Improving Clinical and Experimental Normothermic *Ex situ* Liver Perfusion For Future Application

by

Mariusz Bral

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

of

Doctor of Philosophy

in

Experimental Surgery

Department of Surgery

University of Alberta

Abstract

Contributions of this thesis to the field of normothermic machine perfusion

The past decade seen an increasing interest in *ex situ* normothermic liver machine perfusion as a promising alternative preservation strategy for potential expansion of the donor pool. Herein, my intent is to place my own contributions to this field within the greater context of *ex situ* liver perfusion, and trust that the reader may appreciate how these may further potentially advance our appreciation of how NMP technology may be more optimally applied to graft preservation and assessment. The two experimental *ex situ* NMP studies that we published previously addressed basic but important questions concerning NMP circuit priming, and the validity of 'on circuit' transaminase measurement for viability assessment. Before these studies, the kinetics of transaminase production, degradation and clearance within a closed *ex situ* circuit were not known. Our first published clinical NMP series was the first time that this technology was applied in North America. That study demonstrated that NMP technology was safe and feasible in a Canadian setting, and addressed the unique potential of *ex situ* perfusion to disrupt established transplant practice within the context of a geographically vast land. The second published clinical series (Bral shared first authorship), is the first published study to demonstrate both ongoing utility and increased practicality with a 'back-to-base' implementation strategy that avoids a need to transport heavy and complex machinery between the donor and recipient centres. Looking towards the next iterative steps in application of NMP technologies, we applied an *in vivo* murine ischemia reperfusion model as a screening tool to assess the efficacy of selected therapeutics, with the intention of prescreening these compounds for future translational application into an experimental *ex situ*

liver circuit. Most of these publications have been cited in the discourse surrounding NMP, and we trust have formed part of the foundation on which the technology will move into the future.

Preface

Dear Reader,

This thesis, entitled 'Improving Clinical and Experimental Normothermic *Ex situ* Perfusion for Future Applications' is submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Experimental Surgery at the University of Alberta, Department of Surgery. The document compiles a number of experimental and clinical studies that were performed to advance both the basic understanding, and clinical translation of *ex situ* normothermic machine perfusion (NMP). At the time of publication, NMP technology is currently transitioning from a purely experimental, prospective liver preservation platform to an international, clinically applied organ preservation strategy promising future potential for graft repair and modification.

Chapter 1 provides a general introduction to normothermic *ex situ* liver preservation, outlines the history of the field and describes different temperature modalities and preservation strategies. This chapter has completed peer review and has been published in original form in the journal ***Transplantation Reviews*** (Bral M, Gala-Lopez B, Bigam DL, Freed DH, Shapiro AMJ. *Ex situ* liver perfusion: Organ preservation into the future. Transplant Rev (Orlando). 2018;32(3):132-41.), and is reproduced here with permission from the publishers. The manuscript was 90% designed and written by me, with the remaining written by BGL. AMJS performed final edits as senior author. This section also describes the future potential of NMP technology, much of which has yet to reach application.

Chapter 2 aimed to answer a very fundamental question concerning NMP technology. At the time of publication, machine perfusion is still largely experimental, with many unanswered questions concerning varying aspects of the process and circuits. NMP was just entering the clinical realm, and all clinical perfusions since that time have been performed using packed red blood cells with colloid solution to prime the circuit. We sought to answer the question of how low an 'on circuit' hemoglobin level could be tolerated by a liver graft, in order to safely preserve an *ex situ* liver for transplantation using this technology. This manuscript underwent peer review and is published in the journal ***Transplantation*** in its original form (Bral M, Gala-Lopez B, Thiesen A, Hatami S, Bigam DL, Freed DM, et al. Determination of Minimal Hemoglobin Level Necessary for Normothermic Porcine Ex Situ Liver Perfusion. Transplantation. 2018;102(8):1284-92.), and is reproduced here with permission from the journal. The research project was 90% performed by me, including all graft recovery, organ perfusion, sample processing, data acquisition and analysis, and writing the manuscript. SH assisted with the required surgery and perfusion monitoring. DB and DF supervised the study and assisted in revising the manuscript. AMJS designed and led the study, and performed the final edits on the manuscript.

Chapter 3 further contributes to the fundamental understanding of how *ex situ* technology can ultimately be applied. From its inception, one of the most promising applications of NMP was the possibility of dynamic, real-time 'on circuit' graft assessment. In an era where there are increasing pressures to use more marginal grafts to offset deaths on the transplant waitlist, such 'viability assessment' was perceived to be of vital importance, with the overarching premise that this would facilitate lowering of liver graft discard rates without increasing the risk of primary non-function, thereby increasing the number of lives saved. To this end, many surrogate viability and functional markers were applied, including 'on circuit'

transaminases, bile output, bile quality, perfusate pH, and vascular stability. Despite the frequency with which 'on circuit' transaminases were reported in the literature, no group attempted to show how a healthy liver on an *ex situ* circuit processes a high transaminase burden. This study underwent peer review and is now published in the journal ***PLOS One*** (Clearance of transaminases during normothermic ex situ liver perfusion. PLoS One. 2019;14(4):e0215619. Epub 2019/04/25. doi: 10.1371/journal.pone.0215619.), and is reproduced here with permission from the journal. This study adds to the body of 'viability assessment' publications, and further clarified the utility of 'on circuit' transaminases as a marker of graft health. This study also maintained livers viable on NMP continuously for a duration of 48 hours, a unique accomplishment which further demonstrated the potential of NMP for prolonged preservation period. This study was 85% performed by me, including all graft recovery, organ perfusion, sample processing, data acquisition and analysis, and writing the manuscript. NA and SH assisted with surgery and perfusion monitoring. DB and DF supervised the study and manuscript revisions. AMJS designed and led the study, and performed the final edits on the manuscript.

Chapter 4. Shifting into the clinical realm, we published our first series of NMP preserved and transplanted livers grafts after peer review in the ***American Journal of Transplantation*** (Bral M, Gala-Lopez B, Bigam D, Kneteman N, Malcolm A, Livingstone S, et al. Preliminary Single-Center Canadian Experience of Human Normothermic Ex Vivo Liver Perfusion: Results of a Clinical Trial. Am J Transplant. 2017;17(4):1071-80). This paper is reproduced herein with permission from the publishers. This series was unique, in that it was only the third publication in the world at the time describing the clinical translation of this technology, and was the first time that NMP was used in any clinical study in North America. Our series provided valuable insights into the adaptation of NMP to the real-world hospital setting, and contributed to the

knowledge concerning pre-transplant graft assessment. We were also fully transparent about one instance of graft loss, which heightened the awareness of the steep learning curve in early adoption of such a novel technology. We were also the first group to point out the utility of NMP to change the logistics of the transplant process, allowing for more rested teams to perform transplants during daytime hours. This study was 75% performed by me, including most organ perfusion monitoring, sample processing, data acquisition and analysis, and writing the manuscript. BGL also monitored the perfusions, participated in data acquisition and analysis. All co-authors contributed to manuscript revision. AMJS designed and led the trial, participated in creating the manuscript and performed the final edits.

Chapter 5. As our experience and confidence grew, we continued to embrace NMP in the clinical setting, and began to explore the boundaries of how this technology could positively impact our routine practice of liver transplant surgery. Although the Organox *metra* device was designed for portability, we rapidly discovered in practice in our North American centre where grafts are typically flown by plane to Edmonton from distant donor sites, that there would be huge challenges in moving this 'portable' machine by plane or even between hospitals by ambulance within our city. We therefore began to explore the possibility of a 'back-to-base' approach where a liver could be reperfused *ex situ* before transplantation back in Edmonton. One important concern that surfaced relatively early in our experience was the uncertainty of whether liver grafts that had accrued cold preservation time in static cold storage (SCS) could be perfused or would even benefit from NMP. A single previously published experimental porcine study had demonstrated that any amount of cold ischemia was detrimental prior to NMP, which effectively provided the rational for designing portable NMP technology. Such an approach generated considerable additional cost and logistical problems, particularly in a large geographic catchment area such as Western Canada. As an alternative strategy, we

transported clinical liver grafts in SCS to our recipient center, where NMP was initiated. The paper resulting from our series, which was part of a non-randomized trial, underwent successful peer review and has been published in ***Liver Transplantation*** (Bral M, Dajani K, Leon Izquierdo D, Bigam D, Kneteman N, Ceresa CDL, et al. A 'Back-to-base' Experience of Human Normothermic Ex Situ Liver Perfusion: Does the chill kill? Liver Transpl. 2019). This clinical study is the first to demonstrate that a modest accrual of cold ischemic time (median 3 hours) did not compromise graft or patient outcomes, led to improved graft function, and facilitated transition of liver transplantation surgery into more palatable daylight hours. This study was 60% completed by me, including data accrual and analysis, and writing the majority of the manuscript. KD also participated with data collection, manuscript writing and revision, and we share first authorship of the final publication. All co-authors participated in manuscript revision. AMJS designed and supervised the trial and performed final edits on the manuscript.

Chapter 6 provides a general overarching discussion of topics addressed throughout, with particular emphasis on the future potential applications NMP technology. The most recent developments concerning prospective graft modulation are presented, including minimization of ischemia reperfusion injury, de-fatting of steatotic grafts, altering the immunogenic potential of a graft, as well as application of mesenchymal stem cells and nanoparticles to *ex situ* perfusate. A closing section provides general conclusions, and highlights the future potential of *ex situ* perfusion as a platform to engender highly personalized transplant medicine and advanced graft repair.

The manuscript included in the appendix was recognized as being relevant to the thesis work as a whole, but not necessarily linked to the main chapters. It connects the current status of normothermic perfusion research with future potential translational protectant strategies.

Appendix A- Based on our experimental and clinical *ex situ* perfusion experience, it became clear to us that one of the most prescient and promising advantages of NMP technology was the future implementation of 'on circuit' graft modulating or modifying strategies. Such interventions hold the most potential of truly altering the transplant landscape, possibly significantly expanding the donor pool, or personalizing the matching of organs to recipients. With a view towards the future, this work describes our investigation of a rational selection of three protective additives in a murine ischemia reperfusion injury (IRI) model. All selected compounds had previously demonstrated efficacy in improving experimental and/or clinical islet engraftment and function. The positive findings demonstrated that F573 mitigates IRI in a murine model, based on improved markers of cellular injury, decreased evidence of apoptosis, as well as an improved tissue cytokine profile. Moving forward, this study serves as the basis for adding F573 to NMP perfusate, with the hope of mitigating or even repairing IRI on-circuit. This study was 75% executed by me, including almost all surgeries, data collection and analysis, and writing the manuscript. RP assisted in surgery, performed drug administration, assisted in sample processing, and data collection. ND and BM assisted in sample processing and some data collection. AMJS designed and supervised the study, and provided final manuscript revisions.

Mariusz Bral

Dedication

This thesis is dedicated to my wife Lainna and my newborn son, Linus, who is just starting to look around. You are both my Heart and my World.

Acknowledgements

It is with much gratitude that I would like to acknowledge the following individuals for their immeasurable contribution to my training and education: Dr. Darren Freed, Dr. David Bigam, Dr. Norman Kneteman, Dr. Khaled Dajani.

