion is oxidized into N-acetyl-p-benzoquinone (NAPQI). NAPQI is a strong oxidizing compound that generates oxidative stress and causes cellular dysfunction by forming acetaminophen-protein adducts [9]. NAPQI can be further metabolized into non-toxic compound by glutathione dependent pathways [10]. However, when the main pathways of acetaminophen metabolism become saturated and glutathione stores are depleted, accumulation of NAPQI can lead to hepatocyte death and severe liver injury [9].

Pre-clinical models used to study acetaminophen toxicity in the liver vary from hepatocytes cultured in vitro, to small (e.g., rodents) and large animal (e.g., dogs, primates, pigs) models. More

advanced models, including use of bio-artificial liver and 3D cell culture, have also been employed [10,11]. Porcine models are typically preferred as large animal models, due to their similarity to humans in terms of liver anatomy, histology and metabolism, as well as logistic considerations (e.g., size, handling, availability) [13,14]. Previous methods to induce ALF with acetaminophen in pigs have required the animals to be maintained under anesthesia for the duration of the experiment (typically >24 hours), which are expensive, technically challenging, and confounded by ethical considerations [15,16].

Ex vivo normothermic machine perfusion (NMP) may provide an alternative approach and more ethically acceptable platform for modeling ALF by isolating the injury to the target organ rather than the entire animal. NMP is a technology that aims to maintain normal liver physiology and metabolism outside of the body [17]. It was developed with the intent of preserving and assessing grafts before transplantation, such as marginal grafts donated after cardiovascular death. Its clinical utility was demonstrated in a randomized controlled trial published in 2018 by Nasralla and colleagues, where minimal liver injury was observed in grafts subjected to NMP, despite lower discard rate and longer preservation time, when compared to traditional cold static storage [18]. Related preclinical research has focused on graft treatment while on the *ex vivo* circuit, including 'de-fatting' steatotic livers, gene therapy, and eradication of hepatitis C [19–22] Several other exciting potential applications of NMP outside of transplantation, such as use in toxicology studies, cancer research, and liver support remain to be explored [23].

Development of reproducible, translatable ALF models are needed to facilitate the search for novel treatments. An ALF model in an isolated organ has several potential advantages over in vivo models, including improving animal welfare and extending treatment duration. However, the impact that the lack of other, potentially compensatory mechanisms, provided by other organ

systems will have is unknown, as are the effects of interaction with artificial circuit components. Herein, we describe our approach to creating a large animal *ex vivo* model of ALF using acetaminophen, as well as another common agent, carbon tetrachloride, and describe the limitations we encountered.

3.2.2 – Materials & Methods

This study used domestic swine, 3-4 months of age, weighing 40-50kg, at which point their livers are similar in size to an adult human (~1,200g), while still being easily manageable in our surgical facility. Only female animals were used as male animals are routinely castrated to prevent aggression and boar taint (if used for meat), which can lead to visceral fat accumulation, among other physiological changes. Animals were supplied by the Swine Research and Technology Centre at the University of Alberta. Animals were obtained from different litters, randomly housed, and allocated to treatment groups. This study was conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with approval from the Animal Care and Use Committee (Health Sciences) for the University of Alberta (AUP00001036), which ensure the ethical treatment of research animals.

Liver Procurement

Hepatectomy was performed in fasted, anesthetized pigs, as in our previously established protocol [24,25]. In brief, animals were premedicated with 20mg/kg of ketamine (Ketaset; Zoetis Canada Inc., Kirkland, QC) and 0.05mg/kg of atropine (Atro-SA; Rafter 8 Products Inc., Calgary, AB), followed by endotracheal intubation using direct laryngoscopy. During the procedure,

animals were maintained on inhalational anesthesia (3-4% isoflurane; Frensenius Kabi Canada, Toronto, ON). An orogastric tube was also inserted to decompress the stomach.

Animals were prepped with a 7.5% povidone-iodine solution and draped appropriately. Surgery was conducted under aseptic technique, including the use of sterilized instruments. An 8Fr venous cannula was inserted into the right internal jugular vein for fluid administration. A midline laparotomy was performed to expose the intra-abdominal contents. The bowel was displaced from the abdomen to expose to the infra-renal aorta, which was then dissected and encircled with 0-silk ties (Perma Hand; Ethicon Inc., Bridgewater, NJ) for ease of future cannulation. The bowel was returned to abdomen and rolled laparotomy sponges were placed beneath the liver to elevate it into the surgical field. The fundus of the gallbladder was grasped with an Allis clamp and retracted superiorly to facilitate dissection from the gallbladder fossa. The cystic duct and artery were then ligated and the gallbladder was disposed of. The porta hepatis was exposed and the common bile duct was identified and ligated just prior to it entering the pancreas. The main portal vein was then dissected free of its peritoneal covering and tagged with a silk tie. A portion of the upper intra-abdominal aorta was also dissected and tagged with a silk tie for subsequent clamp placement during abdominal aortic flush.

Blood to be used in the perfusion was collected via the right atrium. To facilitate this, a midline sternotomy was performed and the heart was exposed and freed from the pericardium and associated tissues. The superior portion of the right atrium was encircled with a 4-0 polypropylene suture (Prolene Blu; Ethicon Inc., Bridgewater, NJ). An incision was made in the atrium above the suture and a 28 French two-stage venous cannula was inserted and secured with a vascular torniquet. Blood was collected into a sterile container to the point of complete exsanguination (1.5-2L).

Upon completion of exsanguination, an aortic cross-clamp was placed on the upper portion of the abdominal aorta previously identified to prevent flow of the preservation solution into the chest. The infra-renal aorta was then cannulated and 2L of cold (4ºC) histidine-tryptophan-ketoglutarate solution (Custodiol® HTK; Essential Pharmaceuticals LLC., Durham, NC) was used to flush the intra-abdominal organs. Ice was placed into the abdomen to cool the organs and the inferior vena cava above the diaphragm was incised to vent the flush solution. Following completion of the intra-abdominal flush, the liver was resected and weighed on the back table.

Normothermic machine perfusion (NMP)

Livers were perfused according to our previously established protocol [24,25]. A schematic of our NMP circuit is provided in **Supplementary Figure 3.2.1**. Our custom designed circuit consisted of an EOS ECMO oxygenator (Sorin Group Canada Inc., Burn-aby, BC), a MYOtherm XP heat exchanger (Medtronic of Canada Ltd., Brampton, ON), and two BPX-80 Bi-Medicus centrifugal pumps (Medtronic of Canada Ltd., Brampton, ON). Perfusate draining from the liver was collected in a filtered CARD EVO cardiotomy reservoir (Sorin Group Canada Inc., Burnaby, BC) before recirculation through the pumps and was heated using a CW-05G water bath (Lab Companion, Jeio Tech Inc., Billerica, MA). Hepatic artery and portal venous flows were measured using Transonic Clamp-on Tubing Flowsensors (6PXL and 7PXL; Transonic Systems Inc., Ithaca, NY) and pressures were measured using TruWave pressure transducers (Ed-wards Life Sciences Canada Inc., Mississauga, ON). The centrifugal pumps were computer-controlled to maintain desired hepatic artery pressure and portal venous flow. Inflows of oxygen and carbon dioxide were titrated to maintain a partial pressure of arterial oxygen between 100 and 120mmHg and a partial pressure of carbon dioxide between 35 and 45mmHg.

The circuit was primed with a 1:1 ratio of whole blood to modified Krebs-Henseleit solution (glucose [5mM], sodium chloride [85mM], potassium chloride [5mM], calcium chloride [1mM], magnesium chloride [1mM], sodium bicarbonate [25mM], sodium phosphate monobasic [1mM], sodium pyruvate [5mM], and 8% bovine serum albumin). Piperacillin/tazobactam (3.375g; Sandoz Canada, Boucherville, QC), methylprednisolone (500mg; Solu-Medrol, Pfizer Canada Inc., Kirkland, QC), and sodium heparin (5000U; Fresenius Kabi Canada, Toronto, ON) were added prior to attaching the organ. During perfusion, the organ received infusions of regular insulin (2U/hr; Humulin R; Eli Lilly & Company, Toronto, ON) and sodium heparin (1000U/hr). Piperacillin/tazobactam and methylprednisolone were re-dosed after 12 hours. Perfusate pH and glucose was maintained within physiological range (7.35-7.45 and 6-10mmol/L).

Prior to attaching the liver to the circuit, it was flushed with 2L of normal saline. The hepatic artery and portal vein were canulated with an 8Fr arterial cannula (Medtronic of Canada Ltd., Brampton, ON) and 1/4' polycarbonate tubing connector, respectively. The common bile duct was cannulated with tubing that drained to a reservoir outside of the organ chamber. The perfusate was heated to maintain the organ at 37ºC. Arterial and portal venous flows were gradually increased to physiological values, as the organ warmed.

Experimental protocol

Control livers were run for 24 hours (n=5). Acetaminophen-treated livers (n=6) received doses of acetaminophen (Sigma-Aldrich Canada, Oakville, ON) dissolved directly into the perfusate, starting 1 hour after beginning perfusion. Acetaminophen was dosed at 30-minute intervals up to 12 hours, with the dose titrated to avoid excessive methemoglobinemia as measure via point-of-care blood gas analyis. Acetaminophen-treated livers were intended to run for 24

hours, but were stopped early as necessitated by perfusion parameters and circuit volume. These livers similarly received additives necessary to maintain physiological blood gas parameters. Since we were unable to achieve sufficient toxicity without causing methemoglobinemia, further perfusions were discontinued, which lead to unequal numbers between groups. An additional experimental group treated with carbon tetrachloride (CCl_4; Sigma-Aldrich Canada, Oakville, ON) as the hepatotoxic agent was also attempted, but discontinued after two perfusions, due to circuit and perfusate incompatibility.

Perfusate Biochemistry

Perfusate was sampled from the hepatic artery and portal venous inflows and vena cava outflow every four hours and as needed to measure hemoglobin, methemoglobin, pH, electrolytes, lactate, glucose, and partial pressures of oxygen and carbon dioxide using a point-of-care blood gas machine (ABL Flex Analyzer; Radiometer Canada, Mississauga, ON). Perfusate sampled from the vena cava outflow was also measured for alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and total and conjugated bilirubin using a Beckman Coulter Unicel Dxc800 Syncron (Beckman Coulter Canada LP, Mississauga, ON).

Histology

Incisional liver biopsies were taken at 2, 12 and 24 hours into perfusion from the right lateral lobe, a portion of which was fixed in 10% formalin. These samples were then embedded in paraffin, stained with hematoxylin and eosin, and examined in a blinded fashion by an expert pathologist (AT). Samples were rated on the presence of necrosis, vacuolization, and congestion, as previously reported [26].

Chromogenic & enzyme-linked immunosorbent assays

Both malondialdehyde (MDA) and glutathione were measured in tissue using colorimetric assay kits (ab118970, Abcam PLC, Cambridge, UK; Invitrogen EIAGSHC, Fisher Scientific Co., Edmonton, AB), run according to manufacturer's instructions. Samples and standards were run in duplicate to a 96-well microplate and absorbance was read on a spectrophotometer (Multiskan SkyHigh; Fisher Scientific Co., Edmonton, AB) measuring optical density at 532 and 405nm, respectively. Oxidized low-density lipoprotein (oxLDL) and haptoglobin were measured in perfusate samples using porcine-specific enzyme-linked immunosorbent assays (ELISA) (MBS2508909, MyBio-Source Inc., San Diego, CA; ab205091; Abcam PLC, Cambridge, UK), run according to manufacturer's instructions.

For determination of free hemoglobin (an indicator of hemolysis), perfusate samples were centrifuged (2200×g, 10min, 4°C) and the supernatant in was diluted in Drabkin's reagent (0.61 mmol/L potassium ferricyanide, 0.77 mmol/L potassium cyanide, 1.03 mmol/L potassium dihydrogen phosphate, and 0.1% [vol/vol] TritonX-100 [Sigma-Aldrich Canada, Oakville, ON]). Trilevel Hb controls (StanBio Laboratory, Boerne, TX) were used for quality control. Absorbances were read on a spectrophotometer (SpectraMax 384 Plus, Molecular Devices Corp., Sunnyvale, CA) at 540 nm and free hemoglobin concentration was determined using the calculation previously described in the literature [27].

In vitro testing of acetaminophen metabolites & CCl_4

The effect of acetaminophen metabolites on methemoglobinemia was tested in vitro by adding them to a sample of perfusate (1:1 whole blood with modified Krebs-Henseleit). P-

aminophenol (Sigma Aldrich Canada, Oakville, ON) was added in a concentration of 100µg/mL and let sit at room temperature. Methemoglobin was measured, as described above, at 30 and 90 minutes. N-acetyl-p-benzoquinone imine (NAPQI) (Sigma Aldrich Canada, Oakville, ON) was added to a separate sample of perfusate at a concentration of 125µg/mL and methemoglobin was again measured after 90 and 180 minutes at room temperature. For comparison, acetaminophen (Sigma Aldrich Canada, Oakville, ON) was added to perfusate at a concentration of 10mg/mL and methemoglobin was measured after 60 minutes. The effect of CCl_4 on hemolysis was tested in vitro by adding CCl_4 (Sigma Aldrich Canada, Oakville, ON) to whole blood in a concentration of 10µL/mL and measuring plasma free hemoglobin after 30 minutes at room temperature, as detailed above. Free hemoglobin was measured in porcine whole blood after 30 minutes at room temperature without the addition of CCl_4 for control comparison.

Statistical analysis

Data are reported as medians (interquartile range). The Mann U Whitney test was used to compare single measures between the two groups, as well as repeated measures at different time points. A p-value of <0.05 was considered significant. All analyses were performed using GraphPad Prism v9 (GraphPad Software Inc., San Diego, CA).

3.2.3 – Results

Perfusion parameters

All control livers were perfused for 24 hours (n=5), while acetaminophen-treated livers (n=6) we perfused for a median time of 892 (566-1316) minutes. Peak hepatic artery flow and pressure were 480.1mL/min and 59.9mmHg in the untreated group and 565.3mL/min and

50.1mmHg in the acetaminophen treated group (p=0.931 and 0.046, respectively) (**Supplementary Figure 3.2.2**). Peak portal venous flow and pressure were 902.1mL/min and 7.4mmHg in the untreated group and 759.2mL/min and 8.2mmHg in the acetaminophen treated group (p=0.017 and 0.662, respectively). Median hourly bile production was no different between groups (7.7mL/hr vs. 7.0mL/hr; p=0.610).

Perfusate biochemistry

Acetaminophen-treated livers received a median dose of 8.93g (8.21-9.75g) of acetaminophen, achieving a median peak acetaminophen level of 3780µmol/L (3189-3913µmol/L). The acetaminophen concentration remained above the clinical threshold for toxicity (1000µmol/L) up to 12 hours of perfusion (**Figure 3.2.1**). Peak values of ALT (76 vs. 105U/L; p=0.429), AST (3576 vs. 4712U/L; p=0.429), and LDH (2389 vs. 2496U/L; p=0.537) were not significantly different between groups (**Figure 3.2.2**). There was a trend toward peak bilirubin being higher in the acetaminophen-treated group (45 vs. 57µmol/L; p=0.056). End lactate was significantly higher (1.0 vs. 4.4mmol/L; p=0.011) and end pH was significantly lower (7.42 vs. 7.31; p=0.030) in the acetaminophen-treated group, despite using more THAM for pH correction (33.0 vs. 55.5mL; p=0.004) (**Figure 3.2.3**). There was also a significant difference in perfusate INR, which, over the course of the perfusion, fell by 1.65 points (to 1.65 [1.53-2.45]) in the control group and rose by 0.45 points (to 4.0 [3.93-4.45]) in the acetaminophen-treated group (p=0.029).

Histology

Histological injury scores were similar between groups at the start of perfusion (p=0.900). However, by the end was significantly worse in the acetaminophen treated group (p=0.016)

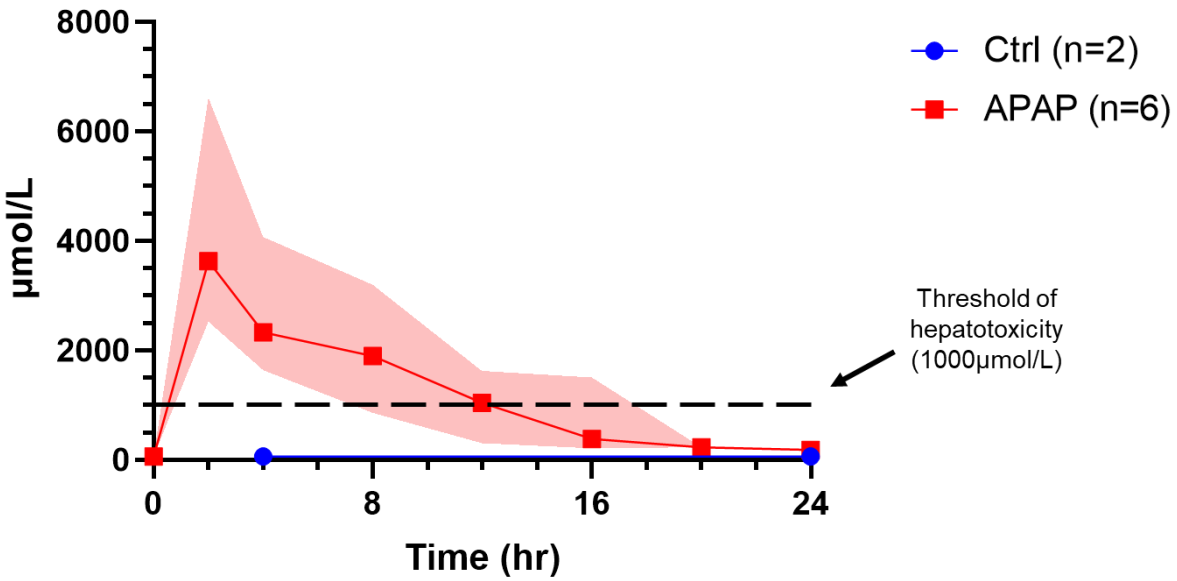

Figure 3.2.1. Acetaminophen (n-acetyl-para-aminophenol, APAP) concentration in per-fusate over the duration of liver perfusion compared to control (Ctrl) livers without addition of APAP. The highlighted area represents the IQR.

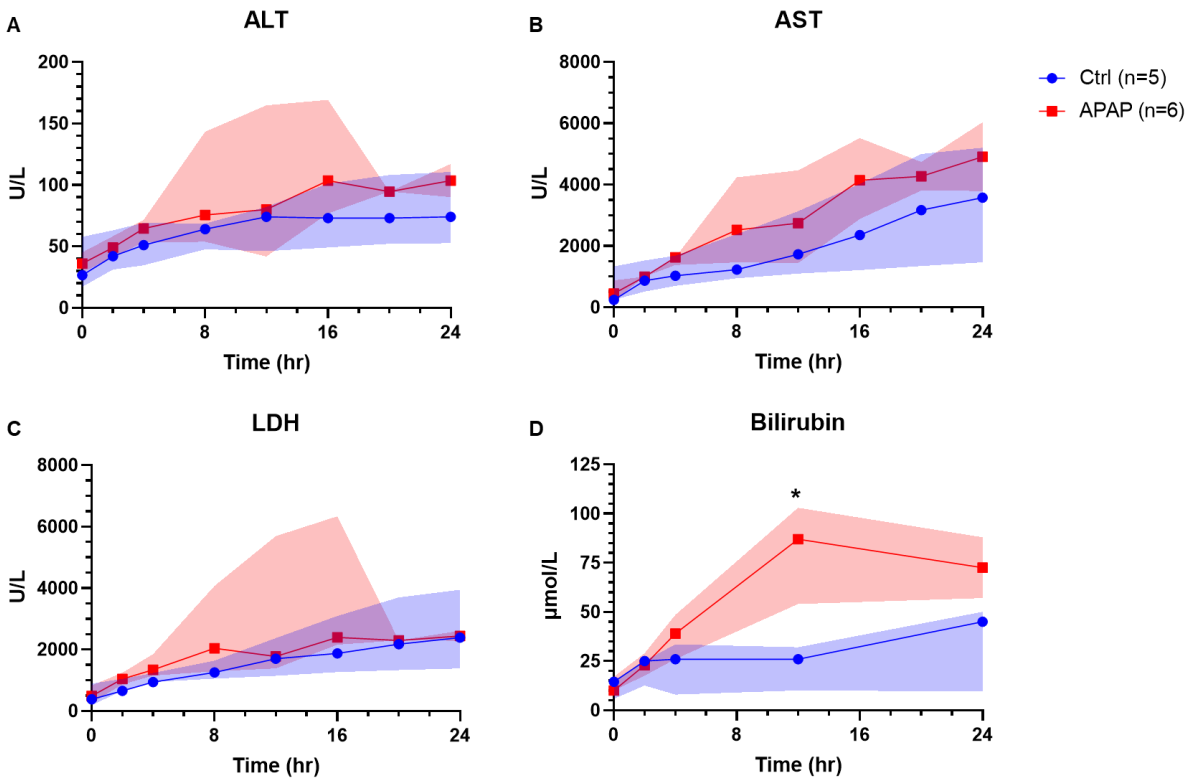

Figure 3.2.2. Perfusate concentrations of (A) alanine transaminase (ALT), (B) aspartate transaminase (AST), (C) lactate dehydrogenase (LDH), and (D) total bilirubin over the duration of perfusion in livers treated with acetaminophen (n-acetyl-para-aminophenol, APAP) compared to those without (Ctrl). The highlighted area represents IQR. * = $p<0.05$

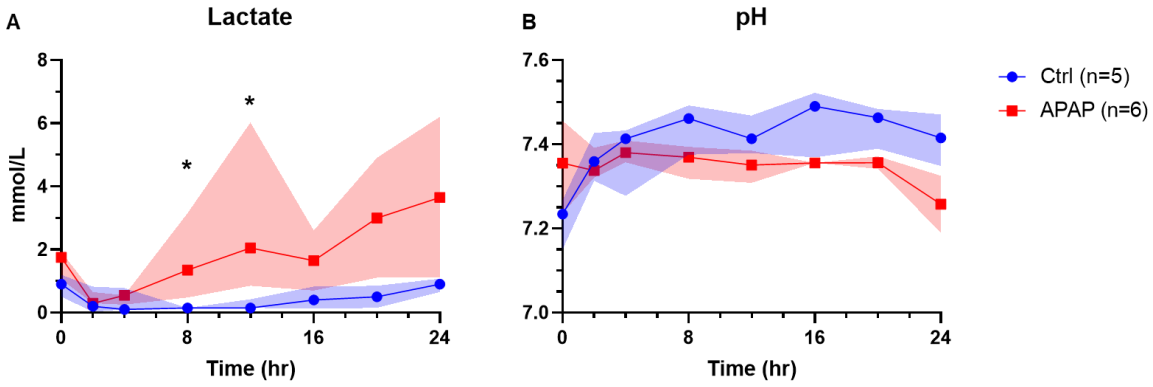

Figure 3.2.3. Perfusate (A) lactate and (B) pH over the duration of perfusion in livers treated with acetaminophen (n-acetyl-para-aminophenol, APAP) compared to those without (Ctrl). The highlighted area represents IQR. * = $p<0.05$

(**Figure 3.2.4**). Specifically, sub-scores of necrosis and vacuolization were significantly higher in the treated group by the end of perfusion (p=0.016 and 0.001, respectively).

Measures of oxidative stress

Tissue MDA as a marker of oxidative stress was significantly increased in the acetaminophen-treated group at the end of perfusion compared to controls (21.9 [21.7-22.5] vs. 30.4 [29.1-31.6] nmol; p=0.036) (**Figure 3.2.5A**). Perfusate oxLDL, a circulating marker of oxidative stress, was also found to be increased in the acetaminophen-treated group at the end compared to the start (1.81 [1.43-2.27] vs. 8.84 [6.95-11.72] nmol/mL; p=0.004), though was not significantly different from end concentration of the controls (4.36 [2.27-7.52] nmol/mL; p=0.191) (**Figure 3.2.5B**). As well, tissue glutathione content, which buffers against oxidative stress, was correspondingly decreased in acetaminophen-treated livers at the end of perfusion (31.0 [24.8-33.1] vs. 7.51 [6.4-12.0] μM/mg tissue; p=0.004) (**Figure 3.2.5C**).

Methemoglobinemia & hemolysis

Methemoglobinemia, a well described complication of acetaminophen toxicity in in vivo animal models, was also observed in our *ex vivo* model (**Figure 3.2.6**) [28]. The median peak methemoglobulin level was 19.3% for acetaminophen-treated livers, compared to 2.0% for control livers (p=0.004). Free hemoglobin, a measure of hemolysis, was significantly lower in controls at the midpoint of perfusion (12 hours) com-pared to similar time points in acetaminophen-treated livers (0.96 [0.72-1.43] vs. 2.37 [2.10-7.65] g/L; p=0.017), though was not significantly different at the end of perfusion (1.41 [1.08-3.02] vs. 2.15 [1.74-7.60] g/L; p=0.247) (**Figure 3.2.7A**).