It is with profound indebted gratitude that I would like to thank Dr. James Shapiro, my thesis supervisor, without whom none of this would be possible. Your tireless approach in always pushing the very edge of what can be done in research and in the clinic is a wonder and inspiration to all of us! A very warm 'thank you!' for all of your thoughtful, constructive guidance over the years, and all of the times that you made yourself available, any time of day. Many thanks for your levity and humour, often in the middle of a transplant, at 2 am: 'Please remember! No PDS!!'. Thank you for all of your kindness and generosity!

It is with much gratitude that I would like to thank the following individuals for their kind, thoughtful support and help over the past years. I have learned so much from you: Rena Pawlick, Dr. Boris Gala Lopez, Dr. Andrew Pepper, Dr. Antonio Bruni, Dr. Nidheesh Dadheech, Dr. Braulio Marfil. Ineffable thanks to my dear friend Scott Arnold, whose attention to detail and excellence is unsurpassed, and who is inseparable from my journey as a surgeon.

I extend my sincere thanks to the staff at SMRI, for all of your support and diligence over the past years.

Thank you, very much.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AKI, acute kidney injury

ALP, alkaline phosphatase

ALT, alanine aminotransferase

AST, aspartate transaminase

ATP, adenosine triphosphate

BE, base excess

CI, confidence interval

CIT, cold ischemia time

COR, controlled oxygenated rewarming

DCD, donation after circulatory death

DRI, donor risk index

EAD, early allograft disfunction

ECD, extended criteria donor

ECMO, extra-corporeal membrane oxygenation

GDA, gastroduodenal artery

HA, hepatic artery

HAR, hepatic artery resistance

HBV, hepatitis B virus

HCC, hepatocellular carcinoma

Hct, hematocrit

HMP, hypothermic machine perfusion

HOPE, hypothermic oxygenated perfusion

HTK, histidine-tryptophan-ketoglutarate

IC, ischemic cholangiopathy

ICU, intensive care unit

I-FABP, L-type fatty acid binding protein

IL-, interleukin

INR, international normalized ratio

IRI, ischemia reperfusion injury

IVC, inferior vena cava

Lac, lactate

LDH, lactate dehydrogenase

MELD, Model for End-stage Liver Disease

MELD-NA, Model for End-stage Liver Disease- sodium

MP, machine perfusion

MRCP, magnetic resonance cholangiopancreatography

MSC, mesenchymal stem cell

NASH, non-alcoholic steato-hepatitis

NMP, normothermic machine perfusion

NRP, normothermic regional perfusion

OLTx, orthotopic liver transplantation

PNF, primary non-function

POD, postoperative day

PRBC, packed red blood cells

PVR, portal vein resistance

RNP, regional normothermic perfusion

SCS, static cold storage

SD, standard deviation

SEM, standard error of the mean

SMA, superior mesenteric artery

SMP, subnormothermic machine perfusion

T-Bili, total bilirubin

TNF-α, Tumor Necrosis Factor-alpha

UNOS, United Network for Organ SHaring

UW, University of Wisconsin

WIT, warm ischemia time

1 Chapter 1.

Introduction

1.1 General Introduction

Since inception and design of the first device, *ex situ* liver normothermic machine perfusion (NMP) has progressed considerably from the first studies performed by Alexis Carrel and Charles Lindberg.

Carrel, born in 1873, was a prolific scientist, philosopher and inventor, who made singularly unique and profound contributions to all disciplines that he chose to engage. One aspect of Carrel's genius was publicly rewarded in 1912, when, at the age of 39, he was the youngest scientist to receive the Nobel Prize in Medicine for his invention of the 'triangulation technique' for suturing of blood vessels with endothelial eversion, a concept which had previously not been recognized as possible. This innovation opened up the future possibility of vascular surgery, and was the key to facilitating the beginnings of kidney transplantation. On other fronts, Carrel actively investigated tissue and cell culture, and his work in trying to keep tissues and organs alive outside of the body predated the current resurgent interest in *ex situ* perfusion by 60 years. Carrel was a controversial figure due to his links with the eugenics movement, however his visionary dream to replace diseased organs with healthy ones did set the stage for the current clinical practice of solid organ transplantation **(Figure 1-1)** [1].

Although prolific in many areas, Carrel seemed to have an overarching pre-occupation unifying many of his endeavours, which was a dream to overcome the process of dying. On a more philosophical and ideological level, Carrel delineated some of these ideas in his 1935 publication '*L'homme, cet inconnu'*, which described a eugenic theory within

which scientists sorted individuals according to their attributes, in the process creating ‘universal efficiency’. This publication was translated into 20 languages and became an international bestseller, despite the intense controversy and at times hostility that it invoked. These ideas unfortunately clouded some of Carrel’s other achievements, and ultimately detracted from the cultural memory of his overall accomplishments.

Carrel’s auspicious meeting with Charles Lindbergh, the famous trans- Atlantic aviator and engineer, created a fruitful union of two exceptional minds. Together, they spent years designing a pump oxygenator for the purpose of organ perfusion. The original perfusion device, which consisted of an elaborate sterile glass circulation flask housed in an incubator, allowed *ex situ* perfusion of organs for the first time in 1935. Perfusate consisted of blood serum, cysteine, insulin, thyroxins, ascorbic acid, phenol red and glutathione and circulation of the fluid was achieved by gas insufflation [1].

In the late 1930’s, around 900 perfusion experiments were performed in Carrel’s laboratory, with notable achievements including the preservation of cat thyroids under normothermic conditions, for up to 30 days. These prescient ideas created the foundation for the ‘rediscovery’ of machine preservation in the 1960’s, and indeed modern NMP devices resemble Carrel and Lindberg’s in many ways.

Figure 1-1 Cover of Time Magazine June 13, 1938 (Image obtained from TIME The Weekly Magazine, Volume XXXI, Number 24, June 1938

url: content.time.com/time/covers/0,16641,19380613,00.html)

In 1956, with the success of the of the world's first kidney transplants between twin recipients, the possibility of organ transplantation was suddenly hailed as the new future. These procedures predated the invention of organ preservation solutions, and indeed any formal definition of brain death, and as a result all initial transplant donor procedures occurred under what is currently known as uncontrolled 'donation after circulatory death' (DCD) conditions, with the implant procedures fraught with massive blood loss and frequent cases of primary non-function (PNF) in most of the early attempts at liver transplantation. Surgical techniques for managing patients with advanced cirrhosis had yet to be developed, and anesthesia as a discipline did not yet have approaches for mitigating coagulopathy. There were no cold preservation solutions and livers were typically flushed with saline.

The first series of clinical liver transplants performed in Colorado between 1963 and 1968 by Thomas Starzl were severely challenged by the circumstances within which they occurred, and there were no survivors (**Figure 1-2)**. Brain death had not been defined as an accepted entity, there were no effective means to preserve organs between donor and recipient surgeries, and the concepts of control of coagulopathy, massive transfusion protocols and other critical anesthetic management techniques were not yet born. The surgical steps in liver transplant surgery were rudimentary and heroic, makings these early attempts almost futile. Both Thomas Starzl and Professor Sir Roy Calne struggled with the surgical challenges of portal hypertension, and as a result, they began to select patients with larger colorectal and hepatocellular metastases as transplant recipients, patients that today would be declined, to avoid portal hypertension, coagulopathy and major blood loss. In 1968, Starzl performed and published the first successful clinical liver transplant, which included donor graft

preservation using machine perfusion [2]. Liver grafts were perfused with low flow, hypothermic diluted homologous blood, insufflated with hyperbaric oxygen at 4 atmospheres, based on previous successful efforts in canine experiments **(Figure 1-3)** [3].

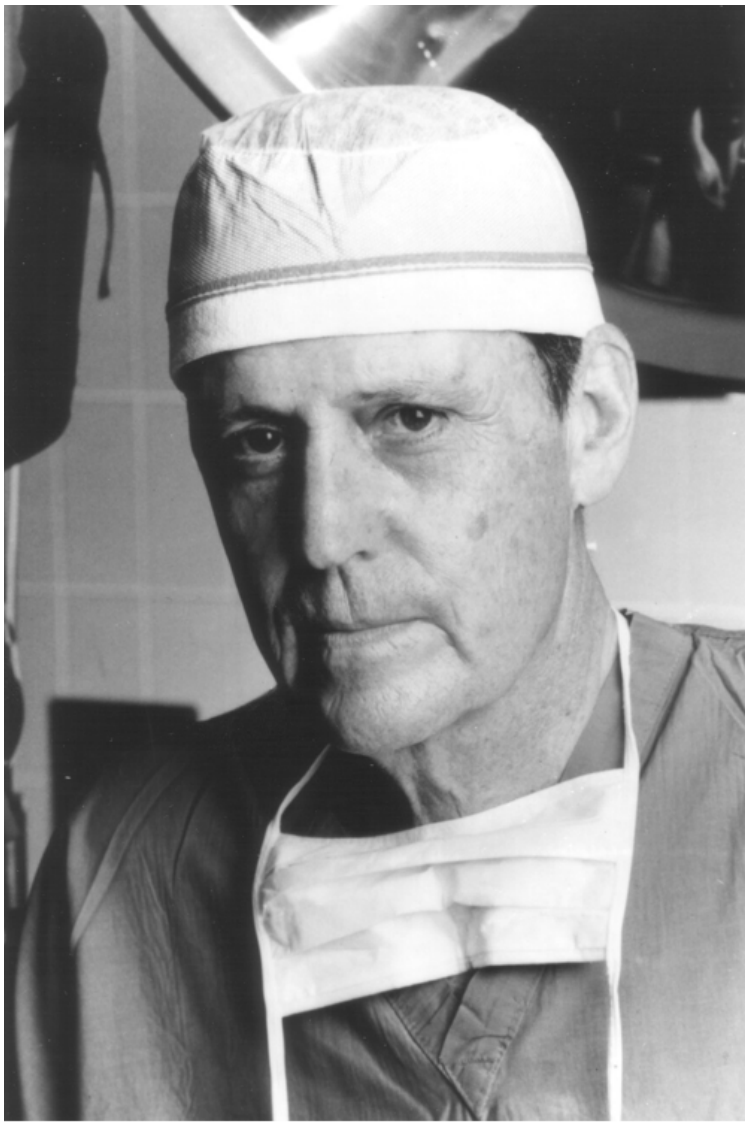


Figure 1-2 Professor Thomas Starzl (Mar 11,1926- Mar 4, 2017). (Photo courtesy of University of Pittsburg)