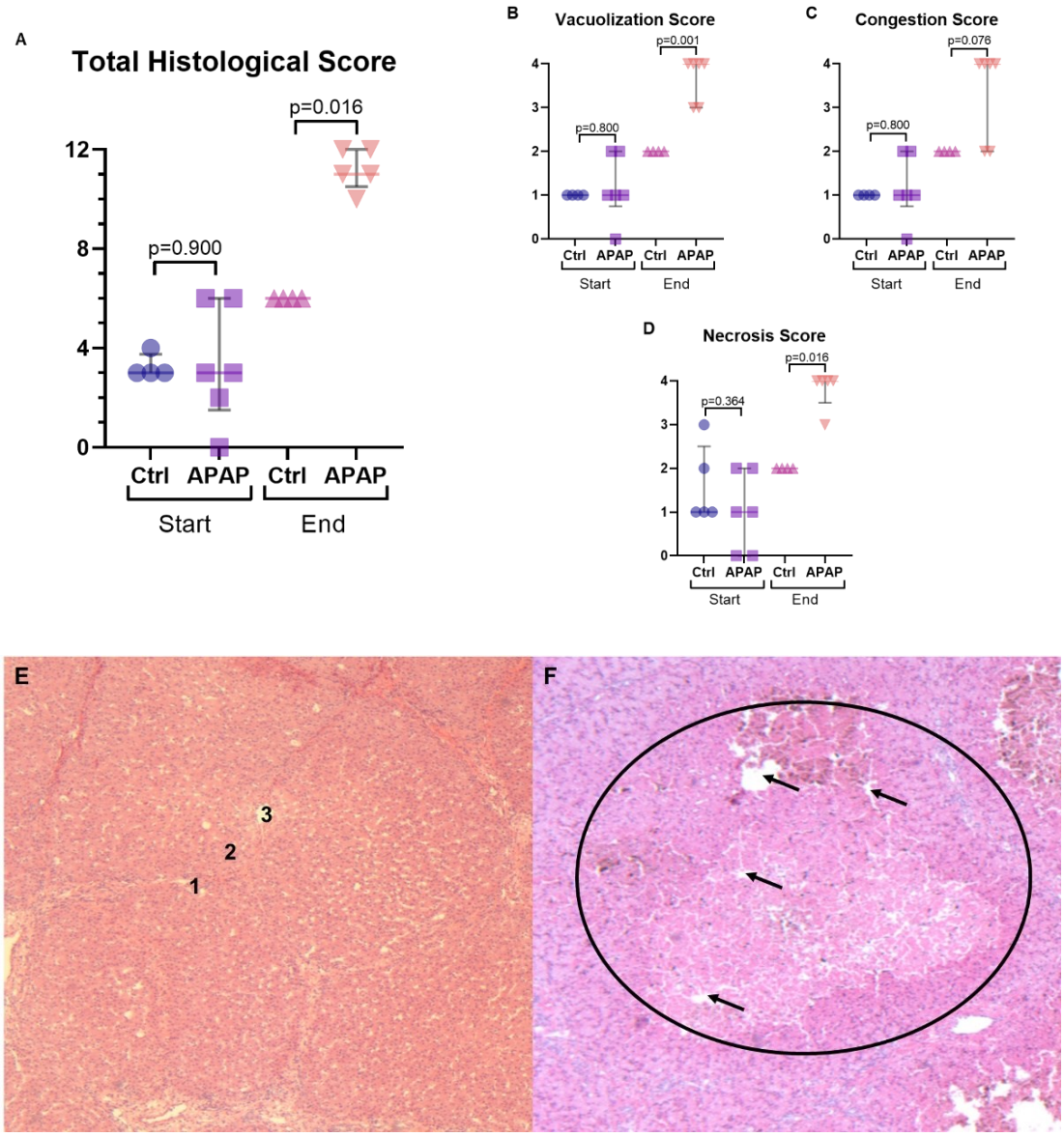

Figure 3.2.4. Histological scoring for control (Ctrl) and acetaminophen-treated (n-acetyl-para-aminophenol, APAP) livers. Comparisons made at the start and end of perfusion based on (A) total histological score and sub-scores of (B) vacuolization, (C) congestion, and (D) necrosis. The data is presented as median and IQR. A representative image showing preserved hepatic architecture is shown (E), with zones 1-3 readily identifiable. This is contrasted with an image from an acetaminophen treated liver (F) showing pan-lobular hepatocyte necrosis and congestion (circle) and diffuse sinusoidal dilation (arrows).

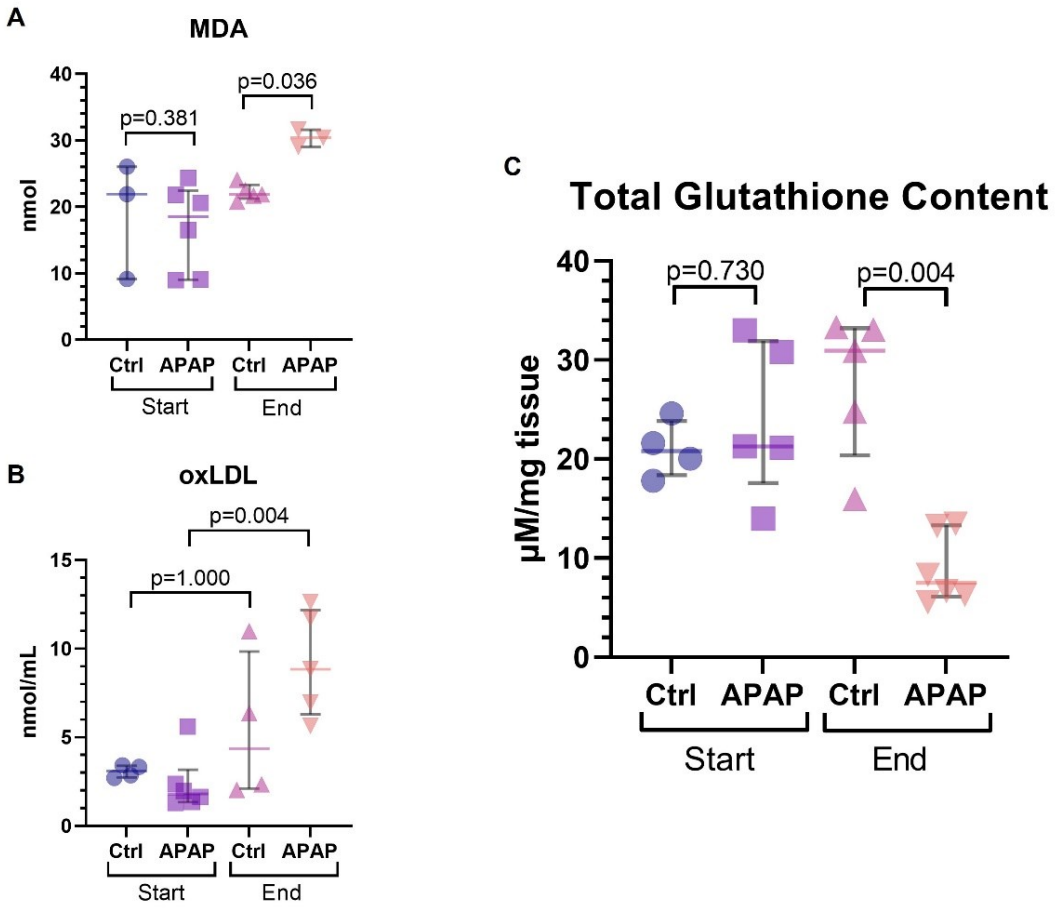

Figure 3.2.5. Markers of oxidative stress during liver perfusion in livers treated with acetaminophen (n-acetyl-para-aminophenol, APAP) compared to those without (Ctrl): (A) tissue malondialdehyde (MDA), (B) perfusate oxidized low-density lipoprotein (oxLDL), and (C) tissue glutathione. The data is presented as median and IQR.

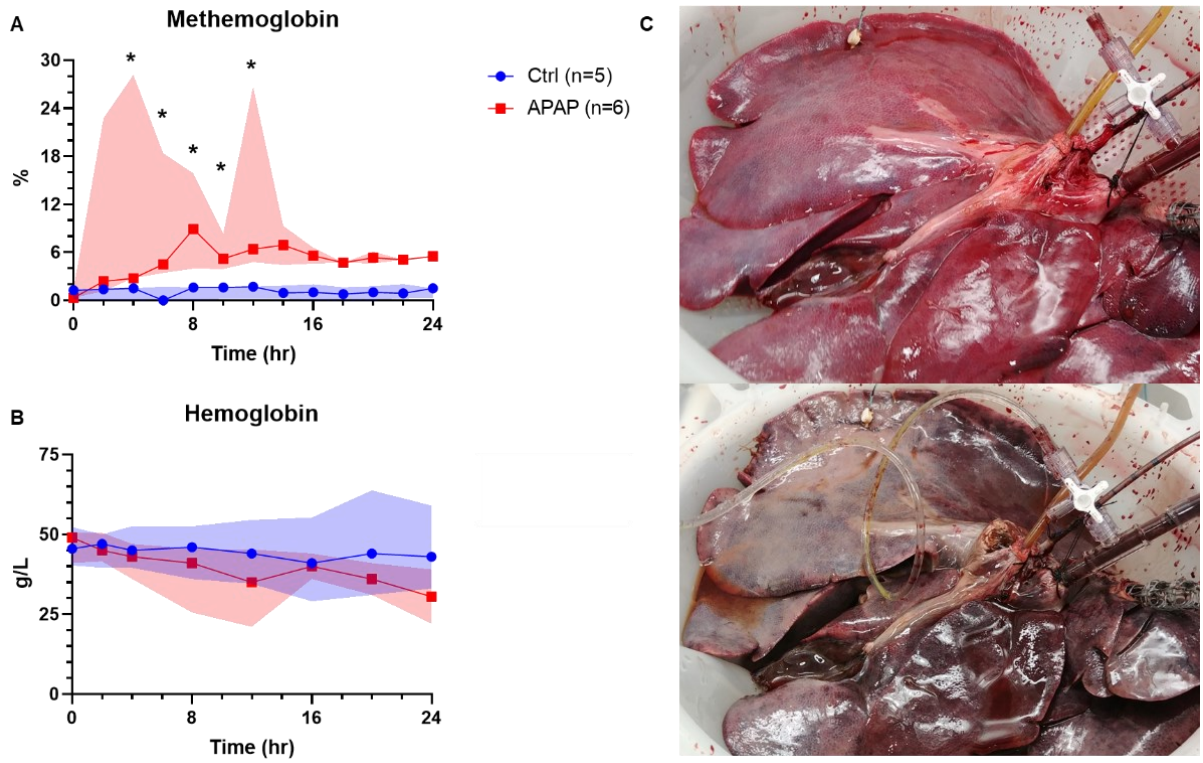

Figure 3.2.6. Effect of acetaminophen of perfusate (A) methemoglobin and (B) hemoglobin concentrations in livers treated with acetaminophen (n-acetyl-para-aminophenol, APAP) compared to those without (Ctrl). The highlighted area represents IQR; * = $p<0.05$. Perfused liver before (top) and after (bottom) development of methemoglobinemia (C).

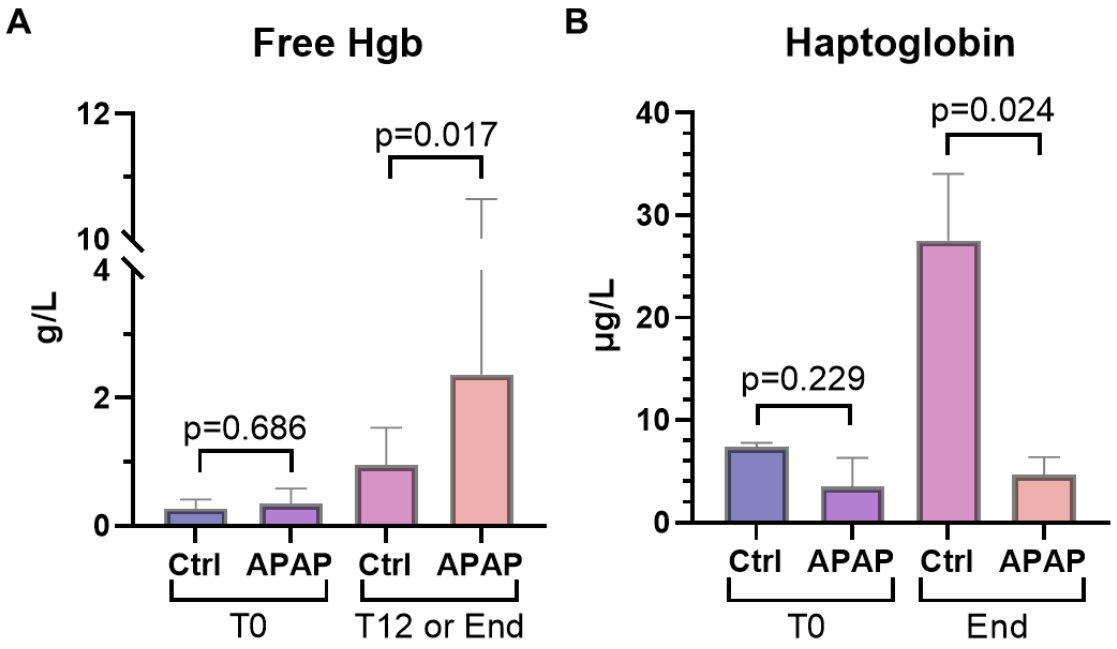

Figure 3.2.7. Perfusate (A) free hemoglobin (Hgb) and (B) haptoglobin over the course of liver normothermic perfusion in livers treated with acetaminophen (n-acetyl-para-aminophenol, APAP) compared to those without (Ctrl). The data is presented as median and IQR.

Hemolysis was further evident in the median drop in hemoglobin over the course of the perfusion, which was 19.0g/L for the acetaminophen-treated group and only 1.0g/L for the control livers (p=0.008). Median perfusate haptoglobin at the endpoint was significantly lower in the acetaminophen-treated group (27.56 [22.73-30.80] vs. 4.67 [2.90-6.09] µg/mL; p=0.024) (**Figure 3.2.7B**).

It had been suggested that the metabolite of acetaminophen responsible for methemoglobinemia was PAP [29]. The addition 100µg/mL of PAP in vitro to a sample of perfusate resulted in significant methemoglobin compared to the control after 30 (1.4% [0.8-1.6%] vs. 6.6% [5.2-9.6%]; p=0.008) and 90 minutes (1.5% [1.5-1.8%] vs. 17.4% [14.9-20.2%]; p=0.008) at room temperature (**Figure 3.2.8A**, **Supplementary Figure 3.2.3**). Adding acetaminophen (10mg/mL) and NAPQI (125µg/mL) to perfusate in vitro did not result in the development of significant methemoglobinemia (**Figure 3.2.8B & C**).

Carbon tetrachloride (CCl_4)

CCl_4 was trialed as an alternative to acetaminophen in two perfused livers, but similar challenges were encountered. The first liver was treated with 10mL of CCl_4 and a second liver ith 5mL. The first was perfused for 1160 minutes and the second for 661 minutes. Both had higher peak ALT (518 and 298U/L), AST (12,929 and 15,271U/L), and LDH (8,912 and 8,486U/L) compared to controls. End pH (6.99 and 7.13) and lactate (10.5 and 9.9mmol/L) were similarly abnormal. Comparisons with controls were not statistically significant owing to the small sample size.

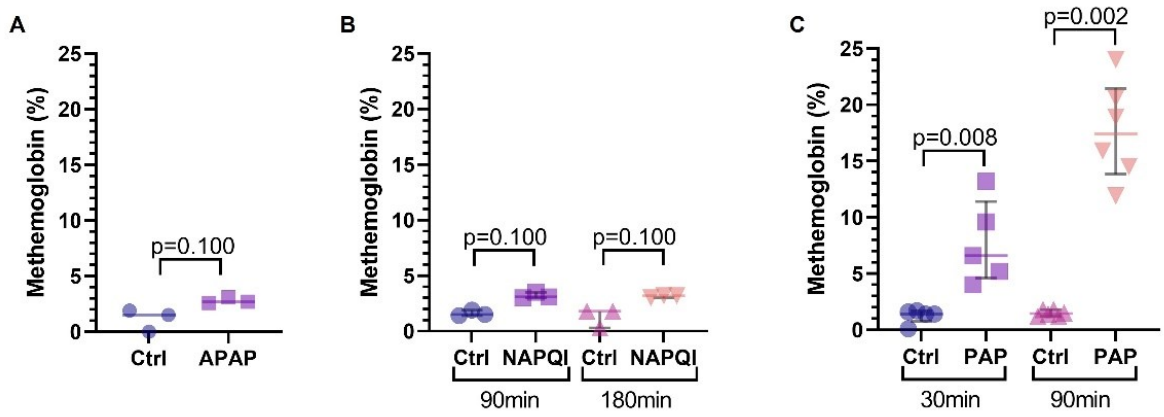

Figure 3.2.8. Effects of (A) acetaminophen and its metabolites, (B) n-acetyl-p-benzoquinone imine (NAPQI), and (C) p-aminophenol (PAP) on the development of methemoglobinemia *in vitro* in whole porcine blood. The data is presented as median and IQR.

Hemolysis was particularly problematic in the CCl_4-treated livers. Free hemoglobin was measured at 8.5 and 5.0g/L by the end of perfusion. Hemoglobin concentration also decreased by25g/L in both perfusions. CCl_4 as the causative agent of hemolysis was again demonstrated *in vitro*. CCl_4 added to whole blood at a concentration of 10µL/mL resulted in free hemoglobin of 13.67g/L (5.41-15.48g/L) after 30 minutes, compared to 0.37g/L (0.30-0.38g/L) in controls (p=0.008) (**Supplementary Figure 3.2.4A & B**). In addition to the hemolysis, incompatibilities with circuit components (mainly the polycarbonate stopcocks and tubing connectors) made further attempts untenable (**Supplementary Figure 3.2.4C**).

3.2.4 – Discussion

Our study set out to develop a large animal model of ALF in an isolated, perfused liver to abrogate ethical challenges, cost and complexity of toxicity studies in the intact pig. We were able to show that the addition of acetaminophen in the *ex vivo* setting resulted in massive liver injury. However, we found that the degree of injury was not well represented by perfusate biochemistry and was confounded by the presence of substantial methemoglobinemia and an overall increase in hemolysis. Additionally, we were able to demonstrate in vitro that the cause of the methemoglobinemia was due to PAP, as opposed to acetaminophen itself or its main hepatotoxic metabolite, NAPQI. We also performed precursory studies with CCl4 as an alternative hepatotoxic agent and identified major limitations with hemolysis for its use in the *ex vivo* setting.

Methemoglobinemia is a well described complication of acetaminophen toxicity in large animal models, including swine and canines [30,31]. However, it is only rarely seen in clinical cases of acetaminophen overdose [32]. This has made it challenging to find relevant preclinical models, as below a certain dose, animals tend to recover without developing lasting liver injury,

while above it, they succumb to the effects of methemoglobinemia. Accumulation of methemoglobin differs from the mechanism by which acetaminophen is known to cause hepatocyte injury and fulminant liver failure, which results from an accumulation of NAPQI by cytochrome P450 metabolism of acetaminophen [33]. In contrast, PAP has been proposed as the metabolite responsible for methemoglobinemia and occurs as a result of deacetylation of acetaminophen [29]. Our in vitro studies confirmed that PAP readily induces methemoglobin formation in blood-base perfusate, whereas exposure to acetaminophen or NAPQI does not. The only swine models that have been able to overcome this challenge with methemoglobinemia have done so using titrated doses of acetaminophen over several hours [15,16].

Despite titrating acetaminophen dosing to methemoglobin level in the *ex vivo* setting, we were not able to avoid onset of experiment-limiting methemoglobinemia (owing to the drop in functional hemoglobin) as the organ continued to be perfused. Methemoglobin develops as a result of the oxidation of the iron molecule in hemoglobin from the ferrous (Fe^{2+}) to the ferric state (Fe^{3+}) [34]. Whether porcine blood is more prone to methemoglobin formation, the additional cellular stress arising from exposure to the artificial components of the *ex vivo* circuit likely further contributed to its development [35]. Supporting this is the observation of methemoglobinemia developing after extended perfusion of discarded hu-man livers [36].

Though the use of CCl_4 in models of both acute and chronic liver injury has been well described, we found it unsuitable as an agent for hepatotoxicity in the *ex vivo* setting [37]. Doses were chosen based on a porcine model of ALF, in which CCl_4 was injected directly into the portal vein [38]. However, this resulted in significant hemolysis, which both confounded the injury pattern and hampered perfusion of the liver in our model. CCl_4 is typically dosed intraperitoneally or *per os*, which may mitigate its hemolytic effects in reported models [37]. We were unable to

find mention of hemolysis amongst studies using CCl_4 to induce liver failure. In addition, CCl_4 proved to be quite a powerful solvent such that its mere infusion through polycarbonate stopcocks resulted in their degradation (**Supplementary Figure 3.2.4**). A corollary of this observation is that CCl_4 may be useful for models of hemolytic anemia.

There are several alternative agents that could be considered to induce ALF. D-galactosamine has commonly been used, often in combination with lipopolysaccharide, to induce ALF in animal models [39]. As an amino sugar it is likely to be compatible with *ex vivo* perfusion components. However, it is not clear the degree to which immune-mediated damage is necessary to induce fulminant liver failure and whether that could be achieved in an isolated organ. In addition, it has been reported to have some batch-to-batch variability in potency, owing to is isolation from a biological source, which may affect model reproducibility depending on the dose required. Thioacetamide and azoxymethane are agents that have been used in vivo in rodent models [40,41]. However, there is limited experience with large animal models. As small molecule, biologically active chemicals, both would first warrant in vitro testing to ensure compatibility with porcine blood and circuit components. A-amanitin, the cyclic peptide produced by the *Amanta* genus of mushrooms, may be a more suitable candidate, as its structure is unlikely to cause unfavourable interactions with blood or artificial materials and, with its mechanism of RNA polymerase inhibition, it would be directly hepatotoxic [42]. There is even some experience with in vivo models in swine and non-human primates [43,44]. However, the cost of the molecule ($250-350 USD/mg) may be somewhat of a deterrent. Alternative measurements of liver injury that are more sensitive may be of use in developing consistency amongst such models, but ultimately they will need to show benefit by some clinically accepted parameter (such as transaminases or lactate clearance) to justify clinical translation.

Among important considerations for future use of isolated perfused organs as models of disease with the aim of achieving reproducibility are the development of standardized protocols for organ procurement, including the use of appropriate cold preservation solutions and cooling techniques if the organ is to be kept cold or minimizing warm ischemia if the organ is to be perfused immediately. As well, appropriate surgical skills are necessary to ensure organ procurement occurs efficiently, and the risks of undue injury are minimized. Similarly, protocols for organ perfusion itself, such perfusion pressures or flows and drug additives, should be consistent across the experiment and researchers should be comfortable with the technical aspects of their setup to allow for basic troubleshooting. If circuit components are to be reused (which is typical practice in the experimental setting), they should be cleaned thoroughly to avoid contamination between experiments and replaced entirely after a certain period of time or number of uses. Otherwise, routine experimental practices, such as maintaining standards for animal care and welfare, randomizing and blinding when possible, and using the same reagents between experiments, should be followed.

Efficient, reproducible large animal models of ALF are needed to develop effective treatments to screen for new interventional therapies that improve patient out-comes and reduce the need for liver transplantation. *Ex vivo* liver NMP offers a new platform on which to implement such models, with the additional benefit of reducing animal suffering and safely extending the duration of experiments. However, our early experience identified several challenging factors that hindered development of a reproducible model using two well-known hepatotoxic agents in this setting. Our finding from the present study provides useful insight to other investigators in the field, and description of this approach will provide additional information for researchers in the field of liver injury to improve their understanding of models of organ injury and aid the

development of efficient animal models. Future studies altering the mode of delivery or trialing novel agents may be able to overcome these barriers to make use of NMP as a model for ALF. Adequately defining ALF in the context of NMP will be further important, as there may be some elements that are not well represented in this setting (e.g., massive inflammatory response).

3.2.5 – References

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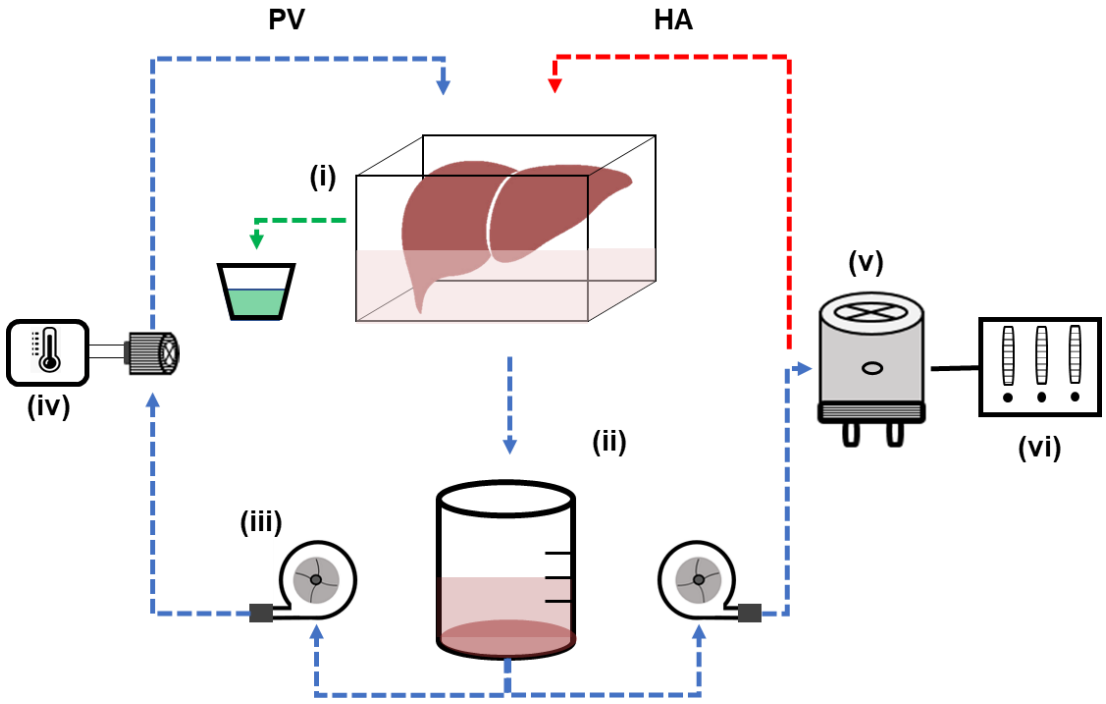
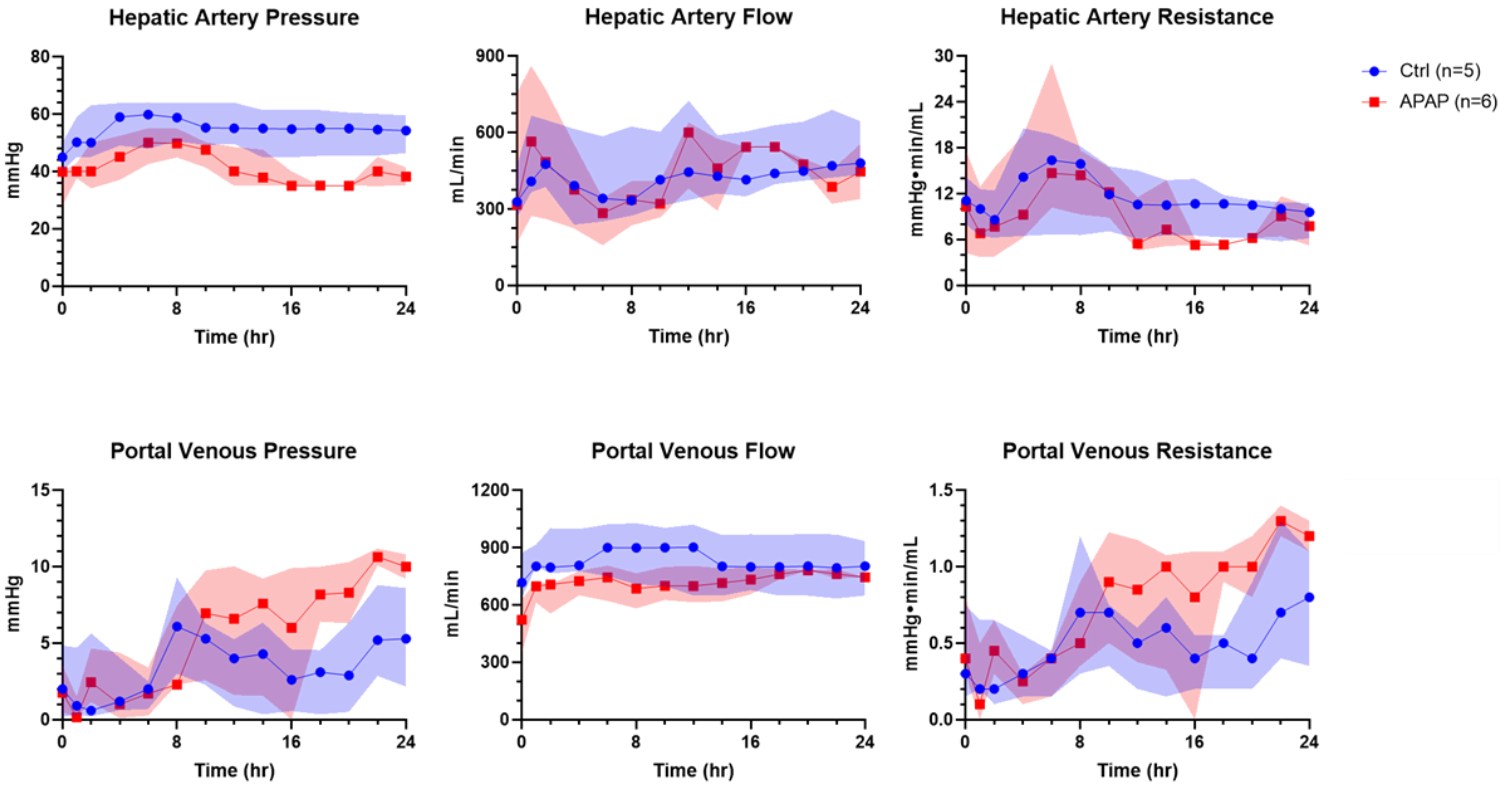
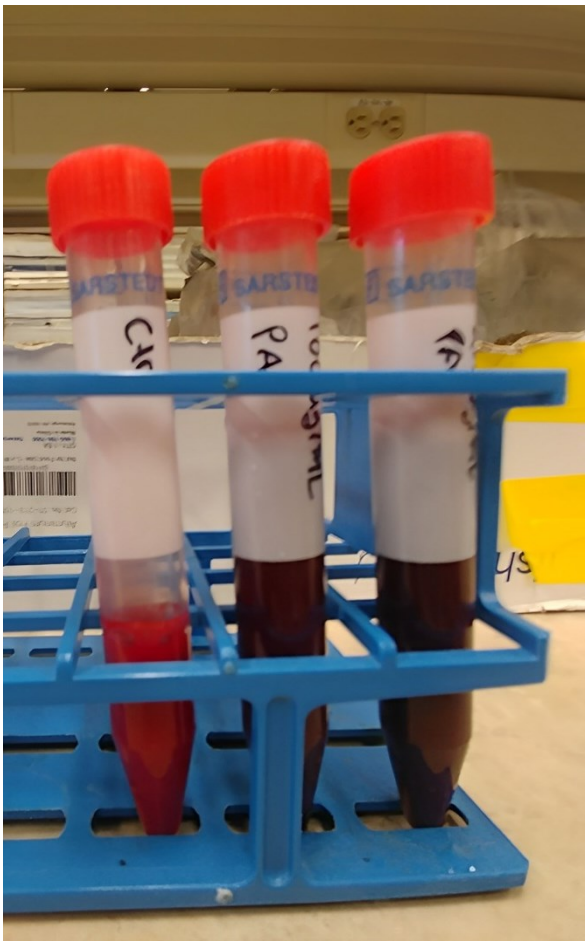
3.2.6 – Supplementary Material



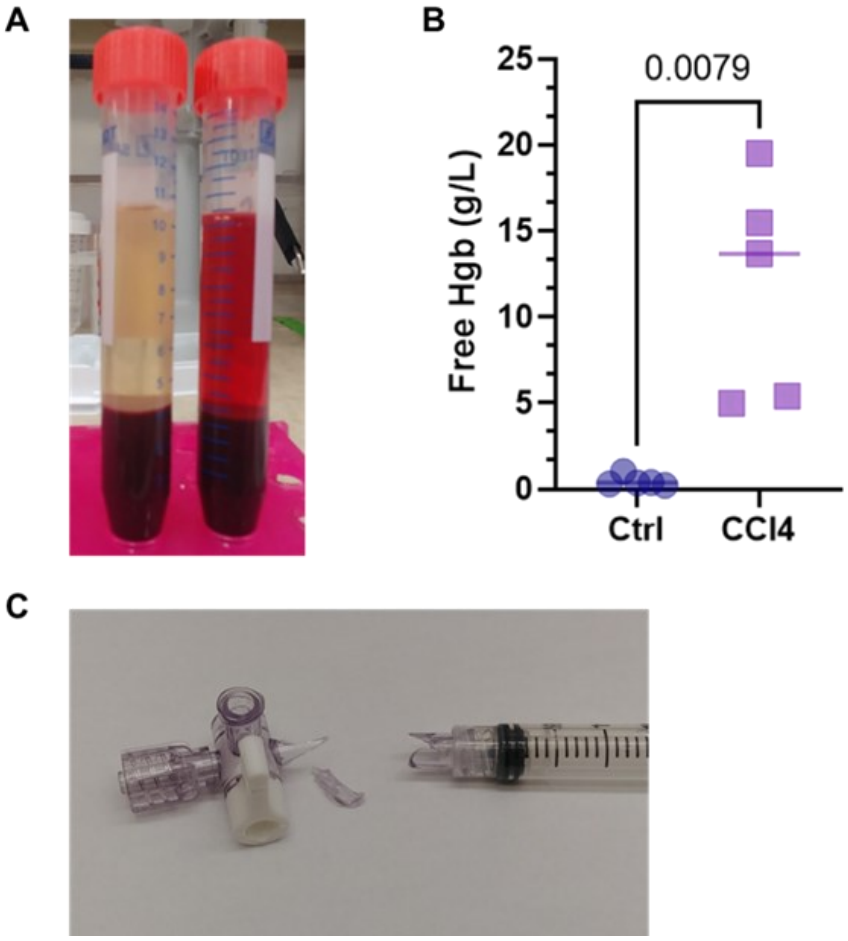
Supplementary Figure 3.2.1. Schematic of experimental *ex vivo* organ perfusion circuit. Perfusate drains from the organ chamber (i) into the reservoir (ii) and is recirculated by centrifugal pumps (iii). The pump on the left circulates perfusate through a heat ex-changer (iv), while the pump on the right circulates perfusate through an oxygenator (v), which is supplied a physiologic gas mixture (vi). HA, hepatic artery; PV, portal vein.



Supplementary Figure 3.2.2. Perfusion parameters for normothermic porcine liver perfusion, with (n-acetyl-para-aminophenol, APAP) or without (Ctrl) acetaminophen as a hepatotoxic agent. The highlighted area represents IQR.



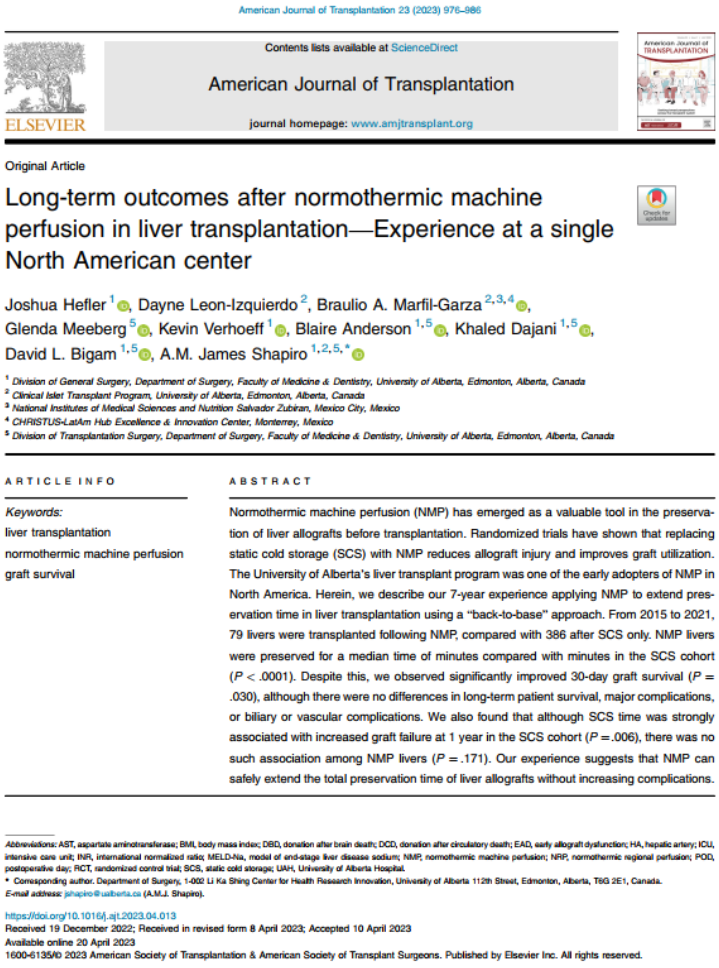
Supplemental Figure 3.2.3. Visualization of methemoglobinemia 90 minutes after the addition of p-aminophenol (PAP) to a sample of perfusate (1:1 Krebs-Henseleit and porcine whole blood). Tubes from left to right contain 0, 100, and 250µg/mL of PAP.



Supplementary Figure 3.2.4. Carbon tetrachloride (CCl4) causes hemolysis in vitro in porcine whole blood samples, as demonstrated (A) visually and (B) by measurement of free hemoglobin after 30 minutes at room temperature. CCl4 also degrades polycarbonate circuit components (C).

CHAPTER 4 – LONG TERM OUTCOMES AFTER NORMOTHERMIC MACHINE PERFUSION IN LIVER TRANSPLANTATION – EXPERIENCE AT A SINGLE NORTH AMERICAN CENTRE

4.1 - Long term outcomes after normothermic machine perfusion in liver transplantation – experience at a single North American centre



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Original Article

Long-term outcomes after normothermic machine perfusion in liver transplantation—Experience at a single North American center

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ABSTRACT

Normothermic machine perfusion (NMP) has emerged as a valuable tool in the preservation of liver allografts before transplantation. Randomized trials have shown that replacing static cold storage (SCS) with NMP reduces allograft injury and improves graft utilization. The University of Alberta's liver transplant program was one of the early adopters of NMP in North America. Herein, we describe our 7-year experience applying NMP to extend preservation time in liver transplantation using a "back-to-base" approach. From 2015 to 2021, 79 livers were transplanted following NMP, compared with 386 after SCS only. NMP livers were preserved for a median time of minutes compared with minutes in the SCS cohort ($P < .0001$). Despite this, we observed significantly improved 30-day graft survival ($P = .030$), although there were no differences in long-term patient survival, major complications, or biliary or vascular complications. We also found that although SCS time was strongly associated with increased graft failure at 1 year in the SCS cohort ($P = .006$), there was no such association among NMP livers ($P = .171$). Our experience suggests that NMP can safely extend the total preservation time of liver allografts without increasing complications.

Abbreviations: AST, aspartate aminotransferase; BMI, body mass index; DBD, donation after brain death; DCD, donation after circulatory death; EAD, early allograft dysfunction; HA, hepatic artery; ICU, intensive care unit; INR, international normalized ratio; MELD-Na, model of end-stage liver disease sodium; NMP, normothermic machine perfusion; NRP, normothermic regional perfusion; POD, postoperative day; RCT, randomized control trial; SCS, static cold storage; UAH, University of Alberta Hospital.

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Title: Long term outcomes after normothermic machine perfusion in liver transplantation – experience at a single North American centre

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4.1.1 – Preface

Normothermic machine perfusion (NMP) has emerged as a valuable tool in the preservation of liver allografts before transplantation. Randomized trials have shown that replacing static cold storage (SCS) with NMP reduces allograft injury and improves graft utilization. The University of Alberta's liver transplant program was one of the early adopters of NMP in North America. Herein, we describe our seven-year experience applying NMP to extend preservation time in liver transplantation using a 'back-to-base' approach. From 2015 to 2021, 79 livers were transplanted following NMP compared to 386 after SCS only. NMP livers were preserved for a median time of 847min compared to 288.5min in the SCS cohort ($p<0.0001$). Despite this, we observed significantly improved 30-day graft survival ($p=0.030$), though no differences in long term patient survival, major complications or biliary or vascular complications. We also found that while SCS time was strongly associated with increased graft failure at one year in the SCS cohort ($p=0.006$), there was no such association amongst NMP livers ($p=0.171$). Our experience suggests that NMP can safely extend the total preservation time of liver allografts without increasing complications.

4.1.2 – Introduction

Despite advances in surgical technique, immunosuppressive therapy, and perioperative care over the past decades, a major limitation of solid organ transplantation remains the availability of suitable organs.[1] This has consequently lead to the increased use of marginal grafts, such as those with prolonged cold ischemia times. In Canada, this has been particularly problematic, as the population is widely distributed (population density of 4 people/km^2) compared to Europe or the United States.[2] In an effort to enlarge the donor pool and improve graft quality for orthotopic liver transplantation, machine perfusion, as an alternative to the standard static cold storage (SCS), has been proposed as a means of extending preservation time, without compromising graft function and potentially improving clinical outcomes.[3]

Several different types of machine perfusion (varying by temperature) have been explored for preservation of liver grafts. Normothermic machine perfusion (NMP) preserves the organ at physiologic temperature (~37ºC), which offers the opportunity for predictive metabolic assessment, beyond preservation.[4] Evidence for its use has been bolstered by the large multicentre, randomized control trial (RCT), published by Nasralla *et al.* using the OrganOx metra® device, which showed decreased organ injury (measured by aspartate transaminase [AST]) despite longer preservation time, and increased rate of graft utilization (i.e., lower rate of organ discard).[5] Markmann *et al.* published similar findings in a subsequent RCT using the Organ Care Systems™ NMP device.[6]

The University of Alberta Hospital was one of the first centres in North America to adopt this new technology in the setting of liver transplantation. Beginning in early 2015, our centre began selectively utilizing NMP for the preservation of liver allografts before transplantation, which allows us to report the largest single centre experience in North America.[7] One limitation

of our own approach has been an inability to transport this heavy and cumbersome technology between donor and recipient centres given the geography and our routine use of small planes to transport livers across vast distances, so we used NMP mostly in a 'back-to-base' manner to avoid that challenge, except in local donors where the cold and transport time was short. While convenient and most practical for us, this may have hampered maximal benefit from this technology as there is an inherent obligate period of SCS before NMP. Herein, we present the clinical outcomes of patients transplanted with livers following NMP over a 7-year period compared with those transplanted after SCS only.

4.1.3 – Methods

Study Design

This study was designed as a phase I/II non-randomized clinical trial to assess the safety of NMP on human livers prior to transplantation, as well as its efficacy in preventing organ injury. It was registered at clinicaltrials.gov with the study identifier NCT03089840. The study was approved by the Health Research Ethics Board at the University of Alberta and Health Canada (IRB 00043239, ID Pro00043239). All liver transplantations were carried out at the University of Alberta Hospital (UAH) in Edmonton, Alberta, Canada from January 1, 2015 to December 31, 2021. The UAH is the primary liver transplant centre for two Canadian provinces (Alberta, Saskatchewan), as well as northern British Columbia and the Northwest Territories, representing a catchment area of close to 6 million people. Follow up was continued until June 30, 2022. Consent was obtained at the time of assessment for liver transplantation and confirmed on the date of transplant. Background characteristics collected included sex, age, body mass index (BMI), model for end stage liver disease-sodium (MELD-Na) score, and reason for transplantation. The

primary outcome was graft survival. Secondary outcomes included mortality, early allograft dysfunction (EAD; defined as having one of more of AST>2000U/L on post-operative day [POD] 1-7, serum bilirubin >170μmol/L on POD7 or INR≥1.6 on POD7), intensive care unit (ICU) and hospital lengths of stay, and vascular and biliary complications. Only significant biliary and vascular complications requiring intervention (i.e., ≥ Clavien-Dindo 3a) were included. Ischemic cholangiopathy was defined as the presence of non-anastomotic biliary strictures occurring with a patent, non-stenotic hepatic artery. Outcomes were compared with liver transplant recipients whose transplant occurred during the same period, but whose graft was preserved by SCS only. Living donor, pediatric, and domino liver transplantations were excluded from the SCS cohort. Five livers subject to NMP that were subsequently discarded were excluded from analysis (**Supplementary Table 4.1.1**).

Normothermic Machine Perfusion

Livers allocated for NMP at the discretion of the operating surgeon (e.g., for considerations of bed or patient availability, to avoid operating at odd hours or when two livers were available at the same time) were preserved using the OrganOx metra® system. Livers were procured in the standard fashion, including *in situ* flush with cold histidine-tryptophan-ketoglutarate (HTK; Custodiol) or University of Wisconsin solution (UW; Bridge to Life). Upon procurement, organs were stored in HTK or UW at 4ºC for transportation from the donor centre to the UAH. NMP was initiated promptly with minimal cold storage for the subset of local donors. Once received at the UAH, the OrganOx was primed with 500mL of Gelofusine (B. Braun Canada Ltd., Mississauga, ON, Canada) and 3 units of type-O packed red blood cells. Boluses of antibiotics (cefuroxime 750mg; GlaxoSmithKline Canada, Mississauga, ON, Canada), heparin (10,000U; Fresenius Kabi

Canada, Toronto, ON, Canada), and calcium gluconate (10%, 10mL; Fresenius Kabi Canada, Toronto, ON, Canada).[8] The liver was then flushed with 1L of Gelofusine and 500mL of 5% human albumin prior to attaching it to the circuit.

During NMP, livers were monitored with point-of-care blood gas analysis, as well as biochemical analysis of perfusate (e.g., alanine aminotransferase, AST, bilirubin) performed by the clinical laboratory, done at the onset of NMP and every 2 hours thereafter. The livers received infusions of heparin (833U/hr; Fresenius Kabi Canada, Toronto, ON, Canada), epoprostanol (8μg/hr; GlaxoSmithKline Canada, Mississauga, ON, Canada), insulin (6.7U/hr; Eli Lilly Canada Inc., Toronto, ON, Canada), sodium taurocholate (0.19g/hr; New Zealand Pharmaceuticals Ltd., Palmerston North, New Zealand), and nutrition (Nutriflex®, B. Braun Canada Ltd., Mississauga, ON, Canada).[8] Glucose was maintained within physiological parameters (5-8mmol/L) by adjusting the Nutriflex infusion and sodium bicarbonate (8.4%; Hospira Inc., Lake Forest, IL, United States) was used to maintain a physiological pH (7.35-7.45). Once the recipient hepatectomy was performed, livers were disconnected from perfusion and flushed with cold Ringer's lactate solution immediately before implantation in compliance with clinical practice.

Perioperative Management

Implantation of the liver graft occurred in the standard fashion in both groups. Livers were transplanted with the standard caval replacement, without bypass or temporary portocaval shunt. Patients were transferred to the intensive care unit immediately post-operatively and subsequently extubated and transferred to a standard ward bed depending on their clinical condition. Standard protocols for tacrolimus-based immunosuppression were initiated post-operatively, with some patients receiving sirolimus due to pre-existing renal dysfunction.

Statistical Analysis

Statistical analysis was performed using Stata 16.1 (StataCorp LLC). Data are presented as medians with interquartile range or proportions, where appropriate. Comparisons between medians were done using Mann-Whitney U tests, while comparisons between proportions used the chi-squared test. To eliminate the effect of confounding on the relationship between storage type and several outcome variables, transplanted livers from the SCS cohort were matched 1:1 with livers from the NMP cohort on the basis of MELD-Na, donor risk index, donor and recipient age, and donor type (i.e., donation after circulatory death [DCD] vs. brain death [DBD]).[9] Exact matching was done with respect to donor type, while other variables were distance matched to achieve values that were as close as possible. Additionally, we employed multivariate logistic regressions using a hypothesis-driven purposeful selection methodology, in which bivariate analysis of variables with a p-value <0.1 or from variables previously deemed clinically relevant to our primary outcome were used to generate a main effects model. Kaplan-Meier survival curves were plotted for graft and patient survival, as well as biliary and vascular complication-free survival, using the log rank test for comparison. The same statistical methods were used to compare matched cohorts and sub-sets of cohorts.

4.1.4 – Results

During the study period, 80 orthotopic liver transplantations were performed following NMP in 79 recipients. A single patient withdrew consent after being transplanted and was excluded from the study. Over the same period at our centre, 386 liver transplants were performed in 370 recipients, excluding pediatric, living donor, and domino graft transplantation. Median follow up

time from first transplant was 3.77 (1.63-4.52; max 7.17) years for patients in the NMP cohort and 3.00 (1.26-5.19; max 7.43) years for patients in the SCS cohort (p=0.965).

Baseline Donor & Recipient Demographics

Most donor characteristics were similar between groups, including age, sex, and BMI (**Table 4.1.1**). However, the groups differed slightly in the proportion of donors who had died due to overdose (19.4% of the SCS cohort vs. 10.1% of the NMP cohort; p=0.049), as well as other causes (e.g., primary central nervous system malignancy, medical assistance in dying), which made up 5.2% of donors in the SCS cohort compared to 13.9% in the NMP cohort (p=0.005). The NMP group had a higher proportion of DCD donors, though this was not statistically significant (13.2% in the SCS cohort vs. 20.3% in the NMP cohort; p=0.104). Median SCS time was significantly longer in the NMP group (288.5 [193-383] minutes in the SCS cohort vs. 359 [301-412] minutes in the NMP cohort; p=0.0001). The median warm perfusion time was 475 (387-620) minutes, leading to a total preservation time of 847 (767-964) minutes in the NMP cohort (compared to 289 [193-383] minutes in the SCS group; p<0.0001) (**Figure 4.1.1**). Recipients were similar in their characteristics, with no difference in age, sex, BMI, reason for transplant or the proportion of retransplant patients between groups. The NMP cohort had a slightly higher proportion of multi-organ transplant recipients (kidney or heart), though this was not statistically significant owing the small numbers in both groups (1.0% in the SCS cohort vs. 3.8% in the NMP cohort; p=0.067).

No significant differences were observed amongst donors or recipients following DCD donation, with the exception of total preservation time, which was higher in the NMP cohort (317 [259-383] minutes in the SCS cohort vs. 965 [734.5-1217.5] minutes in the NMP cohort;

Table 4.1.1. Donor & recipient demographics.

	SCS (n=386)	NMP (n=79)	p value
Donor Characteristics			
Age, years	37 (26-53)	40 (25-56)	0.526
Female (%)	144 (37.3)	27 (34.2)	0.599
BMI	25.8 (22.3-29.4)	25.7 (22.4-29.2)	0.765
Cause of death (%)			
Intracranial event	109 (28.2)	22 (27.9)	0.944
Anoxia/hypoxia	104 (26.9)	21 (26.6)	0.947
Trauma	78 (20.2)	17 (21.5)	0.792
Overdose	75 (19.4)	8 (10.1)	0.049
Other	20 (5.2)	11 (13.9)	0.005
Local (%)	139 (36.0)	23 (29.1)	0.241
DCD (%)	51 (13.2)	16 (20.3)	0.104
Preservation Details			
Functional warm ischemia time, minutes*	19 (12-27)	21.5 (15-25.5)	0.527
Cold ischemia time, minutes	288.5 (193-383)	359 (301-412)	0.0001
NMP time, minutes	N/A	475 (387-620)	N/A
Rewarm time, minutes	40 (35-46)	40 (36-47)	0.280
Total storage time, minutes	288.5 (193-383)	847 (767-964)	<0.0001
Recipient Characteristics			
Age, years	56.0 (45.7-62.4)	58.1 (46.9-63.1)	0.845
Female (%)	118 (30.6)	23 (29.1)	0.798
BMI	27.5 (24.2-32.1)	28.0 (23.8-35.0)	0.578
MELD-Na (at time of transplant)	19 (13-27)	16 (11-23)	0.064
Creatinine, µmol/L	84 (65-111)	84 (65-103)	0.843
Diagnosis (%)			
EtOH cirrhosis	125 (32.4)	25 (31.7)	0.898
Hep C cirrhosis	93 (24.1)	20 (25.3)	0.817
Hep B cirrhosis	17 (4.4)	6 (7.6)	0.233
NASH cirrhosis	62 (16.1)	12 (15.2)	0.847
HCC	132 (34.2)	25 (31.7)	0.662
Autoimmune	23 (6.0)	4 (5.1)	0.757
PBC	26 (6.7)	4 (5.1)	0.581
PSC	37 (9.6)	8 (10.1)	0.882
Retransplant (%)	34 (8.8)	7 (8.9)	0.988
Multi-organ transplant (%)	4 (1.0)	3 (3.8)	0.066

* applies to DCD livers only; values presented with IQR where applicable; BMI=body mass index, EtOH=ethanol, HCC=hepatocellular carcinoma, Hep B=hepatitis B, Hep C=hepatitis C, MELD=model of end-stage liver disease, N/A=not applicable, NASH=non-alcoholic steatohepatitis, NMP=normothermic machine perfusion, PBC=primary biliary cholangitis, PSC=primary sclerosing cholangitis, SCS=static cold storage

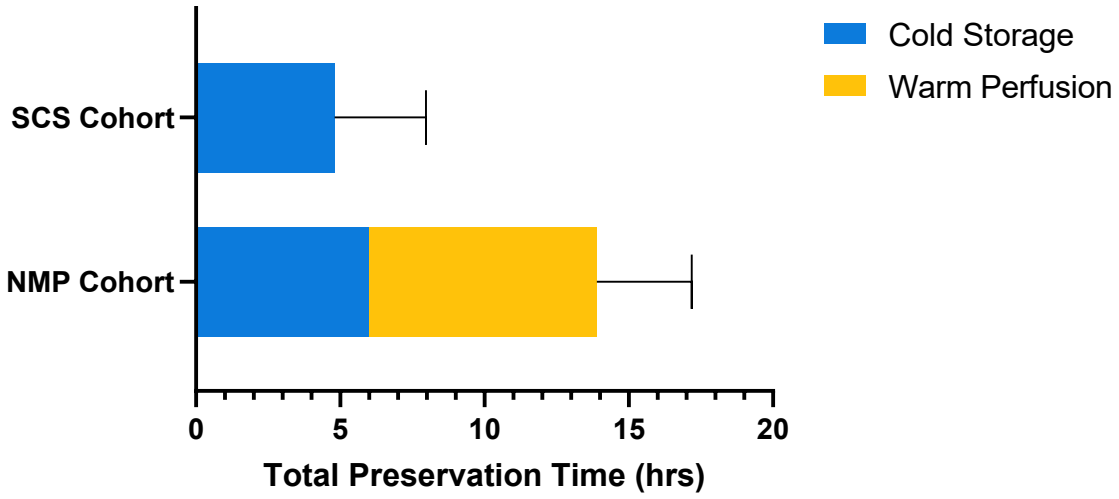

Figure 4.1.1. Median total storage times for liver grafts in the static cold storage only (SCS) and normothermic machine perfusion (NMP) cohorts ($p<0.0001$).

p<0.0001) (**Supplementary Table 4.1.2**). Similarly, local donors and their recipients were no different between cohorts. Both total and cold preservation times were significantly longer for livers in the NMP cohort (223 [140-292] minutes and 818 [689-911] minutes, respectively) compared to those in the SCS cohort (295 [99-209] minutes; p=0.003 and p<0.0001, respectively) (**Supplementary Table 4.1.3**).