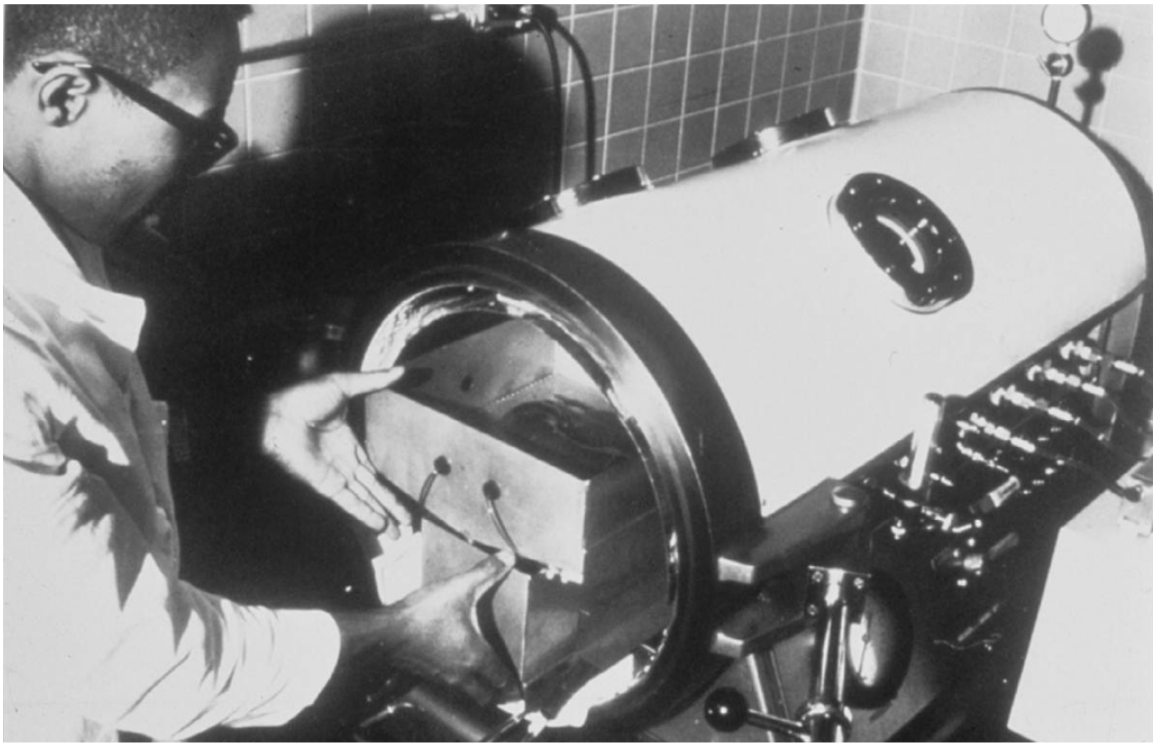


Figure 1-3 Hyperbaric oxygen chamber in which continiously infused dog (or human) livers could be preserved for up to two days. (Image courtesy of 'The official Dr. Thomas E. Starzl website. url: startzl.pitt.edu/transplantation/organs/liver.html.)

From these auspicious beginnings, building on iterative robust experimental evidence, *ex situ* organ machine perfusion has transitioned from a highly experimental treatment, to a therapy that is now being established in the clinical forum as both feasible and safe, laden with potential for future applications. The publication of the large randomized liver normothermic machine perfusion (NMP) UK trial, together with two smaller Canadian series have definitely moved the liver NMP field forward, however the results of a large randomized US trial are still pending and eagerly awaited. The US trial results will require careful evaluation by the Food and Drug Administration (FDA), and a favourable outcome will be essential for NMP technology to gain greater acceptance

and widespread use in the US.

After the initial interest and attempts in the 1960's, efforts in advancing machine perfusion were suspended due to the advent and eventual widespread use of preservation solutions, which offered a convenient and inexpensive option for organ preservation. In counterpoint to the initial organ perfusion machines, which were limited by the technology of the time and were the size of small rooms, preservation solutions appeared as an extraordinary solution to the complexity of machine preservation **(Figure 1-4 A, B)**.

Among the preservation solutions, Collins solution was the first to be initially described in 1969 and then modified in 1976 as the Euro-Collins (EC) solution. These two preservation solutions were formulated to chemically resemble the intracellular environment, and EC contained glucose as an osmotic buffer. They worked well for kidney preservation, but not, unfortunately, for other organs. The introduction of University of Winsconsin (UW) solution, formulated by Folkert Belzer and Hans Sollinger, initially designed to optimize pancreas preservation, ushered in a new era of more widespread success in both liver and pancreas transplantation in the 1980's. Introduced for clinical use in 1987, UW allowed for safe extension of liver graft preservation for up to 15 hours over EC. The simplicity of static cold storage (SCS), combined with the low cost, ease of application and transport, as well as good short and long term outcomes in liver transplantation initially mitigated the need for the much more complex preservation machines, and accounted for the establishment of UW as the clinical standard for organ preservation, as it remains today.

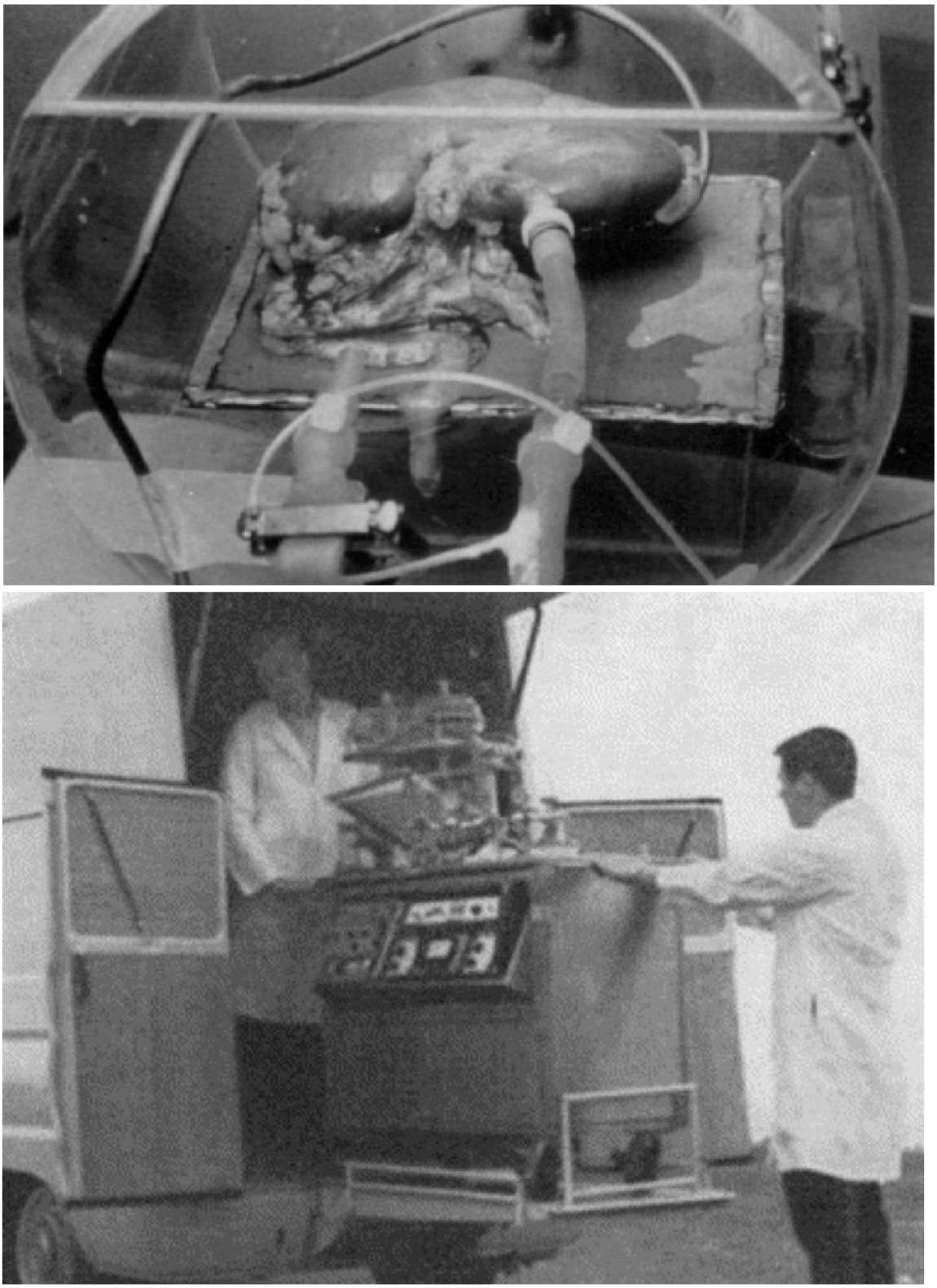


Figure 1-4 A) Early hypothermic kidney perfusion (Permission obtained from Annals of Surgery) **(B) Hypothermic kidney perfusion machine, 1966.** (Photo courtesy of the University of Stanford url: web.stanford.edu/dept/HPST/transplant/html/belzer.html)

As transplantation became firmly entrenched as the only life-saving treatment for end-stage liver diseases, the procedure became a victim of its own success. According to the OPTN/SRTR 2017 Annual Data Report, 13,239 patients were on the waiting list for a liver transplant procedure that year, with 8,082 liver transplants performed. Of those waiting for transplant, 20.3 percent were removed from the list due to death or progression of disease [4]. In the most recent Canadian Organ Replacement Register (2019), in 2017 there were 321 individuals waiting for a liver transplant that year, with 64 deaths on the waitlist (19.9%).

The immense disparity between organ supply and demand, combined with the high waiting list attrition rate drove the transplant surgical community to explore alternative more risky ways to expand the donor pool with introduction of living liver donation, and further through use of marginal, and what were termed 'extended criteria donor' (ECD) livers to overcome this deficiency. Although no standardized definition of ECD exists, commonly accepted factors include: 1) advanced donor age 2) donor BMI > 35kg/m^2, 3) DCD with warm ischemia time more than 30 minutes, 4) donor liver macrosteatosis >30% [5]. Such organs are known to tolerate SCS poorly, with potentially both immediate and long-term inferior outcomes [6].

1.2 Donation after circulatory death liver grafts

During DCD liver procurement, an obligate period of warm ischemia triggers a cascade of deleterious injury pathways, directly proportional to duration of warm-ischemic time (WIT), including danger-associated molecular patterns (DAMPs), accumulation of free radicals, caspase activation leading to graft dysfunction or non-function. These

processes are the rationale for the often reported 30-minute threshold for liver acceptance.

Initial publications reporting the use of DCD liver grafts compared to donation after brain death (DBD) donors described inferior outcomes, attributed to higher rates of hepatic artery thrombosis, higher rates of PNF, and long-term failure due to ischemic cholangiopathy (IC), potentially requiring re-transplantation [7]. Variables including advancing donor age, prolonged cold ischemia time, and donor warm ischemia time have been identified as the important risk factors associated with worse outcomes, particularly IC [7]. Of these, the strongest variable associated with inferior outcomes following DCD graft transplantation was advanced donor age, with studies reviewing UNOS data reporting increased risk with donors older than 55 years [8].