Unadjusted Clinical Outcomes

Primary and secondary clinical outcomes are presented in **Table 4.1.2**. Graft survival after 30 days was significantly improved in the NMP cohort (94.3% in the SCS cohort vs. 100% in the NMP cohort; p=0.030); however, there was no difference at later time points (6 months: 90.1% in the SCS cohort vs. 89.9% in the NMP cohort, p=0.945; 1 year: 89.1% in the SCS cohort vs. 86.8% in the NMP cohort, p=0.571; 3 years: 77.5% in the SCS cohort vs. 79.3% in the NMP cohort, p=0.766). Overall, graft survival was not significantly different between cohorts (p=0.845), as seen in the survival curve (**Figure 4.1.2A, Supplementary Table 4.1.4**). Recipient survival was also thno different between cohorts (p=0.936; **Figure 4.1.2B, Supplementary Table 4.1.4**). Secondary clinical outcomes, including early allograft dysfunction (28.2% in the SCS cohort vs. 25.3% in the NMP cohort; p=0.597) were not significantly different. Median ICU and total hospital lengths of stay were also comparable (3 [2-8] days in the SCS cohort vs. 4 [2-11] days in the NMP cohort, p=0.129 and 17 [12-32] days in the SCS cohort vs. 19 [12-37] days in the NMP cohort, p=0.258, respectively). Major complications (Clavien-Dindo classification ≥3b) were likewise no different between cohorts (47.9% in the SCS cohort vs. 54.4% in the NMP cohort; p=0.292). Biliary and vascular complications requiring intervention (Clavien-Dindo classification ≥3a) within one year were also no different between cohorts (15.4% in the SCS cohort vs. 21.5% in the NMP cohort,

Table 4.1.2. Comparison of clinical outcomes between liver transplantation after static cold storage versus normothermic machine perfusion.

	SCS (n=386)	NMP (n=79)	p value
Crude graft survival			
30 days (n=386/79)	364 (94.3)	79 (100.0)	0.030
6 months (n=385/79)	347 (90.1)	71 (89.9)	0.945
1 year (n=349/76)	311 (89.1)	66 (86.8)	0.571
3 year (n=249/58)	193 (77.5)	46 (79.3)	0.766
5 year (n=168/25)	104 (61.9)	11 (44.0)	0.089
7 year (n=16/3)	12 (75.0)	2 (66.7)	0.764
Crude patient survival*			
30 days (n=369/76)	358 (97.0)	76 (100.0)	0.127
6 months (n=368/76)	344 (93.5)	71 (93.4)	0.985
1 year (n=331/73)	307 (92.8)	67 (91.8)	0.775
3 year (n=237/55)	193 (81.4)	46 (83.6)	0.703
5 year (n=155/22)	104 (67.1)	11 (50.0)	0.116
7 year (n=16/3)	12 (75.0)	2 (66.7)	0.764
EAD (%)	109 (28.2)	20 (25.3)	0.597
Peak AST POD1-7, U/L	695.5 (296.5-1519.5)	813 (358-1610)	0.323
Total bilirubin POD7, µmol/L	37 (21-86)	42 (21-94)	0.705
INR POD7	1.1 (1.0-1.2)	1.1 (1.0-1.2)	0.019
Primary non-function	9 (2.3)	0 (0.0)	0.171
ICU length of stay (days)	3 (2-8)	4 (2-11)	0.129
Hospital length of stay (days)	17 (12-32)	19 (12-37)	0.258
Major complications (Clavien-Dindo ≥3b)	185 (47.9)	43 (54.4)	0.292
Biliary complications within 1yr	60 (15.4)	17 (21.5)	0.193
Anastomotic stricture	39 (10.1)	12 (15.2)	0.187
Leak	20 (5.2)	4 (5.1)	0.966
Ischemic cholangiopathy	4 (1.0)	1 (1.3)	0.857
Biliary complications >1yr (n=312/66)	6 (1.9)	3 (4.6)	0.204
Vascular complications at 1yr	46 (11.9)	13 (16.5)	0.269
HA stenosis	19 (4.9)	7 (10.1)	0.072
HA thrombosis	7 (1.8)	1 (0.9)	0.733
Rejection episode within 1yr	74 (19.2)	16 (20.3)	0.824

* Excludes patients retransplanted during the study period; values presented with IQR where applicable; AST=aspartate transaminase, EAD=early allograft dysfunction, HA=hepatic artery, ICU=intensive care unit, INR=international normalized ratio, POD=post-operative day, NMP=normothermic machine perfusion, SCS=static cold storage

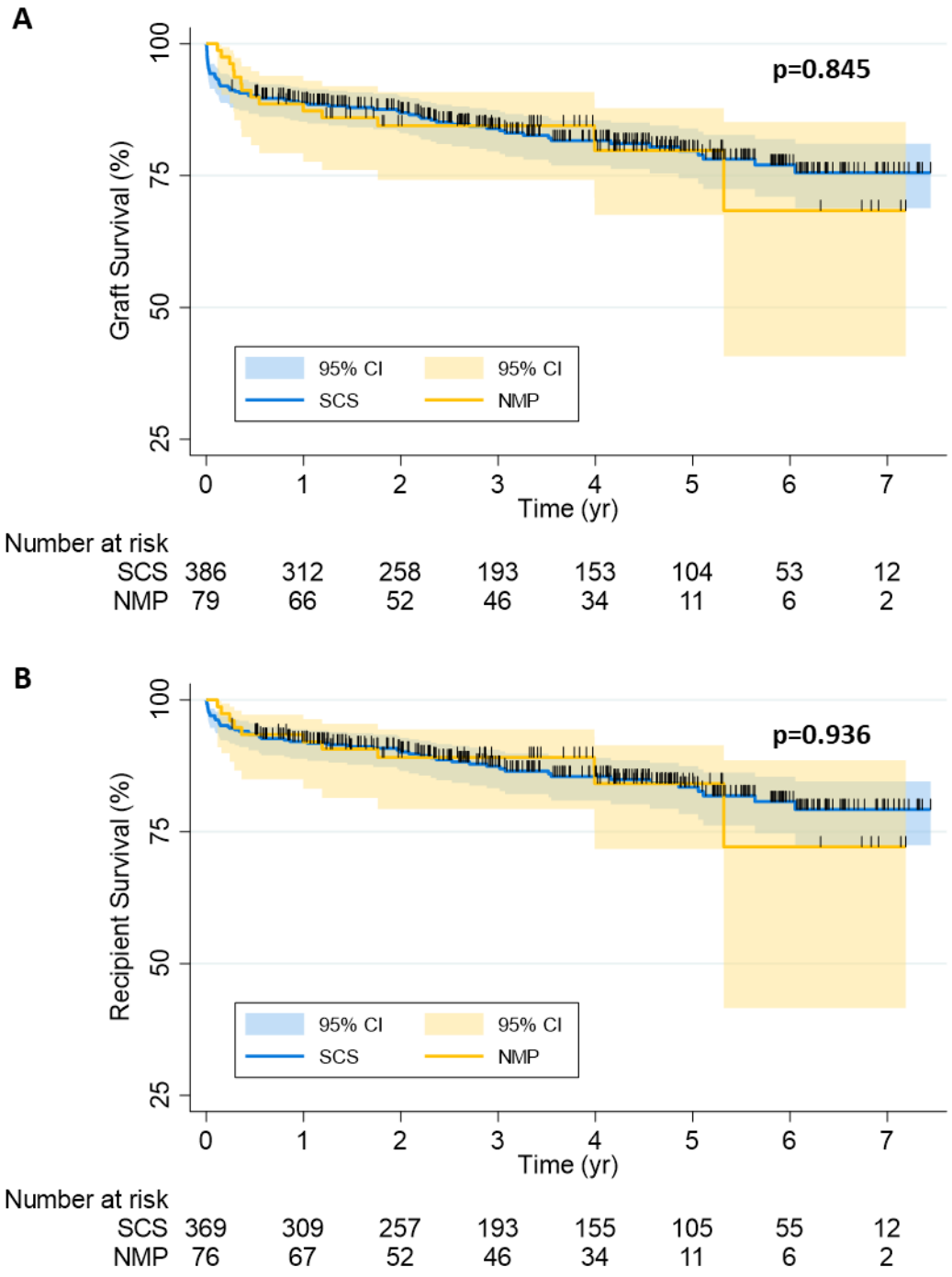

Figure 4.1.2. Kaplan-Meier curve showing graft (A) and recipient (B) survival after either static cold storage (SCS) or normothermic machine perfusion (NMP) preservation.

p=0.193, and 11.9% in the SCS cohort vs. 16.5% in the NMP cohort, p=0.269, respectively). Similarly, timing of biliary and vascular complications requiring intervention were no different (p=0.225 and 0.366, respectively, **Figures 4.1.3A & B**). Specifically, there was no difference in ischemic cholangiopathy between cohorts (1.0% in the SCS cohort vs. 1.3% in the NMP cohort; p=0.857). No patients in the NMP cohort experienced primary graft non-function compared to 9 patients in the SCS cohort; however, this was not significant (p=0.170) owing to the small numbers overall.

Following DCD donation, the majority of outcomes were no different between cohorts (**Supplementary Table 4.1.5**). Peak AST within the first seven post-operative days was significantly lower in the NMP cohort amongst DCD recipients (2600 [1279-2600] U/L in the SCS cohort vs. 1700 [606-2284.5] U/L in the NMP cohort; p=0.045), which is reflected in the decreased rate of EAD (though this did not achieve significance; 63.7% in the SCS cohort vs. 43.8% in the NMP cohort, p=0.136). Although 30-day graft survival was again higher in the SCS cohort, including only DCD donors, this was not statistically significant owing to smaller numbers (92.2% in the SCS cohort vs. 100% in the NMP cohort; p=0.248). Clinical outcomes were similar for local donors between the two cohorts (**Supplementary Table 4.1.6**).

Matched Clinical Outcomes

Several key clinical parameters were used to match transplants in the SCS cohort with the NMP cohort. Matched cohorts were no different in terms of baseline characteristics of donors and recipients, though SCS time and total preservation time remained significantly different (p=0.013 and p<0.0001, respectively, **Supplementary Table 4.1.7**). Thirty-day graft survival was similarly significantly improved in the NMP cohort after matching (92.4% in the SCS cohort vs. 100% in

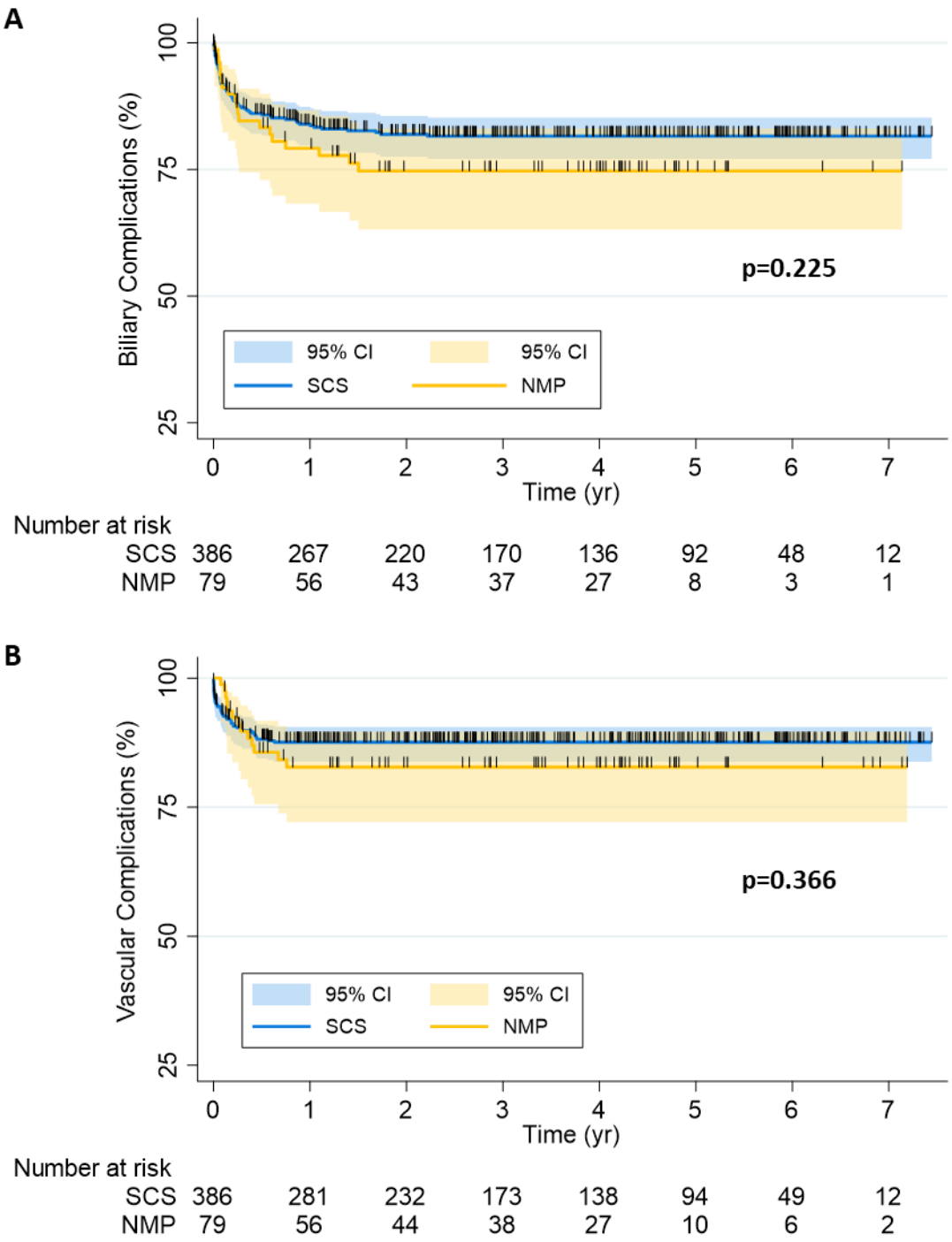

Figure 4.1.3. Kaplan-Meier curve showing time to biliary (A) and vascular (B) complications requiring intervention.

the NMP cohort; p=0.013) (**Table 4.1.3**). Thirty-day recipient survival was also significantly improved comparing matched cohorts (94.7% in the SCS cohort vs. 100% in the NMP cohort; p=0.041). However, other clinical and long-term outcomes remained unchanged. Control for confounding using multivariate logistic regression modeling similarly did not result in significant differences in clinical outcomes between cohorts (**Supplementary Tables 4.1.8 & 4.1.9**).

Impact of Cold Ischemia Time

Cold ischemia time ranged from 53 to 920 minutes in the SCS group and 66 to 688 minutes in the NMP group. Amongst recipients in the SCS cohort, those with graft failure at 30 days, 6 months, and 1 year had significantly longer median SCS times compared to those with graft survival at the same time points (30 days: 286 [190-382] minutes for those without graft failure vs. 387 [295-472] minutes for those with, p=0.003; 6 months: 288 [189-383] minutes for those with graft failure vs. 354 [266-527] minutes for those with, p=0.006; 1 year: 286 [187-381.5] minutes for those without graft failure vs. 354 [281-481] minutes for those with, p=0.005). However, this association was not observed in the NMP cohort, amongst whom SCS times were not significantly longer for those with graft failure at 6 months or 1 year compared to those with graft survival (6 months: 359 [292-410] minutes for those without graft failure vs. 422 [350-523] minutes for those with, p=0.064; 1 year: 359 [292-412] minutes for those without graft failure vs. 378 [332-480] minutes for those with, p=0.171). Neither was NMP time associated with graft failure at 6 months or 1 year in the NMP cohort (p=0.889 and p=0.631, respectively). Both graft and recipient survival were decreased in the SCS cohort for livers with the highest quartile of SCS time (≥397 minutes) compared to those with lower SCS times (p=0.007 & 0.049, respectively,

Table 4.1.3. Comparison of clinical outcomes between liver transplantation after static cold storage versus normothermic machine perfusion matched 1:1.

	SCS (n=79)	NMP (n=79)	p value
Crude graft survival			
30 days (n=79/79)	73 (92.4)	79 (100.0)	0.013
6 months (n=78/79)	69 (88.5)	71 (89.9)	0.776
1 year (n=71/76)	62 (87.3)	66 (86.8)	0.931
3 year (n=48/58)	35 (72.9)	46 (79.3)	0.440
5 year (n=33/25)	18 (54.6)	11 (44.0)	0.426
Crude patient survival*			
30 days (n=75/76)	71 (94.7)	76 (100.0)	0.041
6 months (n=74/76)	67 (90.5)	71 (93.4)	0.516
1 year (n=66/73)	60 (90.9)	67 (91.8)	0.855
3 year (n=47/55)	35 (74.5)	46 (83.6)	0.254
5 year (n=32/22)	18 (56.3)	11 (50.0)	0.651
EAD (%)	26 (32.9)	20 (25.3)	0.293
Peak AST POD1-7, U/L	697 (293-1810)	813 (358-1610)	0.894
Total bilirubin POD7, µmol/L	43.5 (26.5-98)	42 (21-94)	0.590
INR POD7	1.1 (1.0-1.2)	1.1 (1.0-1.2)	0.182
Primary non-function	2 (2.5)	0 (0.0)	0.155
ICU length of stay (days)	3 (2-8)	4 (2-11)	0.163
Hospital length of stay (days)	17 (12-34)	19 (12-37)	0.236
Major complications (Clavien-Dindo ≥3b)	43 (54.4)	43 (54.4)	1.000
Biliary complications within 1yr	12 (15.2)	17 (21.5)	0.304
Anastomotic stricture	8 (10.1)	12 (15.2)	0.339
Leak	5 (6.3)	4 (5.1)	0.731
Ischemic cholangiopathy	1 (1.3)	1 (1.3)	1.000
Biliary complications >1yr	1 (1.3)	3 (3.8)	0.311
Vascular complications within 1yr	10 (12.7)	13 (16.5)	0.499
HA stenosis	3 (3.8)	7 (10.1)	0.118
HA thrombosis	1 (1.3)	1 (1.3)	1.000
Rejection episode within 1yr	14 (17.7)	16 (20.3)	0.685

* Excludes patients retransplanted during the study period; values presented with IQR where applicable; AST=aspartate transaminase, EAD=early allograft dysfunction, HA=hepatic artery, ICU=intensive care unit, INR=international normalized ratio, POD=post-operative day, NMP=normothermic machine perfusion, SCS=static cold storage

Figure 4.1.4A & B). However, this was not observed in the NMP cohort (p=0.362 & 0.606, respectively, **Figure 4.1.4C & D**).

Recipients with EAD also had longer median SCS times in the SCS cohort (281 [166-370] minutes for those without EAD vs. 320 [250-416] minutes for those with; p=0.002), but not in the NMP cohort (364 [323-415] minutes for those without EAD vs. 350 [296.5-390] minutes for those with; p=0.278). Correspondingly, the proportion of EAD was significantly increased for recipients of livers with SCS time in the highest quartile in the SCS cohort (39.1% in the 4^{th} quartile vs. 24.8% in quartiles 1-3; p=0.009), whereas, in the NMP cohort, this was almost reversed (14.8% in the 4^{th} quartile vs. 30.8% in quartiles 1-3; p=0.122). There was also a moderate association between SCS time and peak AST in the first post-operative week (ρ=0.284; p<0.0001) in the SCS cohort, but no association in the NMP cohort (ρ=-0.026; p=0.820). In contrast, the length of NMP storage time, which ranged from 196 to 1347 minutes, was not associated with EAD (475 [390-618] minutes for those without EAD vs. 481 [345-662] minutes for those with; p=0.847) or peak AST (ρ=-0.019; p=0.867).

Looking at a subset of grafts in the SCS cohort with prolonged SCS times (≥8 hours) compared to NMP grafts with similar total preservation times (i.e., ≥8 hours), the impact of cold ischemia becomes apparent. Graft survival was significantly higher at 30 days for NMP livers compared to SCS livers preserved for ≥8 hours (88.6% in the SCS cohort vs. 100% in the NMP cohort; p=0.003), with a trend towards higher graft survival at 6 months (77.3% in the SCS cohort vs. 89.6% in the NMP cohort; p=0.067) and 1 year (72.1% in the SCS cohort vs. 86.5% in the NMP cohort; p=0.055; **Table 4.1.4**). Similarly, recipient survival was significantly higher at 30 days (92.7% in the SCS cohort vs. 100% in the NMP cohort; p=0.018) and 1 year (77.5% in the SCS cohort vs. 91.6% in the NMP cohort; p=0.038), with a trend towards improved survival at 6

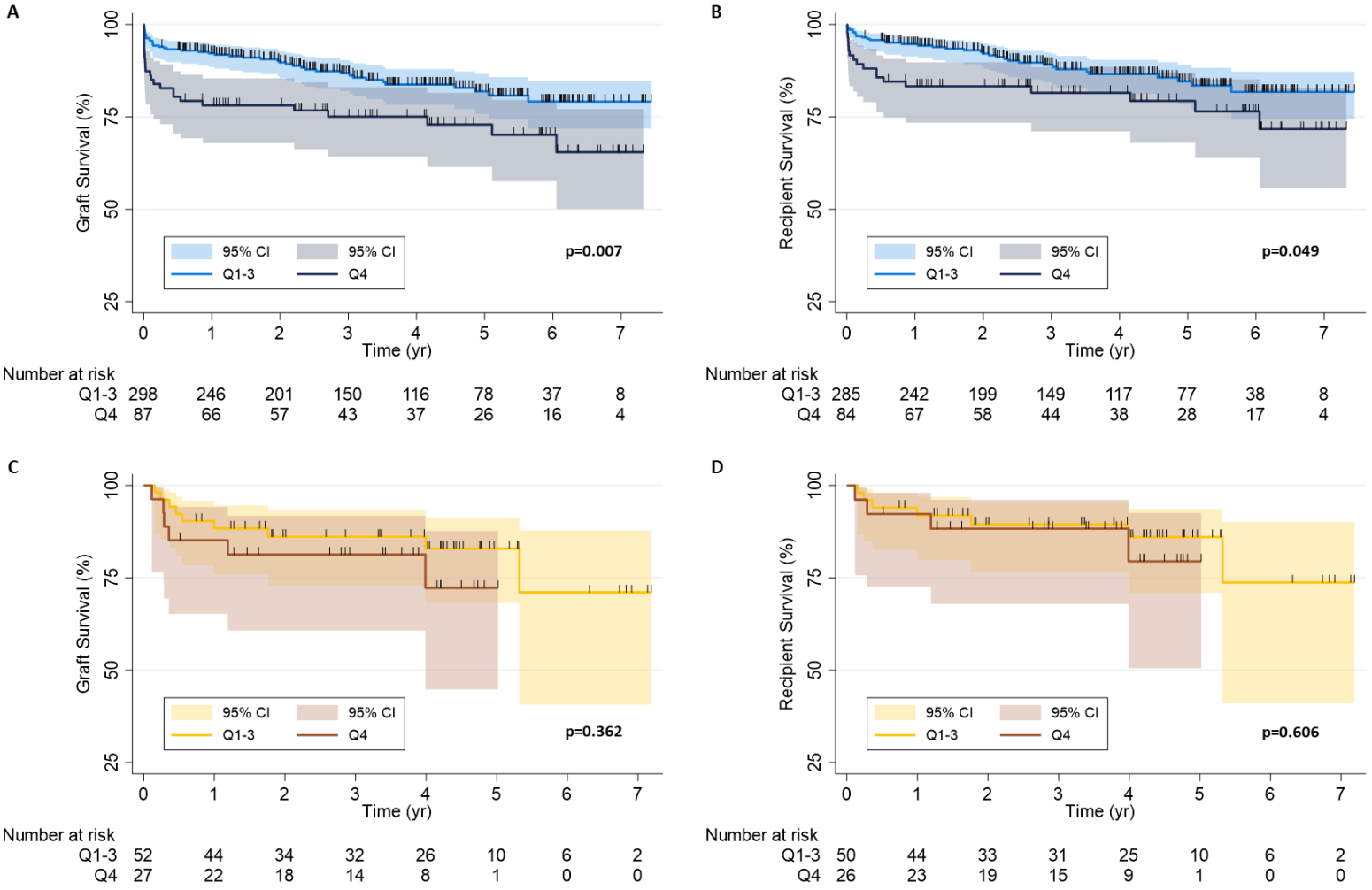

Figure 4.1.4. Graft (A) and recipient (B) survival between livers with the highest quartile of cold preservation time in the static cold storage cohort. Graft (C) and recipient (D) survival between livers with the highest quartile of cold preservation time in the normothermic machine perfusion cohort.

Table 4.1.4. Comparison of clinical outcomes between liver transplantation after static cold storage versus normothermic machine perfusion for transplanted livers with a total preservation time 8 hours or greater.

	SCS (n=44)	NMP (n=77)	p value
Crude graft survival			
30 days (n=44/77)	39 (88.6)	77 (100.0)	0.003
6 months (n=44/77)	34 (77.3)	69 (89.6)	0.067
1 year (n=43/74)	12 (72.1)	66 (86.5)	0.055
3 year (n=34/56)	22 (64.7)	44 (78.6)	0.440
Crude patient survival*			
30 days (n=41/74)	38 (92.7)	76 (100.0)	0.018
6 months (n=41/74)	34 (82.9)	69 (93.2)	0.083
1 year (n=40/71)	31 (77.5)	65 (91.6)	0.038
3 year (n=32/53)	22 (68.8)	44 (83.0)	0.126
EAD (%)	16 (36.4)	19 (24.7)	0.173
Peak AST POD1-7, U/L	1297 (587-2252)	760 (358-1606)	0.043
Total bilirubin POD7, µmol/L	41 (30.5-100)	42 (21-94)	0.547
INR POD7	1.1 (1.0-1.1)	1.1 (1.0-1.2)	0.026
Primary non-function	3 (6.8)	0 (0.0)	0.020

* Excludes patients retransplanted during the study period; values presented with IQR where applicable; AST=aspartate transaminase, EAD=early allograft dysfunction, HA=hepatic artery, ICU=intensive care unit, INR=international normalized ratio, POD=post-operative day, NMP=normothermic machine perfusion, SCS=static cold storage

months (82.9% in the SCS cohort vs. 93.2% in the NMP cohort; p=0.083). Both of these trends for improved graft and recipient survival are apparent using NMP for extended preservation compared to SCS over the duration of the study (**Figure 4.1.5**).