This resulted in a hesitancy by many transplant programs to accept donors 45- 50 years or older [7]. A clear trend of transplanting DCD livers from younger donors was seen nationally in the United States, with only 13% of DCD liver transplants from donors more than 50 years old between 2005 and 2015 [4]. Recently however, a number of groups have shown good outcomes of DCD liver transplantation, including from older donors, with improved outcomes attributed to critical donor selection, minimization of CIT, and the use of tissue plasminogen activator (tPA) thrombolytic delivered via the hepatic artery after venous reperfusion [9]. A recently published study from the UK compared the outcomes of DCD transplants from donors less than, as well as older that 60 years of age and described no difference in graft or patient survival between groups [10]. Studies have shown that the use of these grafts is most optimal for patients who are disadvantaged on the waitlist, with low 'Model for End-stage Liver

Disease' (MELD) scores but significant complications from liver disease [8]. Analysis demonstrated inferior graft survival when advanced age DCD grafts were used in recipients with a MELD greater or equal to 30, on mechanical ventilation, or in the intensive care unit, as well as with incrementally higher donor age, and older doors with diabetes mellitus [8]. Overall cold ischemia time should be minimized as much as possible, and ideally kept below to less than 6 hours [8].

The additional unique problem that affects DCD grafts is the development of ischemic cholangiopathy (IC), which potentially leads to re-transplantation. IC, which is defined by diffuse biliary strictures in the absence of hepatic artery stenosis, occurs at reported rates of 4.5 to 16% [11]. Although the exact cause of cholangiopathy has not been determined, one theory is that microthrombi form in the biliary plexus of ischemic cholangiocytes. In keeping with this theory, a previous report had suggested that the rate of IC could be reduced using tissue plasminogen activator (tPA), describing a much lower rate of diffuse intrahepatic strictures (3.5% vs 21.2%) [9]. This study, however, used a historic cohort, so we cannot fully know if the tPA intervention was in fact responsible for the improved results. Although intra-arterial tPA during liver transplantation theoretically increases the risk of hemorrhage, in retrospective analysis of 100 consecutive DCD liver transplants using a protocol including tPA, there were no systemic tPA related bleeding complications, and no difference in intraoperative transfusion requirements [12]. This was attributed to the relatively small, single tPA dose of 2 mg, administered directly into the hepatic artery after portal vein reperfusion (near normothermic conditions), and adequate concentrations of plasminogen, which is necessary for tPA activity [12]. The application of tPA during e*x situ* NMP of DCD grafts may be an additional strategy to consider, and has not been published yet.

Interestingly, in a recent retrospective analysis by Watson *et al*, demonstrated that the application of normothermic regional perfusion (NRP) donors in a controlled DCD situation eliminated the incidence of IC post-transplant [13]. NRP was performed on 70 DCD donor livers, of which 43 were transplanted. These results contrast with the 11.1% DCD IC rate in livers having undergone NMP in the randomized trial by Nasralla *et al* . This seems to suggest that NRP may be more effective at preventing IC than NMP [14]. Although the reasons for this are not clear, we speculate that, in a NRP situation, the bile duct and attendant arteries are still intact, which may help preserve flow to the biliary tree, thereby protecting biliary integrity. In regard to biliary complications, increased complications were observed in older DCD donors, however they were mostly anastomotic strictures, treated with dilation [13].

1.3 Experimental *ex situ* liver perfusion

In the context of the organ shortage and the issues surrounding SCS preservation, interest in *ex situ* technology was propelled forward by the need to achieve better outcomes with such marginal grafts, as well as to improve graft utilization rates. At the time that I commenced work on this thesis in 2014, there were relatively few publications in the field, and much remained unknown about the optimal parameters, perfusate composition, optimal timing or efficacy of NMP. From the onset, we have sought to create meaningful, relevant, and impactful contributions to the field of liver preservation for transplant, summarized within this doctoral thesis herein.

A limited number of international groups were able to perform large animal *ex situ* experiments to bridge knowledge gaps that existed in the field, effectively building foundations that progressively demonstrated advantages of liver NMP over static cold storage (SCS) [15-19]. Since 2001, when Schön *et al*. demonstrated the superiority of NMP over SCS in preserving DCD porcine liver grafts, NMP has iteratively built on successive studies that have demonstrated efficacy at improving graft quality, 'on-circuit' assessment of grafts prior to transplant, and offering the potential of ultimately increasing the number of grafts successfully transplanted [15,16,20-22].

In an effort to advance the basic understanding of fundamental elements in NMP, we investigated the minimal hemoglobin level necessary for performing NMP, a salient issue, as all clinical normothermic perfusions are performed with colloid solution and diluted packed red blood cells [23]. The positive findings of this study have implications for improved resource utilization, and potential cost savings.

In a second fundamental contribution to basic *ex situ* knowledge, we designed an experiment to demonstrate the 'on circuit' clearance of liver transaminases, enzymes which continue to be widely monitored as surrogate markers of graft injury during NMP. Over perfusions lasting 48 hours, we showed that healthy livers are able to clear transaminases from perfusate, shedding further light on the utility of these enzymes for graft viability assessment [24].

To this date, however, it remains clear that many aspects concerning the optimal application and interpretation of normothermic *ex situ* technology to organ preservation and repair remain unknown, many aspects of which merit further directed study.

1.4 Clinical normothermic *ex situ* perfusion

Building on robust iterative experimental evidence, normothermic *ex situ* machine perfusion transitioned into the clinical forum with the first-in-human NMP series of liver transplants performed successfully in the UK by the Oxford group. This study demonstrated for the first time the safety and feasibility of NMP technology in the hospital setting and showed non-inferiority of NMP to SCS, with NMP-perfused livers demonstrating statistically lower opening aspartate aminotransferase (AST) levels compared to SCS controls [25].

In parallel, we felt that the necessary way to move forward was to validate the feasibility and safety of liver *ex situ* perfusion in the North American setting. After overcoming logistic and bureaucratic challenges, we were the first in North America to transplant an NMP perfused liver on February 25, 2015. Our initial series of NMP livers was published (Bral first author), and at the time was one of only 3 reported clinical experiences worldwide [26].

Publication of the First Randomized Normothermic Machine Perfusion Trial

The landmark publication of the first randomized NMP clinical trial by Nasralla *et al.* further established this technology as a viable alternative to cold storage, with clear superiority over SCS [14]. Randomizing 270 accepted donors to either preservation in SCS or NMP, Nasralla *et al.* demonstrated that there was no difference in post-transplant outcomes such as biliary strictures, hospital days, or graft and patient survival between groups. In grafts subjected to continuous application of NMP during the preservation period, Nasralla *et al*. found significantly decreased liver injury post transplant in the NMP group (evidenced by lower peak AST levels), as well as a 74% lower incidence of early allograft dysfunction compared to SCS controls (**Figure 1-5)**. Early allograft dysfunction (EAD) was defined as the presence of one or more of the following biochemical markers post-transplant: 1) Total bilirubin >170 mol/L on day 7, 2) INR>1.6 on day 7 and 3) Peak AST >2000 IU/L in the first 7 days. The implications of these results are rather impressive, as it has been shown previously that recipients of EAD grafts have a much higher incidence of graft loss (26.1% vs. 3.5%, relative risk= 7.4, $p<0.0001$) and patient mortality (18.8% vs. 1.8%, relative risk= 10.7, $p<0.0001$) [27]. Excellent 1 year graft survival (95%) was observed in the NMP arm, although no different from the control SCS group [14].

Most importantly, the groups demonstrated a 50 percent lower graft discard rate in the NMP arm compared to the SCS control, which equated to a 20% percent higher transplant rate (**Figure 1-5)** [14]**.** This very important outcome demonstrated that NMP may in fact, increase the donor pool and increase the number of transplanted patients, saving lives on the transplant waitlist [14]. In the US, based on the OPTN/SRTR 2017

Annual Report, 8,082 liver transplants were performed that year, which could theoretically have been increased to 9,698 if NMP preservation was used ubiquitously, if the statistical increment derived from the UK-European study can be generalized to the US donation pool. In Canada, based on the Canadian Organ Replacement Register, in 2017, 464 livers were transplanted, and a 20% increase could augment the number of liver transplants to a theoretical 556. Such increased transplant partly offsets, although not completely the waitlist attrition rate.

Table 3 | Trial outcomes

	NMP ($n=121$)[a]	SCS ($n=101$)[a]	Effect (95% CI)[b]	*P* value
Peak AST				
ITT[c]				
Adjusted	488.1 (408.9–582.8)	964.9 (794.5–1,172.0)	0.5 (0.4–0.7)	0.0000
Unadjusted	484.5 (406.4–577.6)	973.7 (795.2–1,192.3)	0.5 (0.4–0.6)	0.0000
Test for interaction by donor type				0.012
Subgroup analysis by donor type				
DBD	526.2 (427.3–647.9)	880.2 (708.5–1,093.5)	40.2% (19.3–55.7%)	0.0009
DCD	389.7 (278.0–546.4)	1,458.1 (944.7–2,250.5)	73.3% (53.7–84.6%)	0.0000
PP analysis	498.6 (414.8–599.4)	982.9 (810.4–1,192.2)	0.5 (0.4–0.7)	0.0000
Secondary outcomes				
Discard rates[d]	16 (11.7%)	32 (24.1%)	−12.4% (−21.4 to −3.3%)	0.008
Primary non-function[e]	1 (0.8%)	0 (0.0%)	NA	NA
Post-reperfusion syndrome	15 (12.4%)	32 (33.0%)	−20.6% (−31.6 to −9.6%)	0.0002
Post-reperfusion lactate[f]	3.6 (2.6–4.2)	4.1 (3.2–5.0)		0.018
Early allograft dysfunction	12 (10.1%)	29 (29.9%)	0.263 (0.126–0.550)	0.0002
Biochemical liver tests[f] (average value over day 1–7)				
Bilirubin (μmol l^{-1})				
Days 1–7	38.5 (21.0–73.2)	49.1 (26.0–85.5)		0.029
30 days	13.0 (8.0–22.1)	13.0 (9.1–21.0)		0.479
6 months	9.1 (6.0–15.1)	9.1 (6.0–13.0)		0.671
AST (IU l^{-1})				
Days 1–7	167.5 (98.0–320.7)	318.5 (152–611.5)		0.0000
30 days	20 (14–35)	22 (15–40)		0.707
6 months	23 (18–33)	23 (18–37)		0.931
γGT (IU l^{-1})				
Days 1–7	268.1 (156.3–408.3)	301 (201.1–443.9)		0.157
30 days	178 (109.5–410.0)	200 (96.0–397.5)		0.949
6 months	47 (28–144)	47 (26–128)		0.452
INR				
Days 1–7	1.2 (1.2–1.4)	1.2 (1.2–1.4)		0.644
30 days	1.1 (1.0–1.2)	1.1 (1.0–1.2)		0.735
6 months	1.1 (1.0–1.2)	1.1 (1.0–1.1)		0.167
Creatinine (μmol l^{-1})				
Days 1–7	92.8 (60.1–121.1)	97.2 (67.2–143.2)		0.139
30 days	82.2 (66.3–104.3)	90.2 (72.5–121.1)		0.019
6 months	99.9 (81.3–117.6)	99.9 (83.1–134.4)		0.265
Lactate (mmol l^{-1})				
Day 1–7	1.3 (1.0–1.7)	1.1 (0.9–1.6)		0.130
Other outcomes				
Need for RRT (number (percentage) of patients)				
Day 1–7 after transplant	26 (21.5%)	19 (18.8%)	2.7% (−7.9 to 13.2%)	0.621
30 days	27 (22.3%)	20 (19.8%)	2.5 (−8.2 to 13.3%)	0.648
6 months	27 (22.3%)	21 (20.8%)	1.5% (−9.3 to 12.4%)	0.784
Duration of RRT day 1–7[f]	4 (2–6)	5 (4–6)		0.346
Length of hospital stay[f]	15 (10–24)	15 (11–24)		0.926
Length of ICU stay[f]	4 (2–7)	4 (3–7)		0.339
Graft survival at 1 year	0.950 (0.893-0.977)	0.960 (0.897–0.985)		0.707
Patient survival at 1 year	0.958 (0.902–0.982)	0.970 (0.909–0.990)		0.671

CI, confidence interval.
[a]Total number of livers transplanted and analysed overall. Primary outcome analysed on $n=220$ due to unavailability of AST values during the first seven days after transplant. Specific outcomes may have different denominators due to some missing data.
[b]Effect reported is: Percentage reduction (from geometric mean ratio) for peak AST; odds ratio for early allograft dysfunction; difference in proportions (%) for discard rates, post reperfusion syndrome and need for renal replacement therapy (RRT); not reported for outcomes for which medians are reported, for survival scores and for tests for interactions of subgroup analysis (only *P* values are reported).
[c]Intention to treat (ITT) analysis was adjusted for donor type and transplant centre.
[d]Denominators for the discard rates is the total number of livers retrieved ($n=270$ (NMP, $n=137$; SCS $n=133$)).
[e]Test not performed due to few events and no events in one arm.
[f]Median and IQR are reported, a non-parametric Mann–Whitney *U*-test was used.