4.1.5 – Discussion

Our study represents one of the earliest and longest duration single centre experiences with liver transplantation after NMP. We found that the addition of NMP following traditional SCS had no adverse effect on multiple clinical outcomes, including mortality, and biliary and vascular complications, and showed clear benefit in terms of early allograft survival. While extended preservation time under SCS conditions results in higher rates of graft failure and mortality, our study shows that extending preservation time with protection of NMP does not impede clinical outcomes.

The efficacy of NMP over SCS has been demonstrated convincingly by two multicentre RCTs, using different NMP devices, with one-year follow up (i.e., OrganOx metra® and Organ Care Systems™ Liver).[5,6] In these studies, livers were recovered in the traditional fashion and placed on the NMP circuit as soon as possible. This resulted in the NMP groups having significantly lower SCS time, despite having longer total preservation times. In contrast, our study had significantly longer SCS times in the NMP cohort, in addition to having overall longer preservation times, reflecting our 'back-to-base' more practical approach, which, while less ideal for preventing preservation injury, was adopted for practical reasons. This may explain why we did not find significantly lower rates of EAD in the NMP cohort, although an additional sub-analysis of local donors (**Supplementary Table 4.1.6**) with shorter SCS times did not alter longer

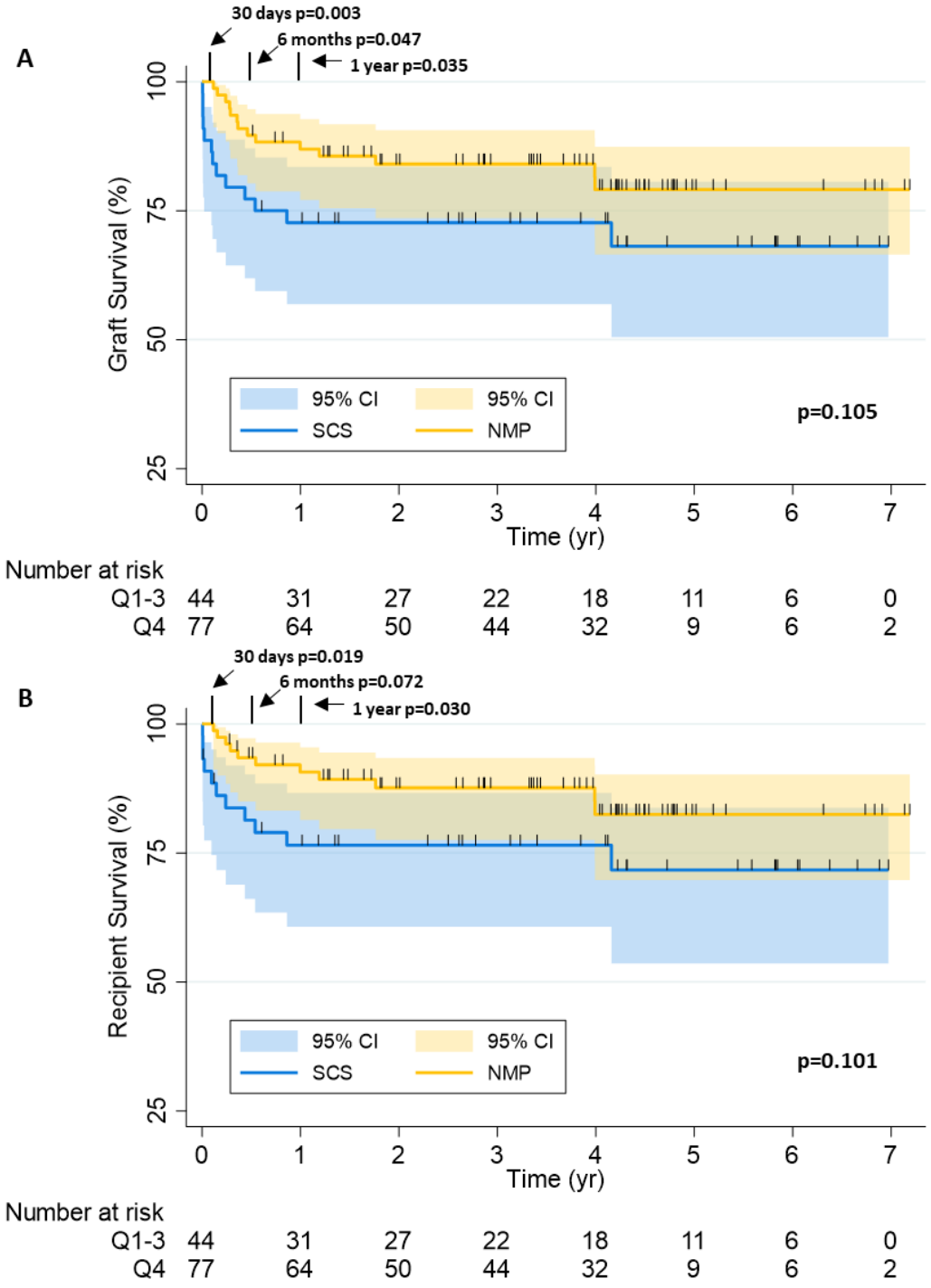

Figure 4.1.5. Graft (A) and recipient (B) survival curves for livers preserved for at least 8 hours prior to transplantation, compared between static cold storage (SCS) and normothermic machine perfusion (NMP) cohorts.

term outcomes in our series. In addition, our study also included retransplants (i.e., patients receiving second or third liver transplantations) and multi-organ transplants, which are more prone to complications.[10] Interestingly, we did find improved 30-day graft survival and no instances of PNF in the NMP cohort, despite longer cold and total preservation times. The fact that graft survival was no different at later time points emphasizes that the impact of NMP is greatest early on. Over time, these initial effects are likely diluted by other circumstances arising in the post-transplant period.

The results from our centre reflect a practical way of applying NMP in a North American geographically dispersed environment, avoiding the additional logistical challenges of transporting heavy and complex equipment by plane and ground ambulance at each end. We demonstrate how NMP can be used to safely extend the preservation of a liver allograft to avoid operating at late hours or accommodate delays due to other surgeries or ICU bed availability (which was problematic during the COVID-19 pandemic). This strategy is also useful with challenging recipients, such as more complex reoperative, retransplantation or multi-organ transplantation, where explantation of the diseased organ(s) may be prolonged.[11] Unlike extended preservation under SCS conditions, extending preservation with NMP does not increase the risk of graft failure or recipient mortality, which is evident in our SCS cohort. However, application of NMP does not positively improve long-term outcomes in our experience.

Though clinically approved NMP devices are designed to be portable, they are still more cumbersome than using cold storage and require trained personnel to operate. The OrganOx metra®, in particular, is designed to be portable in the back of small van-type ambulances used in the UK and Europe. Because of our unique geographic circumstances (being situated nearly 1,200km from the next closest liver transplant centre), a high percentage of organs arrive from

distant sites by air and, consequently, we have adopted a 'back-to-base' approach for integrating NMP into our liver procurement strategy.[12] Though shorter SCS times are associated with improved outcomes, it is probably not necessary or cost-effective to transport every liver under NMP and it has not been clearly established which livers would benefit most from NMP. The observation that extended SCS was associated with decreased graft and patient survival in the SCS cohort, but not in the NMP, is a unique finding from our study that suggests NMP may be particularly beneficial in these organs. This hypothesis merits consideration in future clinical trials. Normothermic regional perfusion (NRP), in which abdominal organs are perfused within the body following controlled DCD, may offer an alternative or complimentary approach with DCD livers, as it avoids the challenges of transporting the organ while it is being perfused.[13] Retrospective studies from several centres in Europe have reported decreased biliary complications and reduced graft loss with this approach.[14,15]

An additional consideration with NMP is the impact of the artificial *ex vivo* environment on the organ, which, while an improvement over SCS conditions, can produce still proinflammatory responses.[16] These effects may be more pronounced on injured livers, such as those donated after circulatory death. Current commercial devices also cannot fully replicate the *in vivo* environment, lacking, for instance, mechanisms to eliminate metabolic waste (i.e., as a replacement for the kidney), as well as hormonal and neurologic input. Still, it seems that the liver, particularly a healthy one, can be maintained reliably up to 24 hours with commercially available devices, with emerging modifications continuing to push the limits of preservation.[17]

Limitations of this study include its non-randomized nature. While our use of 1:1 matching to account for some potential confounders did not change our conclusions, it is possible that residual confounding exists that may have affected outcomes. Given the retrospective nature of

our SCS cohort and that biliary lesions are not routinely assessed at our centre in the absence of symptoms, we were unable to comment on the effect of NMP on biliary lesions not requiring intervention (i.e., asymptomatic). Though not statistically significant, the lower MELD-Na score in the NMP cohort may reflect the tendency not to use NMP in more urgent cases in the setting of relatively new technology. However, there were no exclusion criteria based on MELD-Na and we did, in fact, have 9 patients in the NMP cohort with MELD-Na greater than or equal to 30 at the time of transplantation. In addition, our study did not seek to assess any specific indications for liver NMP, nor did it test the broad application of NMP in liver transplantation. Our study better reflects how NMP can be integrated into a mature North American transplant program.

Solid organ transplantation has been made successful because of ongoing commitment to innovation and development in all aspects of donor and recipient care, including surgical technique, storage solutions, and immunosuppressive therapies. NMP provides another tool to facilitate liver transplantation, whether it is used as a substitute or in addition to traditional methods of SCS preservation. Our experience complements previous RCTs by showing that NMP can safely extend preservation without compromise to graft or patient survival.

4.1.6 – References

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4.1.7 – Supplementary Material

Supplementary Table 4.1.1. Reasons for discard of perfused organs that were not transplanted.

Study ID	Reason for Discard
ORGXD01	Failed to clear lactate; no bile production
ORGXD02	Rising lactate; no bile production
ORGXD03	Rising lactate; no bile production
ORGXD04	Moderate macrovascular steatosis (50%) on biopsy
ORGXD05	Harvesting injury on biopsy; no bile production

Supplementary Table 4.1.2. Donor and recipient demographics for donation after circulatory death.

	SCS (n=51)	NMP (n=16)	p value
Donor Characteristics			
Age, years	47 (32-57)	50.5 (25.5-56)	0.686
Female (%)	18 (35.3)	5 (31.5)	0.766
BMI	24.3 (21.6-27.2)	23.6 (20.4-27.4)	0.665
Cause of death (%)			
Intracranial event	11 (21.6)	3 (18.6)	0.809
Anoxia/hypoxia	19 (37.6)	5 (31.3)	0.662
Trauma	8 (15.7)	3 (18.6)	0.773
Overdose	5 (9.8)	0 (0.0)	0.193
Other	8 (15.7)	5 (31.3)	0.170
Local (%)	12 (23.5)	4 (25.0)	0.904
Preservation Details			
Functional warm ischemia time, minutes	19 (12-27)	21.5 (15-25.5)	0.527
Cold ischemia time, minutes	317 (259-383)	355.5 (296-441.5)	0.174
NMP time, minutes	N/A	534 (384-825)	N/A
Rewarm time, minutes	40 (35-47)	38 (35.5-42.5)	0.234
Total storage time, minutes	317 (259-383)	964.5 (734.5-1217.5)	<0.0001
Recipient Characteristics			
Age, years	54.2 (47.8-63.0)	62.7 (54.7-64.8)	0.065
Female (%)	18 (35.3)	8 (50.0)	0.292
BMI	28.4 (24.8-32.8)	29.8 (22.6-38.1)	0.613
MELD-Na (at time of transplant)	20 (11-25)	20 (10-26.5)	0.939
Creatinine, µmol/L	79 (60-107)	86.5 (73-111)	0.567
Diagnosis (%)			
EtOH cirrhosis	19 (37.3)	5 (31.3)	0.662
Hep C cirrhosis	15 (29.4)	6 (37.5)	0.543
Hep B cirrhosis	4 (7.8)	2 (12.5)	0.569
NASH cirrhosis	53 (15.7)	4 (25.0)	0.397
HCC	22 (43.1)	7 (43.8)	0.966
Autoimmune	3 (5.9)	0 (0.0)	0.321
PBC	4 (7.8)	1 (6.3)	0.832
PSC	2 (3.9)	1 (6.3)	0.694
Retransplant (%)	1 (2.0)	0 (0.0)	0.573

values presented with IQR where applicable; BMI=body mass index, EtOH=ethanol, HCC=hepatocellular carcinoma, Hep B=hepatitis B, Hep C=hepatitis C, MELD=model of end-stage liver disease, N/A=not applicable, NASH=non-alcoholic steatohepatitis, NMP=normothermic machine perfusion, PBC=primary biliary cholangitis, PSC=primary sclerosing cholangitis, SCS=static cold storage

Supplementary Table 4.1.3. Donor and recipient demographics for local liver donation.

	SCS (n=139)	NMP (n=23)	p value
Donor Characteristics			
Age, years	36 (26-51)	38 (26-56)	0.677
Female (%)	47 (33.8)	8 (34.8)	0.928
BMI	26.6 (23.2-30.3)	27.6 (24.7-33.2)	0.228
Cause of death (%)			
Intracranial event	34 (24.5)	6 (26.1)	0.867
Anoxia/hypoxia	41 (29.5)	3 (13.0)	0.100
Trauma	30 (21.6)	5 (21.7)	0.987
Overdose	28 (20.1)	5 (21.7)	0.860
Other	6 (4.3)	4 (17.4)	0.016
DCD (%)	12 (8.6)	4 (17.4)	0.192
Preservation Details			
Functional warm ischemia time, minutes*	25.5 (14.5-28.5)	20.5 (17-23.5)	0.468
Cold ischemia time, minutes	145 (99-209)	223 (140-292)	0.003
NMP time, minutes	N/A	612 (387-689)	N/A
Rewarm time, minutes	39 (34-45)	40 (34-45)	0.902
Total storage time, minutes	295 (99-209)	818 (689-911)	<0.0001
Recipient Characteristics			
Age, years	57.6 (47.2-63.0)	58.1 (46.5-62.9)	0.786
Female (%)	37 (26.6)	5 (21.7)	0.621
BMI	28.7 (24.7-32.4)	29.2 (24.6-36.0)	0.387
MELD-Na (at time of transplant)	19 (11-25)	17 (13-28)	0.561
Creatinine, µmol/L	83 (64-100)	92 (62-103)	0.734
Diagnosis (%)			
EtOH cirrhosis	45 (32.4)	6 (26.1)	0.548
Hep C cirrhosis	37 (26.6)	5 (21.7)	0.621
Hep B cirrhosis	6 (4.3)	2 (8.7)	0.369
NASH cirrhosis	29 (20.8)	5 (21.7)	0.924
HCC	52 (37.4)	7 (30.4)	0.520
Autoimmune	7 (5.0)	3 (13.0)	0.139
PBC	12 (8.6)	0 (0.0)	0.143
PSC	16 (11.5)	1 (4.4)	0.299
Retransplant (%)	14 (10.1)	1 (4.4)	0.380
Multi-organ transplant (%)	0 (0.0)	3 (13.0)	<0.0001

values presented with IQR where applicable; BMI=body mass index, EtOH=ethanol, HCC=hepatocellular carcinoma, Hep B=hepatitis B, Hep C=hepatitis C, MELD=model of end-stage liver disease, N/A=not applicable, NASH=non-alcoholic steatohepatitis, NMP=normothermic machine perfusion, PBC=primary biliary cholangitis, PSC=primary sclerosing cholangitis, SCS=static cold storage

Supplementary Table 4.1.4. Kaplan Meier estimates of graft and patient survival at various time points.

	SCS (n=386)	NMP (n=79)	p value
Graft survival (% ± std. error)			
30 days	94.3±1.2	100	0.031
6 months	90.2±1.5	89.9±3.4	0.980
1 year	88.9±1.6	87.3±3.7	0.793
3 year	84.5±1.9	84.7±4.1	0.969
5 year	80.7±2.3	81.0±4.7	0.975
7 year	76.8±2.9	73.7±8.2	0.845
Recipient survival* (n=369/76; % ± std. error)			
30 days	97.0±0.9	100	0.130
6 months	93.5±1.3	93.4±2.8	0.988
1 year	92.1±1.4	92.1±3.1	0.971
3 year	87.9±1.8	89.4±3.6	0.775
5 year	84.4±2.1	80.5±4.3	0.910
7 year	80.5±2.9	77.7±8.4	0.936

* Excludes patients retransplanted during the study period

Supplementary Table 4.1.5. Comparison of clinical outcomes between liver transplantation after static cold storage versus normothermic machine perfusion in donation after circulatory death.

	SCS (n=51)	NMP (n=16)	p value
Crude graft survival			
30 days (n=51/16)	47 (92.2)	16 (100.0)	0.248
6 months (n=51/16)	46 (90.2)	14 (87.5)	0.758
1 year (n=44/15)	41 (93.2)	13 (86.7)	0.434
3 year (n=25/12)	16 (64.0)	10 (83.3)	0.228
Crude patient survival*			
30 days (n=50/16)	49 (98.0)	16 (100.0)	0.569
6 months (n=50/16)	47 (94.0)	15 (93.8)	0.971
1 year (n=44/15)	43 (97.7)	14 (93.3)	0.417
3 year (n=21/11)	16 (76.2)	10 (90.9)	0.311
EAD (%)	33 (64.7)	7 (43.8)	0.136
Peak AST POD1-7, U/L	2600 (1279-2600)	1700 (606-2284.5)	0.045
Total bilirubin POD7, µmol/L	47.5 (26-105.5)	52.5 (35.5-111)	0.466
INR POD7	1.1 (1.0-1.2)	1.2 (1.1-1.35)	0.079
Primary non-function	2 (3.9)	0 (0.0)	0.421
ICU length of stay (days)	4 (2-8)	4 (2.5-9)	0.932
Hospital length of stay (days)	20 (13-32)	20.5 (18-34.5)	0.500
Major complications (Clavien-Dindo ≥3b)	33 (64.7)	9 (56.3)	0.542
Biliary complications at 1yr	18 (35.3)	7 (43.8)	0.542
Anastomotic stricture	10 (19.6)	6 (37.5)	0.143
Leak	6 (11.8)	0 (0.0)	0.150
Biliary complications >1yr	3 (5.9)	1 (6.3)	0.957
Ischemic cholangiopathy	2 (3.9)	1 (6.3)	0.694
Vascular complications at 1yr	9 (17.7)	5 (31.3)	0.243
HA stenosis	4 (7.8)	3 (18.6)	0.213
HA thrombosis	0 (0.0)	0 (0.0)	--
Rejection episode within 1yr	9 (17.7)	2 (12.5)	0.628

* Excludes patients retransplanted during the study period; values presented with IQR where applicable;
AST=aspartate transaminase, EAD=early allograft dysfunction, HA=hepatic artery, ICU=intensive care unit, INR=international normalized ratio, ITBL=ischemic type biliary lesion, POD=post-operative day, NMP=normothermic machine perfusion, SCS=static cold storage

Supplementary Table 4.1.6. Comparison of clinical outcomes between liver transplantation after static cold storage versus normothermic machine perfusion in local liver donation.

	SCS (n=139)	NMP (n=23)	p value
Crude graft survival			
30 days (n=139/23)	137 (97.8)	23 (100.0)	0.477
6 months (n=139/23)	132 (95.0)	21 (91.3)	0.478
1 year (n=123/23)	115 (93.5)	21 (91.3)	0.703
Crude patient survival*			
30 days (n=134/22)	138 (99.2)	22 (100.0)	0.684
6 months (n=134/22)	130 (97.0)	20 (90.9)	0.168
1 year (n=118/22)	114 (96.6)	20 (90.9)	0.225
EAD (%)	26 (18.7)	8 (34.8)	0.079
Peak AST POD1-7, U/L	416 (205-1020)	923 (326-2190)	0.041
Total bilirubin POD7, µmol/L	32.5 (20-69)	40 (21-101)	0.301
INR POD7	1.1 (1.0-1.2)	1.1 (1.0-1.2)	0.324
Primary non-function	0 (0.0)	0 (0.0)	--
ICU length of stay (days)	3 (2-6)	7 (3-39)	0.001
Hospital length of stay (days)	16 (11-32)	32 (17-86)	0.003
Major complications (Clavien-Dindo ≥3b)	57 (41.0)	14 (60.9)	0.075
Biliary complications at 1yr	21 (15.1)	4 (17.4)	0.779
Anastomotic stricture	13 (9.4)	3 (13.0)	0.583
Leak	8 (5.8)	1 (4.4)	0.785
Vascular complications at 1yr	16 (11.5)	2 (8.7)	0.691
HA stenosis	7 (5.0)	1 (4.4)	0.888
HA thrombosis	3 (2.2)	0 (0.0)	0.477
Rejection episode within 1yr	31 (22.3)	5 (21.7)	0.952

* Excludes patients retransplanted during the study period; values presented with IQR where applicable; AST=aspartate transaminase, EAD=early allograft dysfunction, HA=hepatic artery, ICU=intensive care unit, INR=international normalized ratio, ITBL=ischemic type biliary lesion, POD=post-operative day, NMP=normothermic machine perfusion, SCS=static cold storage

Supplementary Table 4.1.7. Donor and recipient demographics after 1:1 matching.

	SCS (n=79)	NMP (n=79)	p value
Donor Characteristics			
Age, years	42 (27-55)	40 (25-56)	0.958
Female (%)	23 (29.1)	27 (34.2)	0.494
BMI	25.1 (21.8-28.7)	25.7 (22.4-29.2)	0.373
Cause of death (%)			
Intracranial event	25 (31.7)	22 (27.9)	0.602
Anoxia/hypoxia	25 (31.7)	21 (26.6)	0.484
Trauma	12 (15.2)	17 (21.5)	0.304
Overdose	12 (15.2)	8 (10.1)	0.339
Other	5 (6.3)	11 (13.9)	0.114
Local (%)	20 (25.3)	23 (29.1)	0.592
DCD (%)	16 (20.3)	16 (20.3)	1.000
Preservation Details			
Functional warm ischemia time, minutes	21.5 (18-26)	21.5 (15-25.5)	0.773
Cold ischemia time, minutes	312 (251-385)	359 (301-412)	0.013
NMP time, minutes	N/A	475 (387-620)	N/A
Rewarm time, minutes	39 (34-46)	40 (36-47)	0.234
Total storage time, minutes	312 (251-385)	847 (767-964)	<0.0001
Recipient Characteristics			
Age, years	55.9 (48.2-63.0)	58.1 (46.9-63.1)	0.923
Female (%)	24 (30.4)	23 (29.1)	0.862
BMI	26.6 (23.1-31.5)	28.0 (23.8-35.0)	0.175
MELD-Na (at time of transplant)	20 (14-28)	16 (11-23)	0.045
Creatinine, µmol/L	89 (69-115)	84 (65-103)	0.240
Diagnosis (%)			
EtOH cirrhosis	23 (29.1)	25 (31.7)	0.729
Hep C cirrhosis	17 (21.5)	20 (25.3)	0.573
Hep B cirrhosis	3 (3.8)	6 (7.6)	0.303
NASH cirrhosis	11 (13.9)	12 (15.2)	0.822
HCC	20 (25.3)	25 (31.7)	0.378
Autoimmune	6 (7.6)	4 (5.1)	0.513
PBC	4 (5.1)	4 (5.1)	1.000
PSC	6 (7.6)	8 (10.1)	0.576
Retransplant (%)	7 (8.9)	7 (8.9)	1.000
Combined (%)	2 (2.5)	3 (3.8)	0.649

values presented with IQR where applicable; BMI=body mass index, EtOH=ethanol, HCC=hepatocellular carcinoma, Hep B=hepatitis B, Hep C=hepatitis C, MELD=model of end-stage liver disease, N/A=not applicable, NASH=non-alcoholic steatohepatitis, NMP=normothermic machine perfusion, PBC=primary biliary cholangitis, PSC=primary sclerosing cholangitis, SCS=static cold storage

Supplementary Table 4.1.8. Adjusted odds ratios for selected clinical outcomes between liver transplantation after normothermic machine perfusion versus static cold storage only.

	Adjusted OR*	95% CI	p value
Graft failure at 1yr	1.13	0.52-2.46	0.759
Recipient mortality at 1yr	0.95	0.35-2.58	0.927
Early allograft dysfunction	0.63	0.35-1.15	0.133
Major complications (Clavien-Dindo ≥3b)	0.60	0.25-1.41	0.241
Biliary complications at 1yr	1.46	0.77-2.73	0.256
Vascular complications at 1yr	1.39	0.71-2.75	0.339

* Odds of outcome in NMP cohort compared to SCS cohort

Supplementary Table 4.1.9. Summary of univariate analyses used to build multivariate logistic regression model.