Figure 1-5 Trial outcomes of the randomized trial of normothermic preservation in liver transplantation (Reproduced with permission from Nature Volume 557, May 3, 2018)

These findings however, may need to be interpreted with caution, as the study has been openly criticized for its design [28]. The study, which was mostly conducted with DBD livers, demonstrated a SCS control group 1-year survival of 96%. By comparison, successful 1-year survival outcomes using SCS for DBD grafts without fibrosis, steatosis, and with short warm ischemia times have been reported as 92% [29]. This seems to indicate that the UK randomized trial used mostly high quality grafts with low risk recipients. Currently, it is not well known whether the application of machine perfusion, and at what temperature, truly imparts any benefits to low risk grafts. Further, the study has been criticized for not having clinically relevant endpoints [28]. The primary endpoint using a surrogate marker for liver injury (AST), has shown inconsistent correlation to graft outcome after transplantation, particularly in DCD liver grafts, and has predictive value only when highly elevated (> 5000 U/L) [30]. Lastly, and most concerning, the study has also been criticized for a possible selection bias in regard to discarding grafts accepted for transplantation [28]. Nasralla *et al.* report that in the cold storage group, one quarter of accepted livers were discarded by the recipient centers, although initially accepted, and randomized to the trial by the donor surgeon. In the NMP group, by contrast, only 12% of grafts were discarded [14]. This discordance between accepting a liver graft for the trial, but not for implantation, may be source of selection bias, and may distort trial results. With this in mind, the demonstration by this important study that NMP can increase the donor pool and the number of transplanted patients, although encouraging, needs to be followed up by further, rigorous, properly conducted studies using clinically relevant endpoints [28].To date, there have been no published studies that have directly compared different machine perfusion temperature modalities (eg. NMP vs HMP), nor reported graft discard rates in HMP.

Following this important publication, we performed and published (Bral shared first authorship) our second clinical series of transplanted NMP livers, seeking to answer the question whether immediate initiation of NMP at the donor center followed by graft and machine transport to the recipient center was indeed necessary. In a large geographic catchment area such as ours in Canada, obviating a need for transportation of heavy, complex, labour-intensive and expensive equipment to and from donor centers, simplifies logistics and reduces cost considerably. This was the first published study in the world demonstrating that in a clinical setting a modest accrual of cold ischemic time (CIT) (median 3 hours) prior to NMP was safe compared to initiating NMP immediately, and did not compromise either short or long-term graft outcomes [31].

1.4 Subnormothermic vs normothermic *ex situ* perfusion

Although no standardized perfusion temperature set-point for subnormothermic machine perfusion (SMP) exists, SMP provides warm, oxygenated perfusate below physiologic conditions (25°C- 34°C) . This preserves some physiologic function, potentially allowing for graft viability assessment, while simultaneously possibly conferring some preservation advantage from lower metabolism. Although limited in number, previous studies have shown efficacy of SMP in experimental perfusions, with decreased AST, bilirubin levels, and improved endothelial cell function, and decreased bile duct injury [32,33]. Bruinsma et al perfused 7 human livers discarded from the transplant process using SMP at 21°C, demonstrating minimal liver injury, lower lactate levels, and increased adenosine triphosphate levels, with improved bile output and composition. SMP has not been applied to any clinical series of liver transplants [34].

To date, there is no comparative study of SMP and NMP technology, and indeed, there is limited information guiding what is the optimal perfusate temperature, or, for that matter, at what initial temperature perfusion should be initiated. The advantage of maintaining a graft in normothermia, with full metabolism is optimal for viability assessment, however, no one group has yet convincingly demonstrated at what rate full metabolic function should be recruited. Although lower temperatures may add protective benefit, this does in some measure compromise viability assessment, for example, due to lower graft bile output. The Zurich group has postulated that lower temperatures may be advantageous during machine perfusion, imparting slowing of electron transfer, and more efficient ATP re-synthesis, potentially due to less proton and electron leak [28]. Additionally, machine failure at lower temperatures would facilitate a more controlled rescue to cold storage, which could be more fraught with difficulty at 37ºC during plane or road transportation theoretically. This has not borne out as a practical concern of notable significance to date.

1.5 Future perspectives

Looking to the future, *ex situ* liver perfusion is rich with opportunity as an innovative platform for liver graft repair and modification through the addition of therapeutics to manipulate grafts in manners that heretofore have not been possible. This *ex situ* perfusion platform can potentially serve to minimize ischemia reperfusion injury, de-fat steatotic livers, induce genetic and immunologic modification of liver grafts, and facilitate application of stem cell and a nanoparticle therapeutics. The rapidly expanding understanding of these technologies, dovetailing with progressive improvements in NMP machines, creates the potential for highly

personalized transplant medicine, and a future where organs are never discarded, merely reused between those in need.

The following comprehensive review article, peer-reviewed and originally published in ***Transplantation Reviews*** (Bral M, Gala-Lopez B, Bigam DL, Freed DH, Shapiro AMJ. *Ex situ* liver perfusion: Organ preservation into the future. Transplant Rev (Orlando). 2018;32(3):132-41.) , further describes the contextual evolution of liver *ex situ* normothermic machine perfusion technology, as well as the level of understanding and prospective advances of the field at the time of commencment of this doctorate.

Transplantation Reviews 32 (2018) 132–141

Contents lists available at ScienceDirect

Transplantation Reviews

journal homepage: www.elsevier.com/locate/trre

Ex situ liver perfusion: Organ preservation into the future☆

Mariusz Bral, Boris Gala-Lopez, David L. Bigam, Darren H. Freed, A.M. James Shapiro *

Department of Surgery, University of Alberta, 2D4.43 Walter D MacKenzie Health Sciences Centre, 8440 112 St, Edmonton, Alberta T6G2B7, Canada
Members of the Canadian National Transplant Research Program (CNTRP), 2D4.43 Walter D MacKenzie Health Sciences Centre, 8440 112 St, Edmonton, Alberta T6G2B7, Canada

ARTICLE INFO

ABSTRACT

In recent years, remarkable progress has occurred in the development of technologies to support ex situ liver perfusion. Building upon extensive preclinical studies in large animal models, pilot and randomized clinical trials have been initiated, and preliminary outcomes suggest more optimal protection of both standard and extended criteria liver grafts. There currently exists an incredible opportunity and need to further refine this technology, determine appropriate viability measures to predict usable liver grafts, and to explore potent protective additive strategies to further optimize the quality of extended criteria organs. These findings will have major bearing in expanding the limited liver donor pool, and may save lives where up to a quarter of listed patients die on wait-lists. Herein we offer a brief overview of the history and current status of ex situ liver perfusion, and discuss future directions that will likely have major impact on the practice of clinical liver transplantation.

1. Introduction

Since the first successful liver transplant in 1968, liver transplantation has become the established therapy for select patients with end-stage liver disease. The worldwide organ supply and demand mismatch is now the biggest challenge facing the practice of transplantation, and many potential recipients are denied the opportunity for life-saving therapy through lack of precious donor resources. In 2015 in the United States, there were 7127 adult liver transplants performed, and over 75,000 patients living with a transplanted liver [1]. According to the OPTN/SRTR 2015 Annual Data Report, 14,046 patients were on a waiting list for a liver transplant in that year, with 1673 deaths on that list, and a further 1227 individuals removed due to becoming too sick to undergo the procedure [1]. In parts of Canada presently, variance in organ donor rates result in up to one quarter of patients dying on the liver transplant waiting list. This organ shortage, coupled with marked disparity in regional access to donors, is the predominant driving force for increased acceptance of higher risk organs worldwide.

Clinicians worldwide have sought ways to expand the donor pool through use of living donors, sub-optimal extended criteria donors (ECD), and most recently, organs from donation after circulatory death (DCD). It has been well described that these organs in particular do not tolerate the current static cold storage (SCS) preservation process well, and confer a higher risk to the transplant recipient. Importantly, marginal livers do not tolerate the compounding injury of prolonged cold preservation, with the duration of cold ischemia correlating closely with post-transplant function [2,3]. Prolonged cold ischemia exacerbates any pre-existing donor liver injury, creating a difficult to quantify but escalating risk for potential recipients. Further, the build-up of metabolic by-products during SCS serves to exacerbate the pre-existing graft damage by promoting ischemia-reperfusion injury (IRI) at the time of reperfusion. Implantation of such grafts may have devastating consequences [4]. Despite the risk, in recent years DCD and ECD livers have been transplanted in substantial and escalating numbers, with higher rates of primary non-function (PNF), early allograft dysfunction (EAD), ischemic cholangiopathy and inferior long-term graft survival [4–6].

In DCD procurements, donor organs are subjected to an unavoidable period of poor or absent perfusion, which directly results in anoxic tissue injury. This makes DCD organs particularly vulnerable to cold ischemic damage, and highlights the inadequacy of SCS in preserving these grafts. The higher incidence of increased complications ultimately

☆ Funding: MB is the recipient of the American Society of Transplant Surgeons (ASTS) 2015 Scientist Scholarship. AMJS holds a Canada Research Chair in Transplantation Surgery and Regenerative Medicine, and a Senior Clinical Scholarship from Alberta Innovates Healthcare Solutions. Funding from the University Hospital Foundation at the University of Alberta is gratefully acknowledged. Funding from Astellas Pharma Canada is gratefully acknowledged.