	Graft Failure at 1 year	Recipient Mortality at 1 year	Early Allograft Dysfunction	Major Complications (≥3b Clavien-Dindo)	Biliary Complications at 1 year	Vascular Complications at 1 year
Donor age	p=0.7638	p=0.5672	p=0.0283	p=0.2073	p=0.7275	p=0.6829
Donor sex	p=0.3074	p=0.4015	p=0.6506	p=0.6764	p=0.1081	p=0.6405
Donor BMI	p=0.6946	p=0.5784	p=0.6000	p=0.4863	p=0.8600	p=0.6197
Donor cause of death	p=0.1471	p=0.6638	p=0.0135	p=0.8062	p=0.4030	p=0.1898
Death from intracranial event	p=0.6143	p=0.5570	p=0.1137	p=0.5485	p=0.8378	p=0.3013
Death from anoxia/hypoxia	p=0.0371	p=0.0218	p=0.0464	p=0.8594	p=0.6242	p=0.7790
Death from trauma	p=0.0701	p=0.1236	p=0.0639	p=0.5691	p=0.8132	p=0.3137
Death from overdose	p=0.1177	p=0.3630	p=0.0016	p=0.0069	p=0.8421	p=0.1062
Donor location	p=0.0443	p=0.0800	p=0.0200	p=0.1082	p=0.6223	p=0.4479
Donor type	p=0.4917	p=0.2013	p<0.0001	p=0.0149	p<0.0001	p=0.0300
Functional warm ischemic time	p=0.7558	p=0.9947	p=0.7867	p=0.7396	p=0.9299	p=0.6464
Cold ischemic time	p=0.0009	p=0.0028	p=0.0108	p=0.0148	p=0.7146	p=0.7146
Normothermic machine perfusion time	p=0.6306	p=0.4880	p=0.8470	p=0.1793	p=0.0132	p=0.5066
Total storage time	p=0.0102	p=0.0198	p=0.0544	p=0.0107	p=0.7004	p=0.4102

Age at transplant	p=0.6923	p=0.9598	p=0.6678	p=0.4905	p=0.5791	p=0.1256
Sex	p=0.6755	p=0.8841	p=0.8034	p=0.0086	p=0.5156	p=0.5594
BMI	p=0.2268	p=0.5326	p=0.4411	p=0.2463	p=0.0715	p=0.7727
MELD-Na	p=0.0362	p=0.0174	p=0.3347	p<0.0001	p=0.0304	p=0.9635
Creatinine at transplant	p=0.0002	p=0.0004	p=0.1455	p=0.0002	p=0.0169	p=0.7056
EtOH cirrhosis	p=0.1067	p=0.1245	p=0.5967	p=0.0240	p=0.2736	p=0.2254
Hepatitis C cirrhosis	p=0.6853	p=0.9301	p=0.4943	p=0.8767	p=0.9426	p=0.6572
Hepatitis B cirrhosis	p=0.1157	p=0.2066	p=0.0806	p=0.0248	p=0.9162	p=0.9614
NASH cirrhosis	p=0.8209	p=0.3232	p=0.4939	p=0.1418	p=0.3545	p=0.8224
Hepatocellular carcinoma	p=0.2081	p=0.3260	p=0.4185	p=0.0068	p=0.2855	p=0.3833
Autoimmune hepatitis	p=0.6129	p=0.9101	p=0.8068	p=0.0576	p=0.0609	p=0.1269
Primary biliary cholangitis	p=0.4245	p=0.8693	p=0.9073	p=0.1654	p=0.1311	p=0.2161
Primary sclerosing cholangitis	p=0.1834	p=0.0595	p=0.2313	p=0.7503	p=0.8438	p=0.8960
Retransplant	p=0.1125	p=0.5979	p=0.7021	p=0.0140	p=0.8723	p=0.0523
Multi-visceral transplant	p=0.0385	p=0.0149	p=0.9532	p=0.2305	p=0.8687	p=0.3094

BMI = body mass index, EtOH = ethanol, MELD-Na = model of end-stage liver disease sodium, NASH = non-alcoholic steatohepatitis

Supplementary Table 4.1.10. Detail cause of death and/or graft failure for study patients.

Study ID*	Transplant Date	Graft Failure Date	Cause
ORGX04	Apr 29, 2015	Aug 23, 2020	Deceased; recurrent HCC
ORGX07	Sep 10, 2015	Dec 7, 2015	Deceased; recurrent hepatitis C
ORGX09	Nov 30, 2015	Sep 4, 2017	Deceased; unknown (died outside of hospital)
ORGX23	Sep 21, 2017	Nov 17, 2017	Deceased; combined liver & kidney transplant, intra-abdominal sepsis
ORGX24-1	Sep 29, 2017	Jan 9, 2018	Retransplantation; hepatic artery & portal vein stenosis resulting in ischemic cholangiopathy
ORGX26	Nov 7, 2017	Nov 3, 2021	Deceased; metastatic breast cancer
ORGX41	Jun 29, 2018	Jun 26, 2022	Deceased; metastatic pancreatic cancer
ORGX46	Nov 25, 2018	Jan 6, 2019	Deceased; previous double lung transplant, systemic fungal infection
ORGX52	Apr 5, 2019	Jul 19, 2019	Deceased; acute tubular necrosis & diaphragmatic paralysis, culminating in respiratory failure
ORGX57	Oct 12, 2019	Feb 19, 2020	Retransplantation; hepatic artery thrombosis with secondary bile duct necrosis
ORGX61	Jan 23, 2020	Apr 2, 2021	Deceased; recurrent HCC
ORGX63	Jul 4, 2020	Jan 19, 2021	Deceased; right heart failure secondary to pulmonary hypertension
ORGX69	Nov 18, 2020	Mar 31, 2021	Deceased; respiratory failure, multi-factorial
ORGX70	Dec 10, 2020	May 27, 2021	Retransplantation; allograft dysfunction secondary to ischemic cholangiopathy
ORGX72	Dec 20, 2020	Dec 19, 2021	Deceased; metastatic small cell lung cancer
SCS15	Aug 3, 2020	Aug 9, 2020	Retransplantation; hepatic artery thrombosis
SCS19	Jul 12, 2020	Jul 25, 2020	Retransplantation; acute portal thrombosis resulting in bile duct necrosis
SCS25	Jun 26, 2020	Jul 4, 2020	Retransplantation; hepatic artery thrombosis
SCS27	Jun 18, 2020	Mar 7, 2022	Deceased; unknown (died outside of hospital)
SCS29	Jun 6, 2020	Mar 26, 2021	Deceased; respiratory failure secondary to COVID-19 pneumonia
SCS31	May 31, 2020	May 18, 2022	Deceased; recurrent metastatic HCC
SCS37	May 8, 2020	May 19, 2020	Deceased; acute liver failure secondary to undiagnosed urea cycle disorder in transplanted graft
SCS38	May 5, 2020	May 8, 2020	Retransplantation; primary non-function
SCS41	Apr 17, 2020	Apr 13, 2022	Deceased; unknown (died outside of hospital)
SCS72	Nov 16, 2019	Nov 29, 2019	Deceased; small bowel, liver infarction

SCS76	Nov 3, 2019	Nov 6, 2019	Retransplantation; primary non-function
SCS86	Oct 15, 2019	Oct 19, 2019	Retransplantation; primary non-function
SCS89	Oct 8, 2019	Oct 12, 2019	Retransplantation; hepatic artery thrombosis
SCS91	Sep 22, 2019	Dec 24, 2020	Deceased; ruptured splenic artery pseudoaneurysm
SCS94	Sep 9, 2019	Dec 6, 2019	Deceased; intra-abdominal sepsis
SCS111	Jul 13, 2019	Sep 4, 2019	Deceased; central pontine myelinolysis & aspiration pneumonia
SCS116	Jul 7, 2019	Jan 14, 2022	Deceased; metastatic adenocarcinoma of unknown origin
SCS138	Mar 23, 2019	Sep 2, 2020	Deceased; cholestatic liver failure secondary to bile duct stricture
SCS144	Mar 8, 2019	Apr 12, 2019	Deceased; sudden cardiac arrest
SCS181	Aug 7, 2018	Aug 15, 2018	Deceased; allograft dysfunction secondary to portal venous & superior mesenteric venous thrombosis
SCS187	Jul 6, 2018	Jul 9, 2018	Deceased; primary non-function
SCS188	Jul 3, 2018	Jul 6, 2018	Retransplantation; primary non-function
SCS194	Jun 14, 2018	Oct 4, 2020	Deceased; sepsis secondary to COVID-19 pneumonia
SCS213	Mar 17, 2018	Sep 30, 2018	Deceased; pneumonia
SCS215	Mar 8, 2018	Mar 14, 2018	Retransplantation; hepatic artery thrombosis
SCS236	Dec 18, 2017	Feb 4, 2018	Retransplantation; biliary reconstruction complicated by hepatic artery pseudoaneurysm & intra-abdominal sepsis
SCS240	Dec 3, 2017	Dec 18, 2018	Deceased; ischemic cholangiopathy
SCS249	Oct 24, 2017	Dec 11, 2017	Deceased; graft versus host disease
SCS257	Sep 30, 2017	Mar 8, 2018	Deceased; variceal bleeding
SCS261	Sep 20, 2017	Sep 30, 2017	Deceased; liver failure secondary to small for size syndrome
SCS263	Sep 18, 2017	Jan 4, 2018	Deceased; respiratory failure secondary to fungal infection
SCS275	Jul 24, 2017	Apr 8, 2020	Deceased; respiratory failure secondary to non-small cell lung carcinoma
SCS284	Jul 3, 2017	Jul 9, 2020	Deceased; recurrent hepatic epithelioid hemangioendothelioma
SCS292	Apr 22, 2017	Mar 1, 2022	Deceased; recurrent metastatic HCC
SCS296	Apr 14, 2017	May 5, 2022	Deceased; recurrent liver cirrhosis secondary to ischemic stricture
SCS299	Apr 4, 2017	Oct 25, 2021	Deceased; unknown (died outside of hospital)
SCS306	Feb 28, 2017	Jan 9, 2018	Deceased; urosepsis
SCS311	Feb 10, 2017	Mar 22, 2022	Deceased; progressive multifocal leukoencephalopathy
SCS313	Jan 31, 2017	Mar 18, 2017	Deceased; intra-abdominal sepsis secondary to recurrent bile leak
SCS331	Nov 8, 2016	Sep 27, 2019	Deceased; unknown (died outside of hospital)

SCS337	Sep 13, 2016	Jan 25, 2019	Deceased; unknown (died outside of hospital)
SCS338	Sep 5, 2016	Nov 26, 2016	Deceased; metastatic HCC
SCS359	Jul 3, 2016	Aug 11, 2016	Retransplantation; acute liver failure secondary to bleeding hepatic artery pseudoaneurysm
SCS361	Jul 1, 2016	Jul 3, 2016	Retransplantation; primary non-function
SCS366	Jun 6, 2016	Aug 21, 2018	Retransplantation; hepatic artery thrombosis resulting in ischemic biliary strictures
SCS375	May 7, 2016	Dec 27, 2021	Retransplantation; recurrent biliary sepsis secondary to ischemic cholangiopathy
SCS388	Apr 5, 2016	Oct 22, 2019	Deceased; spontaneous bacterial peritonitis
SCS391	Mar 26, 2016	Jun 30, 2019	Retransplantation; recurrent primary sclerosing cholangitis
SCS400	Jan 27, 2016	Jan 8, 2017	Retransplantation; chronic rejection resulting in cholestasis
SCS406	Dec 23, 2015	Jan 22, 2018	Deceased; unknown (died outside of hospital)
SCS409	Dec 15, 2015	May 22, 2016	Deceased; chronic ductopenic rejection
SCS416	Nov 28, 2015	Nov 30, 2015	Deceased; primary non-function
SCS423	Oct 17, 2015	Dec 23, 2017	Deceased; recurrent metastatic HCC
SCS431	Sep 1, 2015	Mar 9, 2019	Deceased; recurrent metastatic HCC
SCS432	Aug 23, 2015	Aug 30, 2015	Deceased; hepatic artery thrombosis
SCS463	Apr 15, 2015	May 11, 2018	Deceased; rejection secondary to medication non-compliance
SCS471	Mar 12, 2015	May 9, 2019	Deceased; acute coronary syndrome in the setting of MRSA bacteremia
SCS478	Feb 18, 2015	Feb 18, 2015	Deceased; ST-elevated myocardial infarction
SCS484	Jan 2, 2015	Jan 21, 2021	Deceased; COVID-19 pneumonia
SCS489	Jun 12, 2016	Dec 28, 2016	Deceased; intra-abdominal sepsis
SCS516	Jul 16, 2021	Jul 21, 2021	Deceased; primary non-function
SCS522	Feb 26, 2021	Feb 28, 2021	Deceased; UGIB, sepsis
SCS525	Aug 12, 2021	Sep 13, 2021	Deceased; hepatic artery thrombosis
SCS529	Jul 23, 2021	Jul 28, 2021	Deceased; hyperacute antibody mediated rejection
SCS535	Mar 27, 2021	Apr 28, 2021	Deceased; distributive shock & ARDS in the setting of acute UGIB
SCS543	Aug 20, 2020	Aug 21, 2020	Retransplantation; primary non-function
SCS548	Sep 4, 2021	Jan 6, 2022	Deceased; multi-system infection
SCS554	Dec 17, 2021	Feb 5, 2022	Deceased; hypercarbic respiratory arrest
SCS564	Dec 27, 2021	Mar 26, 2022	Deceased; septic shock, COVID-19 pneumonia

***** 'ORGX' indicates patients in the NMP cohort, while 'SCS' indicated patients in the SCS cohort; ARDS=acute respiratory distress syndrome, COVID-19=corona virus disease 2019, HCC=hepatocellular carcinoma, MRSA= Methicillin-resistant *Staphylococcus aureus*, UGIB=upper gastrointestinal bleed

CHAPTER 5 – *EX VIVO* MACHINE PERFUSION OF THE LIVER – CURRENT STATUS & FUTURE DIRECTIONS

5.1 – *Ex Vivo* Machine Perfusion of the Liver – Current Status & Future Directions

A version of this section is currently being prepared for submission to *Transplantation Reviews*.

Title: *Ex Vivo* Machine Perfusion of the Liver – Current Status & Future Directions

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5.1.1 – Preface

Machine perfusion (MP) is a novel method of organ preservation that has recently gained prominence. MP of the liver exists as one of several modalities, varying by temperature at which the organ is perfused, as well as perfusate composition. Clinical studies have shown that normothermic machine perfusion (in which the organ is perfused at 37ºC; NMP) to be superior when used in place of static cold storage (SCS) by increasing graft utilization and decreasing early allograft dysfunction (EAD). Hypothermic oxygenated machine perfusion (HOPE) has similarly been found in clinical studies to decrease ischemic biliary strictures and EAD in livers donated after circulatory death. Despite these achievements, the potential of MP in liver transplantation goes beyond mere preservation. Currently, MP is being used for transportation, logistic considerations, and graft assessment. Going forward, we will see MP being used for graft modification to enable the transplantation of livers that would otherwise be discarded, such as with therapies to remove senescent cells in grafts from older donors, remove fat from steatotic livers, reduce reperfusion injury, and promote immune tolerance in the recipient. Additionally, MP will be increasingly used to facilitate pediatric liver transplants, liver splitting, and clearance of infection from donor organs. The future may see MP used as a platform for liver regeneration, super-cooling for long-term storage and transport and even reseeding of decellularized scaffolds with patient-derived cells. Immediate research directions should focus on optimizing techniques to assess livers via MP, including the development of biliary specific markers, determining which modality may be superior for preservation or whether integrated devices, capable of perfusion under varying conditions are more suitable. Given the prevalence of graft steatosis and the success of preclinical models, introducing defatting agents in clinical trials should be a priority.

Fundamentally, *ex vivo* machine perfusion refers to the mechanical circulation of a fluid (called the perfusate) through an organ's vasculature outside of the body. Though it has gained prominence in recent years for the preservation of various solid organs prior to transplant, it has been present from the early days of liver transplantation. In the 1960's, Thomas Starzl made use of a perfusion system that maintained livers in a hypothermic, hyperbaric, oxygenated environment, both in his experimental canine models and subsequently in clinical practice, including in two of the three first successful liver transplantations in humans.[1,2] Machine perfusion subsequently rapidly fell out of favour with the development of effective cold storage solutions. Its resurgence has now come as transplant teams seek superior methods of organ preservation in the face of growing organ demand and stagnant supply. But beyond mere preservation, machine perfusion offers opportunities for graft assessment and modification, which have yet to be fully realized and will undoubtedly become more important as demand for organs continues to increase.[3] This review will summarize the current status of liver machine perfusion in the clinical setting, including existing evidence and common strategies for use, and lay out key avenues for future development, highlighting relevant preclinical studies.

5.1.2 – Variations of Machine Perfusion in Liver Transplantation & their Clinical Evidence

Modern devices for perfusion of the liver have largely varied by the temperature at which the graft is maintained (**Figure 1**). Hypothermic machine perfusion (HMP) maintains the organ in the cold (usually 4ºC), circulating a perfusate similar to traditional static cold storage (SCS) solutions.[4] A variation of this, called hypothermic oxygenated machine perfusion (HOPE), provides the organ with oxygen dissolved in the perfusate.[5] At the opposite end, normothermic machine perfusion (NMP) seeks to maintain the liver in an environment as close as possible to the

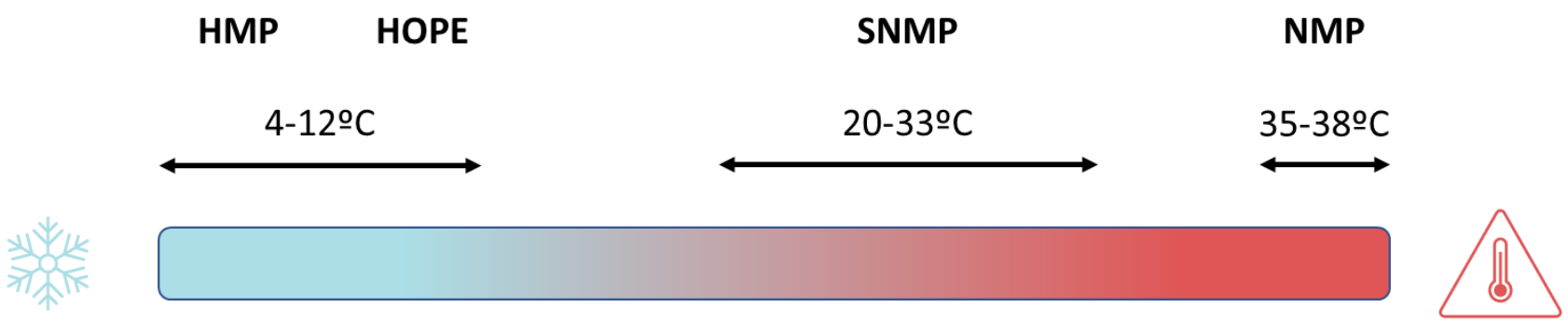


Figure 5.1.1. Temperature differences in variations of liver machine perfusion.

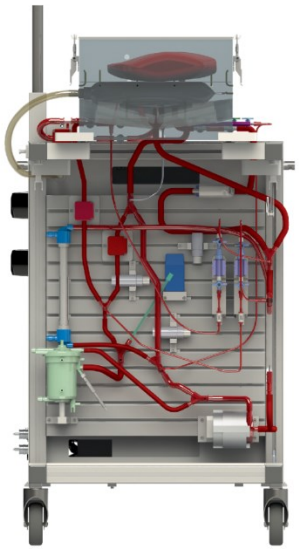
in vivo setting, not only by perfusing at physiologic temperature (i.e., 37ºC), but also making use of a blood-based perfusate and supplementing oxygen, nutrients, and bile salts.[6] In between, there is sub-normothermic machine perfusion (SNMP), which maintains the organ at room temperature or slightly warmer (20-33ºC) and may use a blood-based or acellular perfusate, while supplementing oxygen and nutrients.[7] To date, NMP has the most clinical evidence for use in liver transplantation.

Clinical Evidence for NMP

Two dedicated NMP devices have been produced for commercial use: the OrganOx metra® and TransMedics Organ Care System (OCS™) Liver (**Table 1**). Both are similarly composed of a single centrifugal pump delivering a red blood cell-based perfusate that is split between hepatic artery and portal venous inflows and exits from the inferior vena cava into a reservoir.[8,9] A key technical difference is the use of continuous hepatic artery flow in the former and pulsatile perfusion in the latter (generated by varying pump speed). A third device – the Liver Assist (XVIVO) – is capable of perfusion at a range of temperatures from 12-37ºC and has been used for both NMP and HOPE. Unlike the other two devices, the Liver Assist uses two centrifugal pumps (one delivering pulsatile flow to the hepatic artery and the other delivering continuous flow to the portal vein) and venous outflow accumulates freely in the organ chamber before it is recirculated.[10]

The first clinical NMP studies published used the OrganOx metra® device (**Table 2**). Several preliminary results were published from single institutions in Canada and the UK, which demonstrated safety following transplantation into humans compared to retrospective controls,

Table 5.1.1. Commercial devices for liver machine perfusion.

Device	Manufacturer	Modality	Specifications	Randomized Controlled Trials
LifePort Liver Transporter 	Organ Recovery Systems, Inc.	Hypothermic machine perfusion	- Single roller pump - Perfusion via hepatic artery & portal vein - Circulates Vasosol®	Complete, awaiting publication
Liver4Life 	Wyss Zurich	Normothermic machine perfusion	- Single centrifugal pump - Pulsatile perfusion via hepatic artery & portal vein - Circulates red blood cells combined with fresh frozen plasma, albumin & platelets	Not available

Liver Assist 	XVIVO Perfusion AB	Variable (12-37ºC)	- Two centrifugal pumps - Pulsatile arterial flow - Belzar MPS® UW machine perfusion solution (or similar) for HOPE - Red blood cells with albumin & crystalloid for NMP	van Rijn *et al.* (2021)
Organ Care Systems™ Liver 	TransMedics, Inc.	Normothermic machine perfusion	- Single centrifugal pump - Pulsatile arterial flow - Red blood cells with albumin & crystalloid - Supplemented with sodium taurocholate, other unspecified additives	Markmann *et al.* (2022)

OrganOx metra ® 	OrganOx Ltd.	Normothermic machine perfusion	- Single centrifugal pump - Laminar flow - Red blood cells with Gelofusine® - Supplemented with epoprostenol, heparin, insulin, Nutriflex®, sodium taurocholate	Nasralla *et al.* (2018)
VitaSmart™ 	Bridge to Life, Ltd.	Hypothermic oxygenated machine perfusion	- Single roller pump - Laminar flow via portal vein - Circulates Belzar MPS® UW machine perfusion solution	Ongoing

Table 5.1.2. Summary of clinical studies comparing machine perfusion prior to liver transplantation to standard static cold storage.

Study	Design	No. of Participants	Perfusion Strategy Key Features and Summary of Results
Normothermic Machine Perfusion			
Ravikumar *et al.* (2016)	Retrospective cohort	20 NMP vs. 40 non-perfused controls	- Median NMP time of 9.3h (3.5-18.5h) using the OrganOx metra® - No difference in 30d graft survival (100% NMP vs. 97.5% control, p=1.00) - Significantly lower peak AST in NMP cohort (417 vs. 902 IU/L, p=0.03)
Selzner *et al.* (2016)	Prospective cohort	10 NMP vs. 30 non-perfused controls	- Median NMP time of 480min (340-580min) using the OrganOx metra® - No difference in peak ALT (619 vs. 949 IU/L, p=0.55) - No graft loss in either cohort at 3 months
Bral *et al.* (2017)	Retrospective cohort	10 NMP vs. 30 non-perfused controls	- Median NMP time of 11.5h (3.3-22.5h) using the OrganOx metra® - No significant difference in EAD (55.5% vs. 29.6%, p=0.25) - 30d graft survival not significantly different between groups (90% vs. 100%, p=0.25) - No recipient deaths in either cohort at 1 month
Nasralla *et al.* (2018)	Randomized controlled trial	120 NMP vs. 100 non-perfused controls	- Median NMP time of 547.5min (372.5-710.5min) using the OrganOx metra® - Significant reduction in peak AST (488.1 vs. 964.9U/L, p<0.0001) and EAD (10.1% vs. 29.9%, p=0.0002) - Lower organ discard rates (11.7% vs. 24.1%, p=0.008)
Ghinolfi *et al.* (2019)	Randomized controlled trial	10 NMP vs. 10 non-perfused controls	- Livers from older donors (≥70 years old) perfused for a median time of 250min (195-282min) with the Liver Assist device - 1yr graft survival equivalent between groups (90% vs. 90%, p=1.00) - Equavalent EAD (20% vs. 10%, p=0.53)
Fodor *et al.* (2021)	Retrospective cohort	59 NMP vs. 59 non-perfused controls	- Total preservation time was significantly longer in the NMP group (21 vs. 7 h, p<0.001), using the OrganOx metra® - No difference in EAD (32% vs. 34%, p=0.794) - Fewer ischemic type biliary lesions (3% vs. 14%, p=0.047)

Guo *et al.* (2021)	Non-randomized controlled trial	38 perfused vs. 130 non-perfused controls	- Median perfusion for 240min (160-360min) using the Liver Assist device; initiated during procurement to achieve ischemia-free transplantation - Decreased EAD (50.0% vs. 5.3%; p<0.001) - No difference in patient or graft survival up to 1yr
Gaurav *et al.* (2022)	Retrospective cohort	67 NMP vs .69 NRP vs. 97 non-perfused controls	- Median NMP duration of 460min (330-569min) using either the OrganOx metra® or Liver Assist device - Lower peak ALT in NMP & NRP cohorts - No difference in graft survival at 6 months
Hann *et al.* (2022)	Retrospective cohort	26 NMP vs. 31 historical & 25 contemporary non-perfused controls	- Median NMP duration of 725min (475-934min) using the OrganOx metra® - Significantly high graft steatosis in perfused livers (p=0.006) - No difference in EAD (39% vs. 36% vs. 46%, p=0.743) - Equivalent 6-month graft survival (p=0.934)
Markmann *et al.* (2022)	Randomized controlled trial	153 NMP vs. 147 non-perfused controls	- Mean NMP time of 276.6±117.4min using TransMedics OCS™ Liver - Reduced EAD (18% vs. 31%, p=0.01) & ischemic biliary complications (2.6% vs. 9.9%, p=0.02) in recipients of NMP perfused livers - No difference in recipient survival up to 12 months
Hefler *et al.* (2023)	Prospective cohort	79 NMP vs. 386 non-perfused controls	- Median NMP time of 475min (387-620min) using the OrganOx metra® device - Improved 30d graft survival (100% vs. 94.3%, p=0.030) in NMP livers - No difference in EAD (25.3% vs. 28.2%, p=0.597) or overall graft survival (p=0.845)
Hypothermic Oxygenated Machine Perfusion			
Dutkowski *et al.* (2015)	Retrospective cohort	25 DCD HOPE vs. 50 DCD & 50 DBD non-perfused controls	- Median HOPE time of 118min (101-149min) at 10ºC via portal v. only, using the Liver Assist device - HOPE treated DCD livers had decreased graft injury measured by peak ALT (1239 vs. 2065U/L, p=0.02) & fewer instances of ischemic cholangiopathy (0% vs. 22%, p = 0.013)

			- Improved 1-year graft survival (90% vs. 69%, p = 0.035) compared to non-perfused DCD controls - No difference in outcomes between HOPE DCD livers and non-perfused DBD controls
van Rijn *et al.* (2018)	Retrospective cohort	10 HOPE vs. 20 non-perfused controls, all DCD	- Perfusion for at least 2h at 10-12ºC via portal v. & hepatic a. using the Liver Assist device - Worsening of bile duct stromal necrosis occurred after reperfusion of controls, but not HOPE livers - Fewer non-anastomotic strictures in perfused cohort (10% vs. 35%), but not statistically different (p=0.15)
Patrono *et al.* (2019)	Retrospective cohort	25 HOPE vs. 269 non-perfused controls, all DBD	- Mean perfusion time of 186±46min at at 10-12ºC via portal v. & hepatic a. using the Liver Assist device - No difference in EAD (p=1.00) or biliary complications (p=0.66) - Reduced incidence of AKI in matched sub-set (42.0% vs. 16.0%; p=0.046)
Schlegel *et al.* (2019)	Retrospective cohort	50 DCD HOPE vs. 50 DCD & 50 DBD non-perfused controls	- Median HOPE time of 2h (1.6-2.4h) at 10-12ºC via portal v. only using the Liver Assist device - Decreased graft loss at 1-year (8% vs. 32%, p=0.005) & decreased ischemic cholangiopathy (0% vs. 10%; p=0.0125) in DCD HOPE livers compared to non-perfused DCD livers
Mueller *et al.* (2020)	Retrospective cohort	70 DCD HOPE vs. 70 DCD & 70 DBD non-perfused controls; all transplanted for HCC	- Median HOPE time of 2h (1.7-2.5h); device & other perfusion parameters not reported - Decreased HCC recurrence between perfused DCD & non-perfused DBD cohorts (5.7% vs. 25.7%; p=0.002) - Improved recurrence free survival up to 5yrs (92% vs. 73%; p=0.027)
Czigany *et al.* (2021)	Randomized controlled trial	23 HOPE vs. 23 non-perfused, all extended criteria DBD	- Perfused for a minimum of 1h at 10ºC via portal v. only using the Liver Assist device - Decreased peak ALT (796 vs. 418U/L; p=0.030) & 90d major complications (74% vs. 44%; p=0.036) in HOPE group - 1-year graft survival improved in HOPE group (91% vs. 78%), but not statistically different (p=0.253)