* Corresponding author at: , Clinical Islet Transplant Program, University of Alberta, 2000 College Plaza, 8215–112th St, Edmonton T6G 2C8, Alberta, Canada.
E-mail addresses: mbral@ualberta.ca (M. Bral), galalope@ualberta.ca (B. Gala-Lopez), dbigam@ualberta.ca (D.L. Bigam), dlfreed@ualberta.ca (D.H. Freed), amjs@islet.ca (A.M.J. Shapiro).

https://doi.org/10.1016/j.trre.2018.03.002
0955-470X/

Originally published after peer review in *Transplantation Reviews* 2018; 32: 132-141

Review Article

Ex Situ Liver Perfusion: Organ Preservation Into the Future

Authors: Mariusz Bral, MD,[1,2] Boris Gala-Lopez, MD PhD[1,2] David L. Bigam, MD[1,2], Darren H. Freed, MD PhD[1,2] and A.M. James Shapiro[1,2]

Affiliation:

[1] Department of Surgery, University of Alberta, Edmonton, Canada

[2] Members of the Canadian National Transplant Research Program (CNTRP)

Corresponding Author:

Dr. A.M. James Shapiro, MD, PhD[1,2]

Canada Research Chair in Transplantation Surgery and Regenerative Medicine, Professor, Director of Clinical Islet and Living Donor Liver Transplant Programs, Clinical Islet Transplant Program, University of Alberta. 2000 College Plaza, 8215-112th St, Edmonton T6G 2C8, Alberta, Canada. amjs@islet.ca

1.6 Introduction

Since the first successful liver transplant in 1968, liver transplantation has become the established therapy for select patients with end-stage liver disease. The worldwide organ supply and demand mismatch is now the biggest challenge facing the practice of transplantation, and many potential recipients are denied the opportunity for life-saving therapy through lack of precious donor resources. In 2015 in the United States, there were 7,127 adult liver transplants performed, and over 75,000 patients living with a transplanted liver [1]. According to the OPTN/SRTR 2015 Annual Data Report, 14,046 patients were on a waiting list for a liver transplant in that year, with 1,673 deaths on that list, and a further 1,227 individuals removed due to becoming too sick to undergo the procedure [1]. In parts of Canada presently, variance in organ donor rates result in up to one quarter of patients dying on the liver transplant waiting list. This organ shortage, coupled with marked disparity in regional access to donors, is the predominant driving force for increased acceptance of higher risk organs worldwide.

Clinicians worldwide have sought ways to expand the donor pool through use of living donors, sub-optimal extended criteria donors (ECD), and most recently, organs from donation after circulatory death (DCD). It has been well described that these organs in particular do not tolerate the current static cold storage (SCS) preservation process well, and confer a higher risk to the transplant recipient. Importantly, marginal livers do not tolerate the compounding injury of prolonged cold preservation, with the duration of cold ischemia correlating closely with post-transplant function [2,3]. Prolonged cold ischemia exacerbates any pre-existing donor liver injury, creating a difficult to quantify but escalating risk for potential recipients. Further, the build-up of metabolic by-products during SCS serves to exacerbate the pre-existing graft damage by promoting ischemia-reperfusion injury (IRI) at the time of reperfusion. Implantation

of such grafts may have devastating consequences [4]. Despite the risk, in recent years DCD and ECD livers have been transplanted in substantial and escalating numbers, with higher rates of primary non-function (PNF), early allograft dysfunction (EAD), ischemic cholangiopathy and inferior long-term graft survival [4-6].

In DCD procurements, donor organs are subjected to an unavoidable period of poor or absent perfusion, which directly results in anoxic tissue injury. This makes DCD organs particularly vulnerable to cold ischemic damage, and highlights the inadequacy of SCS in preserving these grafts. The higher incidence of increased complications ultimately translates into higher costs associated with DCD transplantation [7]. As a result, many DCD liver grafts are discarded, with fewer than 78 percent being transplanted overall [1,8].

This current selection pressure to use DCD and ECD livers for transplantation, coupled with the sub-optimal preservation outcomes of these grafts using SCS has led to a resurgence of interest in developing alternate preservation strategies. In recent years, ex situ machine perfusion (MP) has come to the forefront of such endeavours, driving international efforts to explore the potential of this technology in improving viability of DCD and ECD grafts.

1.7 Methodology

This was not intended to be a systematic review. A literature search for published articles regarding MP of livers was performed using PubMed. The final date of the search was January 27, 2018. The searches were conducted using keywords “liver machine perfusion, ex vivo perfusion, ex situ perfusion” combined with terms relevant to machine perfusion such as “normothermic”, ‘subnormothermic”, and “hypothermic”.

1.8 History of *Ex situ* Liver Perfusion

The current state of organ perfusion is built on a foundation of systematic developments in the field **(Figure 1-6)**. Machine organ perfusion was reported as early as 1935, when Alexis Carrel and Charles Lindbergh devised an apparatus used to perfuse cat hearts and thyroids. Between 1935 and 1939, Carrel perfused over 900 organs, and claimed to have preserved a cat thyroid at normothermic temperature for up to 30 days [9]. The initial series of clinical transplanted livers performed by Thomas Starzl in Colorado in 1963 were perfused under hyperbaric conditions with diluted hypothermic oxygenated blood as perfusate [10]. Brettschneider et al. were the first to report 24 hour canine liver perfusion in 1968 [11]. Despite auspicious beginnings, progress in MP lost momentum, largely due to the mechanical complexity of the systems, but also due to parallel progress in the development of cold preservation solutions which facilitated the practice of clinical transplantation [12].

Figure 1: Historical Events in Ex situ Liver Perfusion

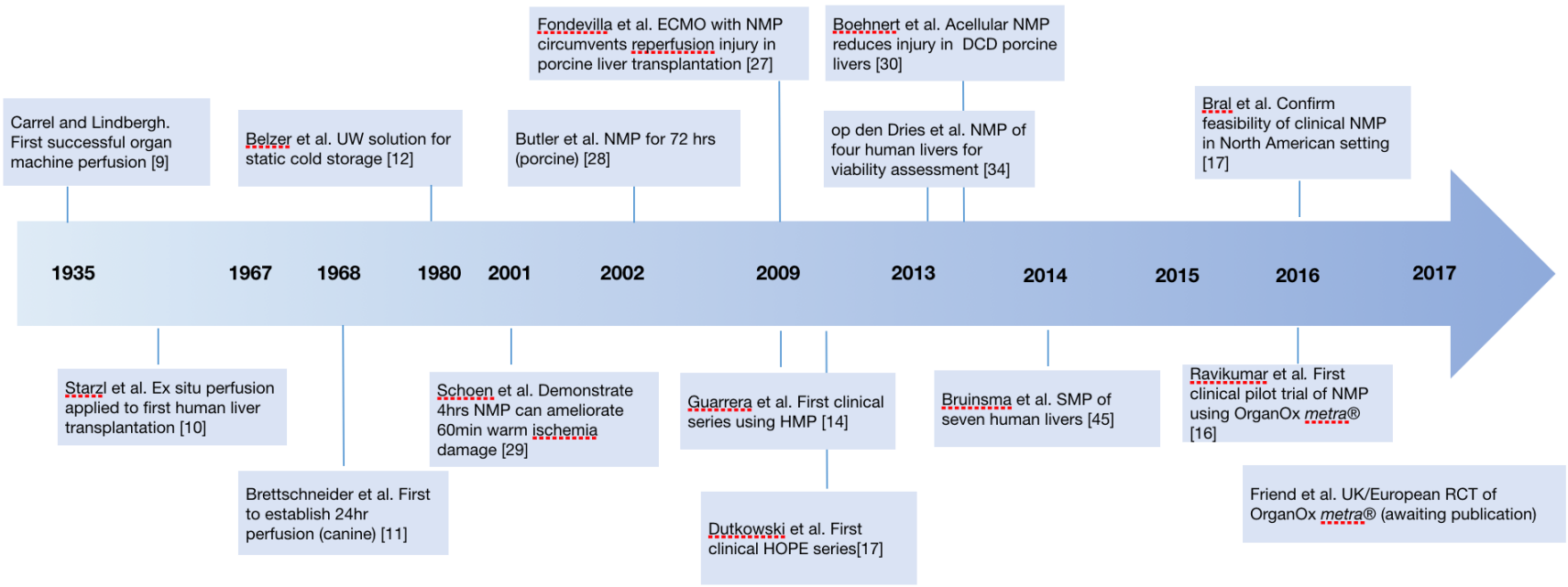


Figure 1-7 Selected historical events in ex situ liver perfusion.

In contrast to SCS, the underpinning principle of MP is maintenance of cellular metabolism in a dynamic physiological environment. Various ex situ liver circuits have been developed, differing in single or dual portal/arterial inflow, pulsatile or continuous inflow waveform, pump-driven or gravity-fed vessel inflow, perfusate temperature, substrate additives, and perfusate composition [13]. Hypothermic, hypothermic oxygenated, subnormothermic, continuous oxygen rewarming (COR) and normothermic perfusion approaches have been explored, each with potential advantages and limitations.

First-in-human trials using ex situ perfusion have been completed, followed by randomized controlled trials (ISRCTN39731134) [14-18]. Preliminary outcomes have established the safety of this technology in the clinical setting, with some studies demonstrating benefit for recipients of ex situ perfused grafts [14,19]. Despite these advances, many open questions remain concerning the application of MP temperature, the timing and duration of MP, as well as any targeted additive strategies or metabolic substrates necessary for a particular liver graft.

To date, limited comparisons between the different MP temperature modes or perfusion timing strategies have been performed [20-22].

1.9 Hypothermic Perfusion

Hypothermic perfusion (HMP) (0°C-12°C) is the simplest mechanical approach, but may confer benefit to the graft by improving liver microcirculation, with mobilization and dilution of metabolic waste. Viability assessment is difficult due to decreased metabolism.

Following supportive evidence derived from porcine experiments [23-25], Guarrera et al. were the first to report a non- randomized series of transplanted human livers perfused under cold (4ºC) non-oxygenated conditions using a modified Medtronic PBS® (Minneapolis, MN) device [14]. HMP was deemed safe in the clinical setting, with recipients of HMP livers demonstrating lower peak enzyme release as well as shorter hospital stay, and less early allograft dysfunction compared to SCS controls from the same time period [26].

Guarrera et al. went on to publish a second non-randomized clinical series of transplanted HMP perfused ECD grafts. When compared to concurrent SCS controls, recipients of perfused livers experienced statistically lower EAD, less biliary complications, and had shorter hospital stays [19].

To date, no HMP study has demonstrated the potential of this technology to extend the preservation period of a liver. These small non-randomized studies are difficult to draw conclusions from, and larger randomized studies are eagerly awaited.
A summary of HMP transplant studies performed are listed in **Table 1**.