De Carlis *et al.* (2021)	Retrospective cohort	37 HOPE vs. 37 non-perfused, all DCD	- NRP followed by HOPE for median time of 120min (42-380min) at 10ºC via portal v. & hepatic a. using the Liver Assist device - Decreased AKI in perfused cohort (p=0.001) - Graft survival at 2 years improved (91% vs. 81%), but not statistically different (p=0.227)
van Rijn *et al.* (2021)	Randomized controlled trial	78 HOPE vs. 78 non perfused, all DCD	- Median HOPE time of 132min (120-153min) at 10ºC via portal v. using the Liver Assist device - Decreased symptomatic non-anastomotic biliary strictures in HOPE group (6% vs. 18%, p=0.03) - No difference in graft survival up to 6 months
Horné *et al.* (2022)	Retrospective cohort	50 HOPE vs. 50 non-perfused, all DBD	- Perfused at 8-12ºC via the portal v. using the Liver Assist device; duration of perfusion not reported - Decreased EAD in HOPE cohort (28% vs. 50%, p=0.04)
Patrono *et al.* (2022)	Retrospective cohort	121 perfused vs. 723 non-perfused	- Median HOPE time of 138min (117-180min) at 10ºC via portal v. & hepatic a. using the Liver Assist device - Decreased early allograft failure (p=0.024) & major complications (≥ Clavien-Dindo 3; p=0.046) with adjusted analysis
Ravaioli *et al.* (2022)	Randomized controlled trial	55 perfused vs. 55 non-perfused, all extended criteria DBD	- Median HOPE time of 145min (120-185min) using the VitaSmart™ machine perfusion device - Decreased EAD in HOPE group (13% vs. 35%, p=0.007) - No instances of retransplantation (compared to 11% of controls; p=0.027)
Rossignol *et al.* (2022)	Prospective cohort	16 perfused hemi-livers vs. 24 non-perfused	- Median HOPE time of 125min (95-165min) at 8-10ºC via portal v. only using the Liver Assist device - Reduction in cold storage time - No difference in clinical outcomes, including graft survival
Schlegel *et al.* (2023)	Randomized controlled trial	85 perfused vs. 85 non-perfused	- Median HOPE time of 95.5min (73-137min) at 8-12ºC via portal v. only using the Liver Assist device - No difference in the primary outcome of at least 1 complication ≥ Clavien-Dindo grade 3 (51.8% vs. 54.1%, p=0.76) - Fewer severe complications overall in HOPE group (p=0.027) & no instances of graft failure due to liver-related complications (vs. 7% of controls, p=0.004)

Hypothermic Machine Perfusion			
Guarrera *et al.* (2010)	Prospective cohort	20 perfused vs. 20 non-perfused	- Mean perfusion for 4.3±0.9h at 4-8ºC via portal v. & hepatic a. using a modified Medtronic Portable Bypass System® - Lower peak AST in HMP cohort (1154 vs. 3339IU/L, p=0.011) - No difference in 1-year graft survival (90% in each group)
Guarrera *et al.* (2015)	Prospective cohort	31 perfused vs. 30 non-perfused, all extended criteria	- Mean perfusion for 3.8±0.9h at 4-8ºC via portal v. & hepatic a. using a modified Medtronic Portable Bypass System® - Decreased EAD in HMP group (19% vs. 30%), though not statistically significant (p=0.384) - Decreased biliary complications (13% vs. 43%, p=0.001) - No difference in 1-year survival (83.8% vs. 80%, p=0.761)
Combined Hypothermic & Normothermic Machine Perfusion			
van Leeuwen *et al.* (2019)	Prospective cohort	11 perfused vs. 36 DCD & 24 DBD non-perfused	- Perfusion via portal v. & hepatic a. using Liver Assist device; 8-12ºC for 1h, then increased 0.5ºC/min to 37ºC - Decrease peak AST in perfused group compared to DCD cohort (751 vs. 2406IU/L, p=0.0004) - Improved 1-year graft survival compared to DCD cohort (100% vs. 80%), though not statistically significant (p=0.207)

with one study showing significantly decreased peak aspartate aminotransferase (AST) in the first post-operative week.[11-14] The first major clinical trial of the OrganOx metra® was published by Nasralla *et al.* in 2018.[8] They reported results of a multi-center, randomized controlled trial (RCT) conducted, which compared clinical outcomes for 137 livers allocated for NMP to 133 allocated to SCS only. Livers in the NMP group were perfused for a median time of 547.5 minutes, leading to a significantly longer total preservation time than the SCS group (465min in the SCS group vs. 714min in the NMP group, $p<0.0001$). They found a 48.5% relative reduction in discard rate (24.1% in the SCS group vs. 11.7% in the NMP group, $p=0.008$) for livers preserved with NMP, as well as significant reductions in early allograft dysfunction (29.9% in the SCS group vs. 10.1% in the NMP group, $p=0.0002$; EAD) and post-reperfusion syndrome (33.0% in the SCS group vs. 12.4% in the NMP group, $p=0.0002$). Despite early benefit, long-term patient and graft survival up to 1 year were no different between groups, though the trial may not have been sufficiently powered to show this results. On the basis of this trial, the *ex vivo* perfusion for liver preservation was approved for use in the UK (up to 24 hours of continuous perfusion) by the National Institutes for Health and Care Excellence (NICE) in early 2019.[15]

A second multicentre RCT using the OrganOx was completed across 15 US centres between 2016 and 2020.[16] Trial results were submitted to the Federal Drug Administration (FDA), which granted the device premarket approval (PMA) in late 2021 for continual use up to 12 hours.[17] The FDA restricted use of this device to 12 hours, owing to the lack of available data from longer perfusions. This study was an open-labeled RCT that randomized 383 livers, 266 of which were transplanted (130 into the SCS group and 136 into the NMP group). As with the European study, the additional warm perfusion time (median of 323min) in the NMP group led to a longer total preservation time compared to the SCS group (523min vs. 303min in the SCS group).

However, unlike the previous study, there was no difference EAD (their primary outcome), which occurred in 20.5% of NMP livers versus 22.8% in the SCS group by intention-to-treat analysis (p=0.275). They did, however, find a decreased incidence of post-reperfusion syndrome in the NMP group (5.9% vs. 14.6% in the SCS group), though statistical significance was not reported.

Additional large single centre non-randomized studies using the OrganOx have been published since completion of the RCTs. Fodor *et al.* from Innsbruck, Austria compared 59 consecutive NMP livers with the same number of propensity matched controls.[18] Livers in the NMP cohort were perfused for a median time of 21 hours (following a median time of 6 hours of cold storage), which was significantly longer than the preservation time in the SCS cohort (median time of 7 hours; p<0.001). They found no difference in EAD, vascular or biliary complications (p=0.794, 0.793, 0.854, respectively). However, unique to this study, they found significantly fewer ischemic type biliary lesions (3% vs. 14% in the SCS cohort; p=0.047).

In a follow up to their report of the first 9 patients, Hefler *et al.* recently published their 7-year experience, which compared 79 NMP livers to 386 livers transplanted after SCS only in the same time period.[19] Livers in the NMP cohort were perfused for a median time of 475 minutes, which combined with a cold storage time of 359 minutes, resulted in significantly longer overall preservation time compared to the SCS cohort (847min vs. 288.5min in the SCS cohort; p<0.0001). They found improved 30-day graft survival in the NMP cohort (100% vs. 94.3% in the SCS cohort; p=0.030); however, graft survival was no different by the 6 month or 1 year mark (p=0.945 and 0.571, respectively). No differences in EAD, or biliary or vascular complications were seen between cohorts (p=0.597, 0.193, 0.269, respectively). The OrganOx metra® is currently under active review by Health Canada, with request for ongoing clinical use for their Special Access program pending final device approval.

The only published clinical trial utilizing the OCS™ Liver device was a multicentre RCT reported by Markmann *et al.* in early 2022.[9] They included clinical outcomes of 151 livers randomized to receive NMP compared to 143 preserved with SCS only across 20 centres in the United States. In their study, livers were perfused for an average time of 276.6±117.4 minutes, leading to a significantly longer total preservation time (338.8±91.5 minutes in the SCS group vs. 454.9±133.9 minnutes in the NMP group). They found a reduction in EAD (31% in the SCS group vs. 18% in the NMP group, p=0.01), which corresponded with a decrease in histological evidence of ischemia/reperfusion injury (18% vs. 6% incidence of moderate to severe lobular inflammation in the SCS and NMP groups respectively, p=0.004). Unlike the previous RCTs using the OrganOx metra®, Markmann *et al.* reported significantly decreased incidence of ischemic biliary complications at 6 and 12 months (8.5% and 9.9% for the SCS group vs. 1.3% and 2.6% for the NMP group at 6 and 12 months respectively, p=0.02). This data sufficed to satisfy the safety requirement for the FDA's premarket approval of the device in September 2021.

Evidence for NMP using the Liver Assist device is limited. Ghinolfi *et al.* published a small RCT comparing 10 NMP livers to 10 livers transplanted after SCS only from donors ≥70 years old.[20] Livers in the NMP group were perfused for a median time of 250 minutes. They reported decreased histological evidence of ischemia/reperfusion injury (on the basis of mitochondrial assessment via electron microscopy), but no difference in clinical outcomes, including EAD, or biliary or vascular complications. Guo *et al.* similarly employed the Liver Assist device in their study of ischemia-free liver transplantation.[21] In their comparison of 38 ischemia-free livers to 130 non-randomized controls, they found a significant reduction in EAD (from 50% in SCS controls to 5.3% in the ischemia-free cohort; p<0.001). One month graft survival was also decreased (97.4% vs. 90.0%), but this did not achieve statistical significance.

Clinical Evidence for HOPE

The VitaSmart™ machine perfusion system (manufactured by Bridge To Life Ltd.) is the only commercial device developed specifically for HOPE. An RCT using the VitaSmart™ device has been published from a single European centre and a larger RCT across 16 US centres is ongoing (NCT05045794). However, the majority of published clinical studies using HOPE have used the Liver Assist device, generally perfusing with a modified University of Wisconsin (UW) solution (Belzer MPS®) at 10-12ºC.

Preliminary studies were published using HOPE around the same time as the early NMP studies.[22,23] These studies focused specifically on DCD donors. The first by Dutkowski *et al.* found significant improvements in EAD (20% vs. 44% in controls; p=0.046) and 1-year graft survival (90% vs. 69% in controls; p=0.032) for 25 livers perfused for a median time of 118 minutes, compared to matched DCD controls.[22] They also found a reduction in ischemic cholangiopathy (defined as intrahepatic strictures in the absence of hepatic artery thrombosis or stenosis), occurred in 22% of DCD controls, but none of the perfused livers (p=0.015). A second study by van Rijn *et al.* compared 10 DCD livers perfused, for a minimum of 2 hours, to matched controls and similarly found a reduction in non-anastomotic strictures (10% vs. 35% in controls), though this did not achieve statistical significance (p=0.15).[23] A subsequent retrospective study, by Patrono *et al.*, looked alternatively at HOPE following donation after brain death (DBD) and found no difference in EAD or biliary complications (32% vs. 325, p=1.00 and 18.2% vs. 24.0%, p=0.66, respectively) compared to matched controls.[24]

The first RCT utilizing HOPE was published by van Rijn *et al.* in 2021.[25] Their trial used the Liver Assist device across 6 European centres for patients receiving livers following DCD. Outcomes of 78 patients receiving livers transplanted after at least 2 hours of machine perfusion

(median time 132 minutes) following SCS were compared to the same number transplanted after SCS only. SCS times were similar between groups (371 minutes in the HOPE group vs. 409 minutes for controls). They reported a significant decrease in ischemic biliary strictures (18% vs. 6%; p=0.03). Post-reperfusion syndrome and EAD were also significantly lower amongst recipients of perfused livers, but no difference was seen in patient or graft survival up to 6 months, though it was not powered specifically to detect this difference.

A second European multicentre RCT was recently published applying HOPE (via the Liver Assist device) to livers from DBD donors.[26] In this study, 177 livers were randomized, resulting in 85 being transplanted following HOPE and the same number transplanted after traditional SCS. Livers in this study underwent HOPE for a median 95.5 minutes (73-137min), leading to a slightly longer overall preservation time of 474 minutes (compared to 427 minutes in the control group). Unlike previous studies of HOPE and NMP, their primary outcome was post-operative complications, specifically the occurrence of at least one complication with a Clavien-Dindo grade ≥3. They found no difference in this respect, with 51.8% of patients in the HOPE group experiencing a complication compared to 54.1% of controls (p=0.76). However, secondary analysis found a fewer number of complications grade ≥3 (p=0.027) amongst recipients of HOPE livers. In addition, no graft loss due to liver-related complications was seen in the HOPE group, whereas this occurred in 7% of the control group (p=0.004) on post-hoc analysis.

Ravaioli *et al.* published their experience using the VitaSmart™ perfusion device.[27] In a single centre RCT, they included 55 patients transplanted following HOPE and 55 after SCS only. Livers from extended-criteria DBD donors underwent perfusion for a median time of 145 minutes (120-185min), for a total preservation time of 400 minutes (compared to 420 minutes for control livers). They found a lower proportion of EAD in the HOPE group (13% vs. 35%; p=0.007), which

corresponded with a lower rate of retransplantation within the follow up period (0% vs. 11%; p=0.027). No difference in biliary or vascular complications was seen.

Clinical Evidence for HMP & SNMP

Despite being one of the first modalities for which clinical data became available, high-quality evidence for the use of HMP (i.e., hypothermia without oxygenation) is lacking. The initial clinical series, published in 2010 by Guarrera *et al.*, found significant reductions in post-operative alanine aminotransferase (ALT), AST and bilirubin in the first 7 days, with a trend towards decreased EAD, but no difference in patient or graft survival.[28] In this series, cold ischemic times were comparable between groups (8.9±2.8h in the control cohort vs. 9.4±2.1h in the perfused cohort), nearly half of which was composed of cold ischemic perfusion (for the perfused cohort). A subsequent study by the same group found no difference in clinical outcomes after HMP of 31 extended criteria donor livers that had been previously declined for transplantation.[29] These studies used a modified version of the Medtronic Portable Bypass System® (intended for cardiopulmonary bypass) circulating Vasosol® (a variant of UW with additional vasodilation and antioxidant agents). The commercial produced device based on these studies, the LifePort Liver Transporter (Organ Recovery Systems), has recently completed a multicentre RCT (NCT03484455) across several centres in the United States, whose results have yet to be released. As with the previous studies, the LifePort Liver circulates Vasosol®.

To date, there have been no clinical studies directly comparing SNMP with SCS, nor are there any ongoing publicly registered clinical trials. SNMP of discarded human livers has been demonstrated to improve oxygen consumption, lactate clearance and adenosine triphosphate (ATP) content over the course of perfusion; however, this was in grafts that were not transplanted.[7]

The few clinical studies utilizing SNMP do so in the context of controlled rewarming, in which the graft temperature is gradually increased from hypothermic, through sub-normothermic and, finally, to normothermic perfusion.[30] The only commercial device capable of delivering SNMP is the Liver Assist device, which has an adjustable temperature between 12 and 37ºC.

Taken together, these trials show that, at a minimum, there are several different modalities of machine perfusion that can preserve liver grafts at least as well as SCS. They provide good evidence that NMP and HOPE, in particular, can be used to safely extend preservation times beyond those typically tolerated in SCS without incurring worse outcomes. NMP may have some additional benefit in mitigating ischemia/reperfusion injury (on the basis of EAD and histologic findings). While HOPE seems to have a distinct benefit in reducing ischemic biliary complications in at risk organs (i.e., DCD livers). The role of machine perfusion in reducing ischemic biliary complications across all categories of organs (i.e., including DBD livers) has not been convincingly shown for either NMP or HOPE and differences between trials using similar techniques with different devices (i.e., OrganOx metra® vs. OCS™ Liver) suggest that a reduction in these complications may not be explained by the temperature of perfusion alone.

Cost-Effectiveness of Liver Machine Perfusion

Liver transplantation itself is a costly endeavour. A 2020 report from the risk management firm Milliman Inc. estimated an average of $878,400(USD) billed per liver transplant in the US, including pre-transplant workup, organ procurement, transplant admission, and 180 days of follow up care.[31] The American Society of Transplant Surgeons estimates that machine perfusion will add $25,000-$50,000(USD) in extra costs per liver transplant.[32] This estimate, of course, depends on which device is used (NMP devices are more costly in terms of consumables), how long the organ

is perfused and whether the device is transported. The potential of machine perfusion to reduce complications, which has been most clearly shown in the case of HOPE for DCD liver transplantation, could offset its additional cost. However, the benefits of machine in facilitating the use of more organs, especially those from extended criteria donors, may not be readily apparent from a pure cost standpoint, as it would be less expensive for potential recipients to exit the waitlist under circumstance other than receiving a transplant.

An analysis of the cost-effectiveness of NMP in the Canadian setting, using the OrganOx metra®, was performed by Webb *et al.* using a combination of local data and literature values (including survival probabilities based on the RCT by Nasralla *et al.*).[33] While the transplantation itself was more expensive with NMP ($118,563[$USD] vs. $93,762[USD]), incorporating NMP allowed for an estimated 15 additional transplants per year, leading to reduced costs and improved quality of life due to a decrease in the number of patients awaiting transplantation and waitlist mortality. Modeling the NMP versus SCS only strategy for 100 hypothetical patients over a five-year period, they found that the mean cost using the NMP strategy was $456,455($USD) compared to $519,222(USD) with the SCS only strategy. The use of NMP also led to increased quality-adjusted life years (3.48 vs. 3.17 with the SCS only strategy; QALY). Contrastingly, in a cost-utility analysis comparing the OrganOx metra® and Liver Assist devices against standard of care in the UK setting, Zimmermann and Carter estimated that use of the OrganOx led to higher costs and lower QALYs, while the Liver Assist led to lower costs, but also lower QALYs.[34] However, they acknowledged uncertainty due to imperfect and missing data. Ultimately, the cost-effectiveness of machine perfusion in liver transplantation will vary greatly depending on in what fashion and under what circumstances machine perfusion is used, an assessment that continues to evolve as more becomes understood about the possibilities of machine perfusion.

5.1.3 – Current Strategies for the Use of Machine Perfusion in Liver Transplantation

Major trials have highlighted the utility of machine perfusion in liver transplantation, but they do not necessarily reflect the way in which it is used clinically.[35] Criteria for use varies from centre to centre, and even from surgeon to surgeon. Particularly with its different modalities, machine perfusion can be adapted to meet one or more of several challenges present in modern liver transplantation. The most consistent evidence across multiple trials is that machine perfusion (whether NMP or HOPE) can be used to extend preservation time without incurring additional injury. Whereas prolonged cold ischemia is strongly correlated with worse outcomes, including graft failure, machine perfusion appears to at least partially mitigate these outcomes.[36]

Transportation

An early consideration in the design of most commercial machine perfusion devices was that they be portable. Attaching the organ to the device at the donor site and continuing perfusion during transportation would maximize perfusion time (limiting cold ischemia in the case of HOPE or NMP) and, potentially, enable transport from more distant centres. Devices from European manufacturers (e.g., OrganOx metra®, Liver Assist) were designed to be portable in the back of an ambulance or ambulance van, whereas those designed with the North American experience in mind (e.g., OCS Liver, LifePort Liver Transporter) accounted for the necessity of flying by fixed wing transportation from donor to recipient centres, recognizing the more disparate geographic challenges involved..[37-40] Despite their innovative designs, machine perfusion devices still remain more cumbersome than traditional SCS techniques, often requiring specific vehicles be available for transport and specially trained personnel. As such, not all centres have made routine use of it for organ transport.[41] Certainly, transport with the device is likely to be more effective in cases of

higher risk donors or when the anticipated transport time would be prohibitively long otherwise, but specific indications have not been widely discussed in the literature. The multicentre RCTs of NMP did specify that machine perfusion was started at the donor site, which is why they were able to achieve significantly lower cold ischemic times.[8,9] However, this was not necessarily the case in subsequent studies and needs to be taken into consideration when interpreting results. HMP may be most suitable to transport given that it has fewer technical requirements and, if the device failed during transport, it would essentially revert to SCS.[4]

Logistics

Even without perfusion during transportation, adoption of a 'back-to-base' strategy, in which perfusion begins upon returning to the recipient centre, has been used to address some logistic challenges of transplantation.[42] For instance, it can be used to avoid operating at late hours, thereby mitigating staff fatigue and burnout and allowing easier accommodation into the operating room (OR) schedule. In the Canadian setting, where distances between liver transplant centres can be vast, machine perfusion can allow one centre to accommodate multiple offers. It can also be used in the cases where the recipient may be delayed, or a post-operative intensive care unit (ICU) bed is unavailable. Bogensperger *et al.* described a case report illustrating this point, in which NMP was used to extend the preservation of a donor liver by 10.5 hours (to a total time of more than 16 hours) both to ensure a negative COVID-19 test of the recipient and accommodate the reduced OR and ICU capacity early in the COVID-19 pandemic.[43] The recipient had good initial liver function and no complications to discharge, despite the prolonged preservation time.

Complicated explanations, including re-transplantations and multi-organ transplants, have also benefited from the additional time afforded by machine perfusion. A case illustrated by

Carvalheiro *et al.* describes the use of NMP in a recipient of a third liver transplantation due to late hepatic artery stenosis in the second graft.[44] In addition to the significant intra-abdominal adhesions expected, this case further complicated by extensive IVC thrombosis extending almost to the right atrium, which required cardiopulmonary bypass and open extraction by a cardiovascular surgery team. NMP lasted for 23 hours for a total preservation time of 30.5 hours. Without NMP to facilitate, this surgery would not have been possible, and, despite his high-risk profile, he recovered well, with the authors reporting no significant complications up to 12 months. A corollary to this that machine perfusion may facilitate the transplantation of sub-optimal grafts that would otherwise be prohibitive in re-transplant patients. This was demonstrated by in a cohort study by Hann *et al.*, who found that use of NMP enabled the transplantation of more steatotic grafts (some of which had been declined by other centres) into re-transplant recipients with no difference in outcomes.[45]

Graft Assessment

Another important way in which machine perfusion is increasingly being used is for assessment of marginal grafts. This has been done mostly with a focus on NMP, given that the liver is the most metabolically active with this technique. Among the most widely cited is the VITTAL study published by Mergental *et al.*, which considered discarded livers to be viable if within 4 hours of perfusion they demonstrated lactate clearance to <2.5mmol/L plus two or more of bile production, pH>7.30, glucose metabolism, hepatic artery flow ≥150mL/min and portal vein flow ≥500mL/min, or homogenous perfusion.[46] In their study, they assessed 31 livers that had been previously declined by all centres in the UK. Using these criteria, they were able to transplant 22 (71%) of the livers and reported 100% graft survival at 90 days. However, of these transplanted

livers 18% developed biliary strictures requiring retransplantation by the end of the follow up period (median time of 542 days). Watson *et al.* similarly proposed lactate clearance, maintenance of pH, consumption of glucose, and perfusate transaminase level in their initial assessment of discarded livers for transplant.[47] Their analysis of 203 perfused livers found that ALT >6000U/L and lactate >2.8mmol/L after 2 hours of perfusion, peak bile pH <7.6, as well as a need to use ≥30mmol of bicarbonate to correct perfusate pH to be associated with EAD.

Several variations have been proposed in the literature, with normalization of lactate and maintenance of perfusate pH consistently included.[48] Still, no criteria have been universally adopted and there are ongoing endeavors to identify to most reliable markers of graft viability. It has been suggested that an ideal viability parameters should assess both hepatocyte and cholangiocyte function and/or health.[38] Bile production in and of itself has not been correlated with improved graft function or reduced biliary complications, though some studies suggest the quality of the bile (e.g., alkaline pH, bilirubin concentration) is important.[49] In their model of rat liver SNMP, Abraham *et al.* even found markers of hepatocyte injury (ALT and AST) in bile to be more sensitive to ischemia duration than the same markers measured in the perfusate.[50]

Additional, novel biomarkers have been proposed including microRNAs (miRNAs) and damage-associated molecular patterns (DAMPs) (**Table 3**). In a study of 12 discarded livers perfused via NMP, Matton *et al.* found miRNAs HDmiR-122 and CDmiR-222 in the perfusate, measured at 30 minutes, correlated strongly with peak AST levels and that their relative ration was predictive of histological injury at 6 hours.[51] Release of flavin mononucleotide (FMN) from damaged mitochondria has also been shown to be higher in NMP perfused primate livers with longer ischemia time and have been associated with delayed function or primary non-function in human kidneys.[52,53] As well, Beetz *et al.* found significantly higher levels of the proinflammatory

cytokine, interleukin-18 (IL-18), and the DAMP, high mobility group box 1 (HMGB1), in porcine livers reperfused with NMP after prolonged cold ischemia, compared to controls.[54] IL-18 has also been found to be decreased in effluent flushed from HOPE perfused human livers compared to that from livers subject to SCS only.[55] In addition, lower levels of the proinflammatory markers, pentraxin-related protein 3, tissue necrosis factor alpha, and angiopoietin-like protein 4, and higher levels of the anti-inflammatory cytokine, IL-10, were observed in effluent from HOPE perfused livers. De Vries *et al.* even observed the release of whole liver cells (including hepatocytes, sinusoidal cell, stellate cells, and resident immune cells) during SNMP of rat livers and found that hepatocyte release most strongly correlated to ischemic injury over the course of perfusion, while initial release of sinusoidal and stellate cells was associated with cold ischemic injury.[56]

Beyond markers of injury, some novel techniques for functional assessment are being explored. Schurink *et al.* applied the liver maximum capacity (LiMAx) test, which measures liver function based on the breakdown of ^{13}C-methacin to $^{13}CO_2$ by the cytochrome p450 1A2 enzyme, to human livers perfused via NMP.[57] Previous studies had identified values for normal (315μg/kg/h) versus poor (140μg/kg/h) liver function.[58] They found a range of 421-1838μg/kg/h in the perfused livers, with clear associations between lactate clearance and peak perfusate transaminases.