Table 1-1. Selected *ex situ* liver perfusion transplant studies

Study	Species/ Sample Size	Cold Ischemia Duration	Warm Ischemia Duration	Perfusion Duration	Temp.	Perfusate	Perfusion Parameters	Transplant Duration	Result
Watson et al. 2017[41]	Human (12)	Median 427 (222-877) min	5-14 min	Median 284 (122-530) min	37 °C	Gelofusine or Steen + PRBC	HA pressure 60 mmHg, PV 9 mmHg	Clinical	NMP livers at high oxygen tension developed reperfusion syndrome and vasoplegia post transplant
Angelico et al. 2016[80]	Human (6)	Median 91 (73-117) min		Median 525 (395-605) min	37 °C	Gelofusine + PRBC	HA pressure 60 to 75 mmHg, -1 to 2 mmHg	Clinical	Lower hemodynamic instability at reperfusion after NMP
Bral et al. 2016 [17]	Human (9)	Median 167 (95-293) min	Median 21.5 (16-26) min DCD	Median 11.5 hrs	37 °C	Gelofusine + PRBC	HA pressure 60 to 75 mmHg, -1 to 2 mmHg	Clinical	Demonstrated safety and feasibility of clinical NMP
Mergental et al. 2016 [49]	Human (5)	Median 422 (387- 474) min		798 (724-951) min	37 °C	5% Human Albumin + PRBC	Mean HA flow 360-654 mL/min	Clinical	Successful evaluation, transplant of 5/6 rejected livers
Selzner et al. 2016 [18]	Human (10)	NA	Median 49 (21-760 min min.	480 (340-580) min	37 °C	Steen + PRBC	Median HA 300 ml, PV flow 1250 ml	Clinical	Comparable outcomes between NMP and SCS grafts
Ravikumar et al. 2016	Human	NA	Median	Median 9.3	37 °C	Gelofusine +	HA pressure	Clinical	Median peak AST lower in the NMP

[16]	(20)		21 min.	hrs		PRBC	60 to 75 mmHg, -1 to 2 mmHg		group. Demonstrated safety and feasibility of clinical NMP
Boehnert et al. 2013 [36]	Porcine (18)	4 hrs	60 min	4hrs	38 °C	Dilute pig blood- Hct 15%	Not reported	8 hrs	Protective. NMP livers- superior biochemical, histologic and radiographic features
Fondevila et al. 2011 [33]	Porcine (18)	4 hrs	90 min	4 hrs	38 °C	autologous blood	HA Flow: '60/40 PV Flow 8 mmHg	5 days	NMP led to significant improvements in injury, inflammation, and synthetic function
Brockmann et al. 2009 [57]	Porcine (36)	5 hrs, 20 hrs	40 min, 60 min	5 hrs, 20 hrs	38 °C	autologous blood	'physiologic pressures'- constant pressure, variable flow	5 days	Protective. Post-transplant survival improved, improved transaminase levels and histology
Schoen et al. 2001 [35]	Porcine (36)	4 hrs	60 min	4 hrs	38 °C	blood + 'balanced electrolyte' solution	HA flow: 100 mmHg, PV flow: 15 cm H2O	7 days	Significant protective effect of NMP.
Spetzler et al. 2016 [31]	Porcine (32)	3 hrs	NA	3 hrs of SMP	33 °C	Steen solution+ erythrocytes (Hct 15%)	HA: 50-60 mmHg, PV 2-4 mmHg	4 days	Protective. SMP livers- lower AST, ALP and bilirubin levels, less apoptosis. Hyaluronic acid was notably better in the SMP group.

Knaak et al. 2014 [30]	Porcine (10)	7 hrs	45 min.	3 hrs SMP	33 °C	Steen + washed erythrocytes (Hct 10-12%)	HA: 60-70 mmHg, PV 4-8 mmHg	7 days.	Protective. SMP livers- better biochemistry, less bile duct necrosis.
Hoyer et al. 2016 [40]	Human (6)	508 (369-870) min	26.5 (18-33) min	90 min	10°-20°C COR	Custodiol®	HA pressure 25 mmHg, PV 2- 4 mmHg	Clinical	Protective. Lower peak AST, ALT post transplant, increased graft and patient survival
Dutkowski et al. 2015 [29]	Human (25)	188 (141- 264) min	18 min (range: 17 - 21 min)	118 (101 – 149) min	10 °C HOPE	KPS-1®	Flow rate: 120- 180 mL/min	Clinical	Protective. HOPE grafts- lower peak ALT, less EAD, less ischemic choangipathy, better 1 year graft survival
Dutkowski et al. 2014 [15]	Human (25)	2.4 hrs	38 min (range: 26- 43 min)	1 – 2 hrs.	10 °C HOPE	KPS-1®	PV: < 3 mmHg	Clinical	Protective. HOPE grafts- lower AST and ALT, shorter ICU stays
de Rougemont et al. 2009 [81]	Porcine (30)	6 hrs	60 min	1 hr	4 °C HOPE	Modified UW solution (starch free)	PV alone: 32 to 36 mL/min	6 hrs	Protective. HMP conferred absolute survival compared to no survival in SCS controls
De Carlis et al. 2016 [48]	Human (7)	Mean 309 (210-370) min	Mean 33.3 (20-45) min DCD	Mean 183 (170- 230) min	10 °C	Belzer solution	HA pressure 25 mmHg, PV 4 mmHg	Clinical	100% patient and graft survival at 6 months. No ischemic cholangiopathy, no PNF

Guarrera et al. 2014 [19]	Human (31)	9.3 ± 1.6 hrs.	45.6 ± 7.3 min.	3 – 7 hrs.	4 – 8 °C	Vasosol®	HA: 5.1 ± 0.2 mmHG, PV: 2.9 ± 0.1 mmHg	Clinical	Protective. Post hoc analysis-less biliary complications, shorter hospital stay in the HMP group
Guarrera et al. 2010 [14]	Human (20)	8 – 9 hrs	-	3 -7 hrs	4 - 6 °C	Vasosol®	HA: 6 PV: 4	Clinical	Protective. HMP livers- shorter mean hospital stay, lower serum injury markers.
Fondevila et al. 2011 [28]	Porcine (6)	Not reported	90 min	4 hrs	4 °C	UW solution	25% of physiologic pressures	5 days	Protective. HMP conferred survival (20%) benefit compared to no survival in SCS controls
Uchiyama et al. 2001 [82]	Porcine (19)	Not reported	60 min	2 hrs	8 °C	Modified Belzer's solution	Continuous HA: 60 and 30 mmHg; pulsatile 30 mmHg	>3 days	Protective. Non-pulsatile HA perfusion at 30 mmHg conferred survival benefit.
Iwamoto et al. 2000 [83]	Porcine (25)	Not reported	60 min	2 hrs	8 °C	UW gluconate solution (UW-G)	Continuous HA: 50-60 mmHg, and 20-30 mmHg.	>2 days	Protective

ALT (alanine aminotransferase), AST (aspartate transaminase), COR (controlled oxygenated rewarming), HA (hepatic artery), Hct (hematocrit), HMP (hypothermic machine perfusion), ICU (intensive care unit), NMP (normothermic machine perfusion), PNF (primary non-function), PRBC (packed red blood cells), PV (portal vein), SMP (subnormothermic machine perfusion), UW (University of Wisconsin

1.10 Hypothermic oxygenated perfusion

Hypothermic oxygenated perfusion (HOPE) is an approach which provides supplemental oxygen to HMP. Substantial evidence derived from animal models demonstrates the advantage of the HOPE approach to re-energize the electron transport chain and replenish cellular adenosine triphosphate (ATP), resulting in less IRI and improved liver function [27,28].

In the clinical setting, Dutkowski et al. reported a non-randomized series of eight DCD livers, perfused with HOPE, and transplanted. HOPE grafts displayed excellent early function, with no evidence of intrahepatic biliary complications at 8.5 months follow-up compared with historical controls [15]. An international matched analysis comparing SCS to HOPE in DCD liver grafts demonstrated decreased peak ALT, less ischemic cholangiopathy, biliary complications and improved 1-year graft survival in the HOPE group. In this study however, the HOPE livers had a relatively short cold ischemia, calling into question the beneficial effect of HOPE in more optimally preserving the grafts [29]. A randomized trial has been initiated comparing HOPE with SCS (NCT01317342, and NCT02584283).

A summary of HOPE transplant studies performed to date is listed in **Table 1**.

1.11 Subnormothermic Perfusion

Subnormothermic machine perfusion (SMP) provides oxygenated perfusate at warm, but not physiological conditions (25°C-34°C). A degree of metabolic function is preserved, and functional viability may potentially be assessed, with the liver graft still benefiting from a lower metabolic demand.

In both standard and DCD porcine liver transplant models, SMP led to decreased post transplant AST, ALP and bilirubin levels as well as improvement in endothelial cell and bile duct injury [30,31]. SMP of seven human livers perfused for 3 hours at 21°C demonstrated minimal liver injury, improved oxygen uptake, lower lactate and increased ATP levels in the grafts. Bile production increased and bile composition became more favourable [32]. To date, liver SMP has not been applied in any clinical series or randomized trials. A summary of SNP transplant studies is provided in **Table 1**.

1.12 Normothermic Perfusion

Normothermic perfusion (NMP) (35°C-38°C) attempts to provide as near a physiologic environment as possible to maintain a liver in a fully metabolic state, providing the opportunity for viability assessment. An oxygen carrier is required, which in most cases are erythrocytes. The possibility of warm ischemia due to machine technical failure mid- perfusion is concerning, as it may result in graft loss.

Large animal models have demonstrated the advantages of NMP compared to SCS [33-36]. Application of NMP to organ preservation in animal models has shown the potential of this modality to prolong preservation time, improve graft function from DCD donors, and for graft functional assessment [35-37].

Initial NMP clinical approaches focused on perfusion of human livers declined for transplantation. The feasibility of human NMP was demonstrated for up to 24 hours, with some preliminary viability assessment [38,39].

The first non-randomized clinical series of transplanted NMP livers was published by the Oxford group from the UK. Outcomes established the feasibility and safety of this technology in the clinical setting [16]. These findings were confirmed in two further non-randomized clinical series [17,18]. Two of these three suggested improved function of NMP liver grafts when compared with historical SCS controls, evidenced by statistically lower AST levels in NMP graft recipients post-transplant [16,18].

A 260 subject multi-center randomized controlled trial comparing NMP with SCS in DBD livers has been completed in Europe, with results pending publication (ISRCTN 39731134). A summary of NMP transplant studies is provided in **Table 1**.

1.13 Controlled Rewarming of Liver Grafts

An alternate approach to MP is one during which the liver graft attains physiologic temperature incrementally over time (36, 37). This has already been implemented clinically, with promising results. In a non-randomzied study, livers treated with controlled oxygenated rewarming (COR) prior to transplantation demonstrated statistically significant reduction in transaminases after transplantation compared with historical controls. Monitoring of glucose levels within the perfusate correlated with post-transplant synthetic graft function [40]. This study had a small sample size, and was underpowered to draw any definitive conclusions from.
The single COR transplant study is in **Table 1**.

1.14 Perfusate Oxygen Tension

Inextricably correlated to the temperature of MP is the possible necessity of, and the tension of dissolved oxygen in the perfusate. Higher temperatures at which organs maintain a higher metabolic rate depend on higher oxygen concentrations for optimal function. This however also creates the potential for increased IRI at reperfusion. A recent study demonstrated that liver grafts perfused at high perfusate oxygen tension levels had more post-reperfusion syndrome and sustained vasoplegia, compared with livers perfused at lower oxygen tensions [41]. There is limited evidence comparing different oxygenation settings during MP, although it has been demonstrated that titrating oxygen along with temperature during graft rewarming results in improved liver preservation, compared to a constant level of oxygenation [42]. These studies emphasize the importance of considering the relationship between temperature and oxygenation parameters in subsequent ex situ MP research.