Non-invasive imaging techniques have also been proposed. Kneifel *et al.* applied hyperspectral imaging (HIS) during NMP of 25 transplanted livers.[59] HIS provides data on organ microperfusion using such measures as oxygen saturation, tissue hemoglobin index (THI), near-infrared perfusion index (NIR), and tissue water index. They found that both oxygen saturation and THI correlated with perfusate lactate. As well, recipients who ended up dying had lower NIRs at 4 hours of perfusion, though this did not achieve statistical significance ($p=0.0784$).[59] Hou *et al.*

described the application of dielectric relaxation spectroscopy (DRS) to NMP perfused porcine livers.[60] Measurements are taken over a range of frequencies, with lower frequencies being sensitive to structural changes, while higher frequencies are indicative of the distribution of polar molecules (i.e., water). They found that differences in conductivity became more apparent over the course of perfusion, with injured livers (subject to either hepatic artery occlusion or prolonged cold storage) showing greater change in conductivity compared to controls.

Most of the novel methods of graft assessment have focus on NMP, particularly in studies human livers. Assessments using HOPE or HMP are limited by the lack of metabolic activity, though perfusate transaminases and vascular resistance can still provide some information on the degree of injury.[61] A caveat is that the full extent of graft injury may not be apparent until the organ is reperfused. Additionally, it is worthwhile to consider whether different criteria may be suitable for specific subcategories of livers, such as those with steatosis or those from older donors.

5.1.4 – Potential Future Strategies for the Use of Machine Perfusion in Liver Transplantation

The greatest potential for machine perfusion lies in expanding the donor pool. This ultimately means making use of grafts that would otherwise go to waste, as well as reducing the incidence of graft loss and re-transplantation. Machine perfusion has the potential to facilitate several strategies for increasing graft utilization (**Figure 2**).

Liver Defatting

Defatting (or de-steatification) protocols have already been well established in animal models. Nagrath *et al.* first reported de-steatification of a rat liver (reducing triglyceride content by 65%) using a defatting composed of forskolin (induces lipolysis by increasing cellular cAMP),

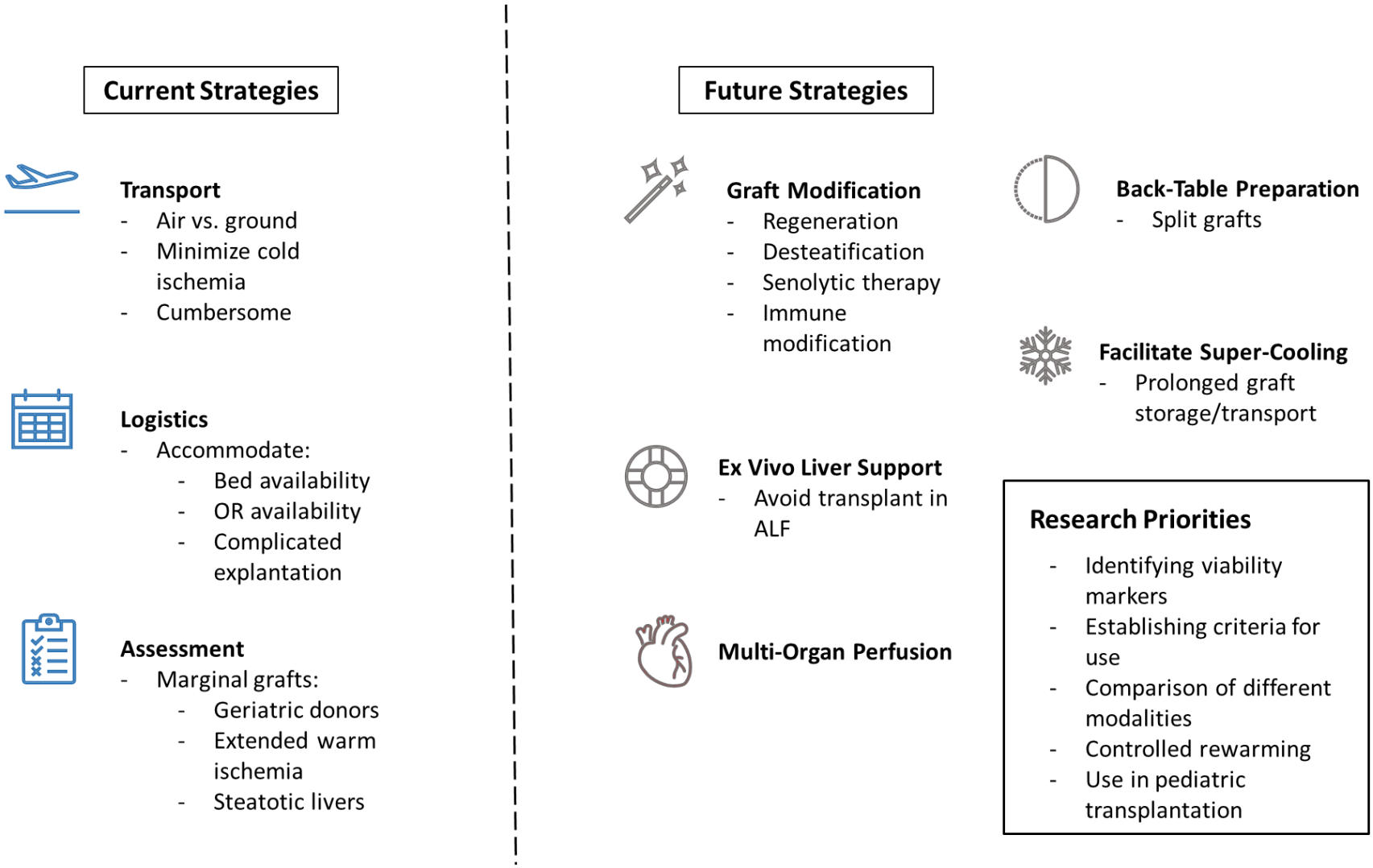


Figure 2. Current and potential future strategies for the use of machine perfusion in liver transplantation.

a PPARα receptor agonist (GW7647), hyperacin (inhibitor of lipid peroxidation), scoparone (inhibitor of TGFβ/SMAD signaling pathway), visfatin (for NAD^+ salvage), and a PPARβ receptor agonist (GW501516).[62] A similar defatting cocktail used in discarded steatotic human livers has been shown to reduce triglyceride content by 38% and macrovascular steatosis by 50% in 12 hours.[63] NMP by itself (i.e., without the inclusion of specific defatting agent) has been shown to reduce steatosis in rat and porcine models.[62,64] That the same finding has not been observed with NMP of discarded human steatotic livers has been attributed to the chronic nature of lipid deposition in humans as opposed to the more rapid accumulation in animal models.[65] The use of NMP for defatting of steatotic livers has the potential to substantially increase the donor pool, given the rising rates of obesity and associated non-alcoholic fatty liver disease across the world. As to whether liver defattinf can be accomplished with other modalities of machine perfusion, Liu *et al.* assessed the efficacy of defatting cocktails during SNMP (at 20ºC) of rat livers and found that the defatting cocktail did not reduce lipid content after 6 hours of perfusion.[66]

Senolytic Therapy

Another key demographic trend affecting the donor pool is the aging population. In the United States, the proportion of senior citizens is expected to exceed that of children (<18 years old) by 2035, a demographic shift that has already been seen in Japan and several European countries.[67] While the liver has a remarkable capacity for regeneration, donor age has been clearly associated with decreased graft survival.[68] Senolytic therapy administered in the context of machine perfusion has the potential to increase the utilization of livers that would otherwise be discarded due to donor age. Senolytics work by decreasing the burden of senescent cells that accumulate through the normal aging process. Not only are these senescent cells themselves less

robust, with compromised mitochondrial capacity and decreased capacity for tissue regeneration, they also induce senescence in adjacent, quiescent cells through production of a senescence-associated secretory phenotype.[69] Senolytics can work through a variety of mechanisms, including inhibition of B-cell lymphoma-2 (BCL-2) family proteins, the 90kDa heat shock protein (Hsp90), and the p53 pathway.[70] There are also a variety of unrelated senolytics that have been identified by high throughput screening of molecular libraries, as well as techniques of targeting other chemotoxic agents to senescent cells, such as by modifying them with a galactose moiety (most senescent cells have increased lysosomal β-galactosidase activity). While there is a lack of evidence from liver transplant models, the administration of senolytics to older donors has been shown to improve graft survival of cardiac allografts in mice.[71] Antiaging glycopeptide has been shown to prevent tacrolimus-related injury in mice transplanted with xenogenic pancreatic islets by protecting against oxidative stress and may have a role in reducing senescence.[72] In 2022, Ferreira-Gonzalez *et al.* found that the administration of two senolytics (dasatinib plus quercetin) during NMP of discarded human livers resulted in better preservation of biliary architecture and retained regenerative capacity of cholangiocytes.[73] Machine perfusion as a platform for senolytic therapy has the advantages of avoiding systemic side effects (e.g., thrombocytopenia seen with BCL-2 inhibitors) and requiring a lower dose.

Mitigation of Ischemia/Reperfusion Injury & Promotion of Immune Tolerance

In addition to modification for the specific indications of steatosis and old age, machine perfusion may serve as a platform for more general modifications, such as those aiming to reduce ischemia/reperfusion injury or promote tolerance in the recipient. Both are complex processes, involving multiple cellular pathways and interactions at different levels of the immune system.

Modulation of these responses could be achieved by gene silencing (e.g., RNA interference [RNAi]), gene editing (e.g., CRISPR/Cas9) or cell therapy. Though these therapies could be applied systemically, machine perfusion can target the intervention to the specific organ, potentially making it more effective and avoiding unintended side effects. As well, using machine perfusion avoids the ethical concerns of treating the donor for the benefit of the recipient.

Various RNAi targets have been studied in animal models of ischemia/reperfusion injury (either transplant or vascular pedicle clamping), including toll-like receptor 4 (TLR4), caspase 3 and 8, Fas receptor, tumour necrosis factor alpha, and various complement proteins.[74] Uptake of small interfering RNA targeting the *FAS* gene during HOPE has been demonstrated to reduce injury in a rat model of liver transplantation.[75] The same group also demonstrated successful delivery of a CRISPR/Cas9 edited gene product by viral transfection during HOPE.[76]

There are several potential cell therapies that could be applied to the liver during machine perfusion. Different types of stem cell therapies have been proposed for liver disease, including hepatocyte-like cells derived from either embryonic or induced pluripotent stem cells, multipotent adult progenitor cells (MAPCs), mesenchymal stem cells (MSCs) and liver progenitor cells (which are capable of differentiating into hepatocytes or cholangiocytes).[77] The benefits of adding hepatocytes or their precursors, particularly if derived from the recipient, to replace older or injured cells are clear. MAPCs and MSCs have unique properties that influence the cellular milieu through the release of anti-inflammatory and pro-tolerogenic cytokines.[78] A study infusing MAPCs into the portal vein of liver recipients at the time of transplant found a significant increase in regulatory T cells (a subset T cell population associated with tolerance; Tregs) post-operatively. Studies have been conducted applying MSCs to normothermically perfused kidneys which have shown engraftment of the cells in the organ, though they failed to demonstrate a positive benefit.[79,80]

Successful engraftment of MAPCs during NMP of discarded human livers has similarly been shown, though it is not clear how they would impact the recipient.[81]

Tregs are a particular cellular therapy that may be useful in promoting operational tolerance. This has been demonstrated in a pilot study of 10 patients who received an infusion of autologous Tregs expanded *ex vivo*.[82] Seven patients were able to remain off immunosuppression for nearly two years of follow-up, with only patients suffering auto-immune causes of liver failure unable to tolerate immunosuppression weaning. *Ex vivo*-expanded Tregs apply during perfusion could target the liver specifically and engraft prior to reperfusion.

Liver Regeneration

The liver is well known for its regenerative capacity, which enables it to return to its former size 5-7 days after partial hepatectomy.[83] There is evidence that the liver retains its regenerative capacity through prolonged NMP.[84] However, NMP does not seem to induce hypertrophy on its own. With the advent of devices for long-term liver preservation (7 days and beyond), more intensive regeneration strategies can be pursued.[85] Hepatic regeneration is a complex process, involving dozens of different genes and mitogenic factors that induce hepatocytes to re-enter the growth phase of the cell cycle, enabling them to proliferate and generate liver mass.[86] Achieving this would be beneficial in several clinical scenarios, most notably auto-transplantation in patients with isolated, unresectable hepatic tumour. However, not enough is known presently about the specific factors that would allow this process to occur in the *ex vivo* setting.

Graft Splitting

Even without regeneration, machine perfusion could enable the increased use of liver partitioning. In this scenario, half of a split graft could be preserved via machine perfusion, while the first half is transplanted. Though this would require careful recipient selection to avoid small-for-size syndrome, it would potentially allow one organ to suffice for two recipients.[87] Already, there are several reports of successful perfusion of partitioned livers in the preclinical setting.[88] Additionally, both NMP and HOPE have been used to facilitate liver splitting.[89,90] Lau *et al.* described the partition of 10 discarded livers during NMP, after which each hemi-liver was perfused separately.[91] With the exception of one technical failure, all of the hemi-livers were successfully perfused for 24 hours and demonstrated lactate clearance, bile secretion, and production of factor V, though these were not transplanted. Rossignol *et al.* reported the transplantation of partial grafts following partition during HOPE.[92] They compared 8 livers split into extended right and left lateral grafts during HOPE to 12 split under static conditions and found that the use of HOPE enabled a reduction in cold storage times for both the adult and pediatric grafts. All grafts split during HOPE were successfully transplanted and no graft loss was seen during the follow up period, though clinical outcomes were not significantly different from the control group.

Ischemia-Free Liver Transplantation

Combining techniques of normothermic regional perfusion (NRP), in which the abdominal organs are perfused in the body prior to resection, with NMP, researchers at the Sun Yat-sen University have developed a protocol that completely avoids liver ischemia.[93] Using the Liver Assist device, cannulation is established prior to hepatectomy, perfusing the portal circulation via

an interposition graft and the arterial circulation via the gastroduodenal artery, so that perfusion can continue while anastomoses are completed in the recipient. Venous drainage occurs via the infrahepatic IVC into the organ chamber (the suprahepatic IVC being clamped). Upon completion of hepatectomy, the organ continues to be perfused in the Liver Assist device and, when the recipient hepatectomy is finished, anastomoses are completed with the organ still being perfused.

The initial report using this technique included 38 ischemia-free livers compared to 130 SCS livers from DBD donors transplanted during the same time period.[94] They found significant reduction in EAD (5.3% vs. 50.0%; $p<0.001$). One-month graft survival was higher (97.4% vs. 90.8%), but this did not achieve statistical significance ($p=0.302$). Their subsequent RCT included 32 patients in the ischemia-free group and 33 in the control.[95] Cold storage time in the control group (6.9 hours) was similar to perfusion time in the ischemia-free group (7.1 hours). As in the initial study, they found significant reduction in EAD (6% vs. 24%; $p=0.044$), as well as reperfusion syndrome (9% vs. 64%; $p<0.001$). However, there was no difference in graft survival. They did find, additionally, a decrease in the incidence of non-anastomotic biliary strictures observed on magnetic resonance cholangiopancreatography at one year (8% vs. 36%; $p=0.014$). It is likely that the potential benefit does not justify use in every case, but may be suited to DCD donors when perfusion can begin at the donor institution.

Infection Clearance

Infectious contraindications to transplantation can also potentially be addressed with machine perfusion. Organs from hepatitis C virus (HCV) positive donors have traditionally been considered marginal and only suitable for transplantation into HCV positive recipients.[96] Although this is starting to change with the increasing use of direct-acting antivirals, machine perfusion

offers a platform for potential HCV eradication.[97] This has been demonstrated in perfused HCV-infected human lungs using ultraviolet irradiation.[98] Machine perfusion can also be used to prevent re-infection, as demonstrated by Goldaracena *et al.*, who found that treatment of a perfused liver with miravirsen enabled the sequestration of miRNA-122 to prevent HCV replication.[99] Uncontrolled systemic infection in the donor is likewise considered a contraindication to organ transplantation that may be mitigated using machine perfusion. In a model of rat kidney perfusion, Liang *et al*. were able to show that carbapenem-resistant *Klebsiella pneumoniae* could be eradicated during either NMP or HMP to prevent infection in the recipient, which demonstrates how machine perfusion can be used to treat problematic infections.[100]

Pediatric Transplantation

Pediatric transplantation has not seen as much activity in machine perfusion as in adults. This is partially due to the lower numbers of pediatric liver transplants performed, as well as pediatric recipients being more likely to receive a living donor transplant.[101] Now that there is good evidence of safety in adult populations, use in pediatric populations is likely to become more widespread. The first report of pediatric liver transplantation following machine perfusion was published by Werner *et al*. in 2018.[102] They described the use of HOPE to perfuse a DCD liver from a 13-year-old donor that was successfully transplanted into a 16-year-old recipient. So far, no comparative studies or case series have been reported in the literature. Adapting machine perfusion to pediatric liver transplantation would require modification of the current regulatory approval. There are some technical challenges to overcome for the successful perfusion of smaller grafts, such as optimizing flow rates, adjusting the infusion of medications, and ensuring

appropriate sized cannulas are available, though these have been somewhat addressed through the research on hemi-liver perfusion.

Normothermic Regional Perfusion

Adjacent to the field of machine perfusion, there has also been a recent surge of interest in normothermic regional perfusion (NRP) for DCD organ procurement. This approach involves perfusion of the abdominal organs *in situ* before retrieval and can be done in combination with or separate from the intra-thoracic organs (i.e., heart and lungs).[103] It is currently standard practice for some European countries; however, uptake in North America has been slow and there are concerns that it may conflict with standards for determining death, which can vary between states and provinces, as well as the general concept of the and the 'dead-donor rule', which specifies cessation of blood flow.[104] NRP has the potential to replace *ex vivo* machine perfusion in certain settings, particularly when machine perfusion is not intended for transport, and may be technically simpler. In a study comparing NRP with NMP and SCS, Gaurav *et al.* found that NRP showed improved 6-month graft survival compared to the SCS group (90% vs. 76%; p=0.006) whereas NMP did not.[105] Also, none of the NRP livers developed non-anastomotic strictures compared to 11% of the NMP and 14% of the SCS livers. Still, a better role for NRP may be to augment rather than supplant machine perfusion. Patrono *et al.* were able to achieve outcomes for DCD livers subject to NRP followed by HOPE comparable to donation after brain death donors (DBD) with 1-year graft survival of 90% versus 95% in the DBD cohort (p=0.82).[106] Of course, NRP has been used in conjunction with NMP to achieve ischemia-free liver procurement, as described previously.[93] A greater role for NRP, with or without *ex vivo* perfusion, will require expanded

regulatory approval (particularly in North America), which is more challenging than for machine perfusion as the technique is applied to the whole donor, rather than a single organ.

Other Considerations

There are several potential applications that are slightly farther away from clinical implementation, but still warrant thought as they may offer solutions to unique clinical problems. With sophisticated NMP systems in already in clinical use, it is worth considering whether this platform could be used to provide extracorporeal liver support. Currently, there are few targeted interventions to support individuals presenting with acute or acute-on-chronic liver failure. The best-known acellular liver support device is the molecular absorbent circulating system (MARS) and, while there is some evidence that it can improve 21-day transplant-free survival in acute liver failure, it has not gained widespread use owing to the relative rarity of the condition, as well as the absence of durable survival benefits compared to liver transplantation.[107] Bio-artificial livers (BALs), which employ some live component (typically individual hepatocytes), have similarly failed to gain widespread adaptation, despite increasingly innovative designs.[108] With the advent of machine perfusion technology capable of maintaining livers for days, there is the possibility to harness the whole organ to support a patient in liver failure through cross-circulation, potentially avoiding the need for a transplant. If done with animal livers, this would provide a potentially limitless supply of organs, though extra safeguards would be required to prevent the transfer of antigens or zoonoses to the patient.[109] Discarded human livers could potentially be used, provided they had retained enough functional capacity. Even if one transplantable liver could be used to avoid transplantation in one patient by this means, it would be advantageous to enable the individual to keep their native liver and avoid a lifetime of immunosuppression.

Machine perfusion could also be used to facilitate super-cooling. This has already been demonstrated in discarded human livers, with this technique enabling 24-hour storage, using glycerol and trehalose as additives.[110] These livers remained viable for three times as long as traditional SCS livers using this technique. However, despite the impressive technical innovation, it has not yet found a clear clinical indication. Already machine perfusion (particularly NMP), by itself, has been used to extend preservation time to 24 hours and beyond. It has yet to be established whether super-cooling provides superior preservation within this timeframe. If super-cooling can be shown to safely extend preservation time beyond what is possible with machine perfusion, to weeks or even months, there are several potential applications, such facilitating long distance transport or enabling long-term storage of regenerated livers, such as those intended for auto-transplantation.

There has been growing interest in reseeding decellularized organs for transplantation, particularly with recent innovations in 3D bioprinting.[111] Machine perfusion has been proposed as a platform for reseeding organ scaffolds and could also function as a means to assess recellularized organs.[112] The great benefit is if this can be done with patient derived cells, as this would obviate the necessity for immunosuppression. However, in addition to overcoming the technical challenges, cost-effectiveness is a major barrier. It is also unknown how residual immunogenicity of decellularized extracellular matrix would affect graft tolerance, if xenogenic sources were to be used.[113]

5.1.5 – Future Research Directions in Liver Machine Perfusion

Keeping in mind, the current and potential uses of liver machine perfusion, several immediate directions for future research make themselves clear. One key issue is the development

of standardized, objective methods to assess liver viability in the *ex vivo* environment. Current assessments are often based on parameters familiar to the clinician (e.g., perfusate biochemistry, blood gases, lactate clearance, bile production and general appearance). However, it is not clear that these are the most sensitive metrics. A viable liver is one that can meet the metabolic demands of the recipient, so the ideal marker should reflect the organs synthetic capacity. Traditional biomarkers, such as ALT and AST, that correspond more to the degree of injury are not necessarily indicative of whether the liver can support the recipient metabolically. Another consideration is whether a specific marker of cholangiocyte health is needed, as these cells are more sensitive to ischemia and bile duct injury can lead to significant complications, including graft loss.

Not only will these biomarkers be useful in assessing marginal grafts, but, as researchers and clinicians continue to push the boundaries of organ modification, they will be essential in determining whether recovered or regenerated organs have become suitable for transplant. De-steatification is the best initial candidate for graft modification, given that graft steatosis is the most common reason livers are declined.[114] Several de-steatification agents have been used alone or in combination, but protocols still need to be optimized for human livers.[115] Further interventions to protect DCD livers from reperfusion injury represents another target that could significantly expand the donor pool. Many agents have been tested in the experimental setting, but it is unknown which or in which combination they will prove most effective.

Another important question to address is the relative merits of different modalities of liver machine perfusion (e.g., HMP, HOPE, SNMP, NMP). Considering the typical duration of organ perfusion, which is superior in preserving the liver? Currently, there are no clinical studies comparing different modalities head-to-head. The case can be made that certain modalities are more suitable depending on the indication (such as HMP for transport or NMP for assessment). It

may be that the optimal solution is to integrate temperature and perfusate varying capabilities into the same device to enable flexibility in perfusion strategy and allow for the possibility of controlled rewarming, as is possible with the Liver Assist device.

Commencing perfusion as soon as possible is key to maximizing the potential benefit of machine perfusion. Improving portability of devices to be more easily transported to the procurement centre will help to facilitate this. No doubt, device manufacturers are addressing technical concerns with their engineering teams. However, further research could be done with existing devices (particularly NMP) to determine whether designs could be simplified (e.g., eliminating infusions, optimizing single pump perfusion, integrating the reservoir with the organ chamber) or perfusate composition could be adjusted (e.g., using alternative oxygen carriers) without compromising the quality of preservation.

As normothermic regional perfusion (NRP) becomes increasingly used for DCD donors, strategies should be developed to integrate it with machine perfusion (i.e., when one or the other or both should be used). NRP and NMP have been shown to have similar outcomes in DCD donors, both improved compared to SCS.[105,116] Additionally, NRP used in conjunction with HOPE for DCDs has been shown to have similar outcomes to DBD livers.[106] When the donor is located in the transplant centre NRP could be used as an alternative to machine perfusion or be integrated with machine perfusion to achieve ischemia-free liver transplantations. NRP has additional ethical considerations, particularly ensuring permanent cessation of brain flow is maintained, and is not legal in all jurisdictions.[103]

Machine perfusion is a valuable technology for modern liver transplant programs. It has demonstrated its superiority over SCS in many settings. However, how to best use machine

perfusion and its different modalities has yet to be fully elucidated. No doubt, indications for machine perfusion will expand as new therapies for graft regeneration and modification develop.

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APPENDIX A – OTHER PUBLISHED WORKS

1. Bral M, Pawlick R, Marfil-Garza B, Dadheech N, **Hefler J**, Thiesen A, et al. Pan-caspase inhibitor F573 mitigates liver ischemia reperfusion injury in a murine model. ***PLoS One***. 2019;14(11):e0224567.
2. Marfil-Garza BA, **Hefler J**, Bermudez De Leon M, Pawlick R, Dadheech N, Shapiro AMJ. Progress in Translational Regulatory T Cell Therapies for Type 1 Diabetes and Islet Transplantation. ***Endocr Rev***. 2021 Mar 15;42(2):198-218.
3. Marfil-Garza BA, **Hefler J**, Dajani K, Kin T, Shapiro AMJ. Total pancreatectomy with islet cell autotransplantation in a 2-year-old child with hereditary pancreatitis due to a PRSS1 mutation. ***Am J Transplant***. 2021 Nov;21(11):3790-3793.
4. Marfil-Garza BA, Pawlick RL, Szeto J, Kroger C, Tahiliani V, **Hefler J**, et al. Tumor necrosis factor receptor superfamily member 25 (TNFRSF25) agonists in islet transplantation: Endogenous in vivo regulatory T cell expansion promotes prolonged allograft survival. ***Am J Transplant***. 2022 Apr;22(4):1101-1114.
5. Hatami S, **Hefler J**, Freed DH. Inflammation & oxidative stress in the context of extracorporeal cardiac & pulmonary support. ***Front Immunol***. 2022 Mar 4;831930.
6. Marfil-Garza BA, Imes S, Verhoeff K, **Hefler J**, Lam A, Dajani K, et al. Pancreatic islet transplantation in type 1 diabetes: 20-year experience from a single-centre cohort in Canada. ***Lancet Diabetes Endocrinol***. 2022 Jul;10(7):519-532.
7. Marfil-Garza BA, **Hefler J**, Verhoeff K, Lam A, Dajani K, Anderson B, et al. Pancreas and islet transplantation: comparative outcome analysis of a single-centre cohort over 20-years. ***Ann Surg***. 2023 Apr 1;277(4):672-680.