1.15 Application of Perfusion Strategies

Much of the discussion surrounding machine perfusion (MP) advantages and limitations concerns the temperature at which the perfusion is performed. Equally important may be the timing at which perfusion strategies are applied during the graft preservation process **(Figure 2).** Different temperature machine perfusion (MP) can strategically be applied to specific graft types for variable durations. In the clinical setting, this is limited by the portability of the commercial perfusion machine.

The terminology used to describe perfusion strategies is derived from a recent consensus of nomenclature pertaining to machine perfusion [43].

1.15.1 End Ischemic Perfusion (post–SCS MP)

The current established practice of liver graft preservation with SCS has been the standard for fifty years, but is not ideal for DCD and ECD grafts. An alternate approach is to transport the graft in SCS to the recipient center, where MP is initiated and continued prior up to the transplant procedure. This is currently referred to as 'end ischemic' perfusion, or post-SCS MP.

In a non-transplant porcine model, findings indicate that this approach is not ideal if NMP is to be used. Reddy et al. demonstrated that livers perfused with NMP after a shorter duration of SCS demonstrated superior bile production and less hepatocellular damage as evidenced by lower transaminases and histology, implying that in an ideal situation NMP should be established as close to donor flush initiation as possible [44] [45]. In a clinical setting, this would mean that the NMP machine would be portable to the donor center.

1.15.2 Pre-SCS MP combined with post-SCS MP

In a variation of end ischemic perfusion, a clinical case report documented the addition of further SCS after initial SCS transport and NMP. Total preservation time was 26 hours (618 minutes of SCS, 8.5 hours of NMP, followed by 412 minutes SCS) [46]. This strategy may prove useful in the future if organs are transported to regional centers for repair or modification, and then transported again to remote centers for implantation.

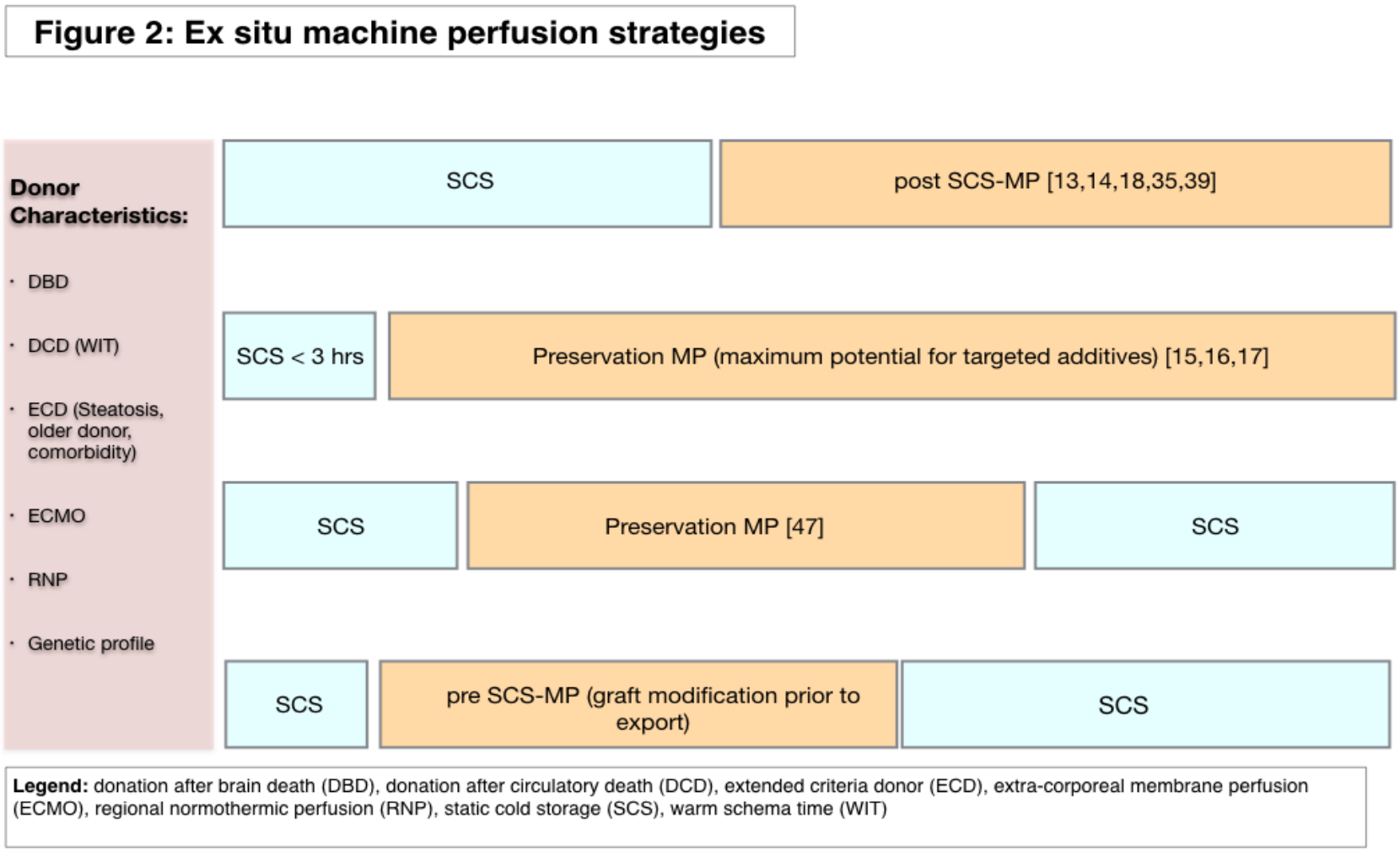


Figure 1-8 *Ex situ* machine perfusion strategies.

1.15.3 Preservation machine perfusion

An alternate approach is to immediately initiate MP after graft procurement. In this strategy cold ischemic time is minimized and the full benefits of machine perfusion are extended. This strategy introduces more complexity, including the logistics of machine perfusion transport and the peril of graft loss mid-perfusion at higher perfusion temperatures. This strategy is referred to as 'preservation machine perfusion' (preservation MP) and the term only applies if the SCS period before or after MP is less than a maximum of 3 hours [43].

1.16 Implementation of machine perfusion strategies

With consideration to the demonstrated utility of each temperature modality, as well as the current logistic constraints of commercially available machines, it may be useful in the future to apply different perfusion strategies to specific grafts [47].

Should an ideal liver graft be procured from a DBD donor, then HOPE, with its potential to maintain the energy of mitochondria would be a good storage option. If a graft is procured from a DCD or ECD donor, or with concerning macrosteatosis, then a warmer perfusion mode may mitigate storage injury if applied with a preservation MP strategy.

In situations where graft viability is of concern, then preservation MP or post- SCS SNP or NMP will be necessary for appropriate graft evaluation prior and up to transplant. If a therapeutic intervention is implemented, then preservation NMP or post-SCS NMP will likely be the most useful strategy to optimize the full metabolic function of the graft. The duration of perfusion will be different in perfusion for evaluation or perfusion for modification or repair, with longer perfusions likely required if treatment strategies are employed.

A further consideration in choosing a preservation strategy is whether extracorporeal membrane oxygenation (ECMO) or normothermic region perfusion (NRP) is administered to the donor prior to procurement. In 2009, Fondevila et al. applied in situ donor vascular cannulation and ECMO, followed by ex situ perfusion in a porcine DCD transplant model. The combination of donor ECMO and ex situ NMP led to less injury and inflammation, and improved synthetic function compared to controls [33]. In the clinical setting, De Carlis et al. applied post-SCS HMP to livers from 7 DCD donors with post-asystole initiation of NRP. No cases of PNF or ischemic cholangiopathy were reported, with 100% patient and graft survival at a median of 6.1 months [48].

1.17 Clinical Devices

To date there are five companies that have developed clinical grade ex situ liver perfusion technology for potential commercial application: 1) OrganOx metra® (OrganOx, Oxford, UK); 2) Liver Assist (Organ Assist, Groningen, Netherlands); 3) Organ Care System (OCS) Liver (Transmedics, Andover, MA. USA); 4.) Medtronic PBS® (Medtronic, Minneapolis, MN, USA); and 5) Life Port Liver Transporter (Organ Recovery Systems, Des Plaines, IL, USA).

The portable OrganOx metra®, which is designed to perform NMP, is primed with 3 units of packed red blood cells, colloid solution, antibiotic, heparin, and calcium gluconate. Prostacyclin, bile salts, insulin, and heparin are infused at a constant rate. Non-lipid total parenteral nutrition is infused depending on the glucose level of the perfusate. Both hepatic artery and portal vein perfusion are constant. Hepatic artery perfusion is pump-driven, portal vein perfusion is achieved by gravity from a suspended soft-shell reservoir, allowing for autoregulation of portal vein flow by the liver. The inferior vena cava is cannulated as part of

the closed circuit. The metra® entered first-in-human trials in 2013, with the results of initial clinical series, and case reports published [16-18]

The Liver Assist is a non-portable device that can accommodate varying perfusion temperatures. It is designed to produce pulsatile flow through the hepatic artery and continuous flow through the portal vein, both using rotary pumps. The liver is fully immersed in the preservation solution, with the vena cava left open to drain freely into a collecting reservoir. The machine is pressure controlled, allowing the liver to auto-regulate variable flow rates. Perfusion parameters of temperature, pressure and flow are displayed in real time. The Liver Assist has been used in a large number of clinical perfusions [41,46,49].

The Organ Care System Liver device, made its first appearance in the clinical forum in early 2016, is a fully automated and portable device, designed for NMP. It delivers pulsatile flow through the hepatic artery, and the vena cava is left to drain feely into a reservoir. The device is currently part of a clinical trial (Liver PROTECT) to determine the safety and efficacy (NCT02522871).

The Medtronic PBS® device was used by the Columbia group to perform HMP of the first clinical series of perfused and transplanted human livers, with good outcomes [14]. The Life Port Liver Transporter has yet to enter the clinical forum.

The relative costs of alternative organ preservation systems remains undefined, and will be likely to remain so until the clinical utility of each technology is proven in practice.

1.18 Future Directions of *Ex situ* Machine Perfusion

Proponents of ex situ liver perfusion refer to the following potential benefits **(Figure 3):**

1.18.1 Prolonged preservation

To date ex situ NMP is the only perfusion modality that has demonstrated potential considerably extend the preservation period well beyond what is currently possible with SCS [17,34,39,50]. Safe prolonged preservation may confer advantage not only to injured organs, but also to transport livers over greater geographic distances (and potentially internationally) for urgent need, size, blood group or human leukocyte antigen matching allocation. The current clinically accepted limit of NMP perfusion is 24 hours, although the consequences of longer perfusions in relation to timing of initiation and underlying graft characteristics are not fully understood.

1.18.2 Improved graft function

Machine perfusion offers the potential to 'recondition' an injured liver before transplantation, reversing ischemic insult sustained during agonal events and surgical retrieval. Maintaining metabolic substrate with normal ATP is a primary goal, and is readily achievable with ex situ machine perfusion [20,40,51-53].

Two HMP non-randomized clinical series have demonstrated that human liver grafts perfused on HMP prior to transplantation demonstrate significantly less EAD, less biliary complications

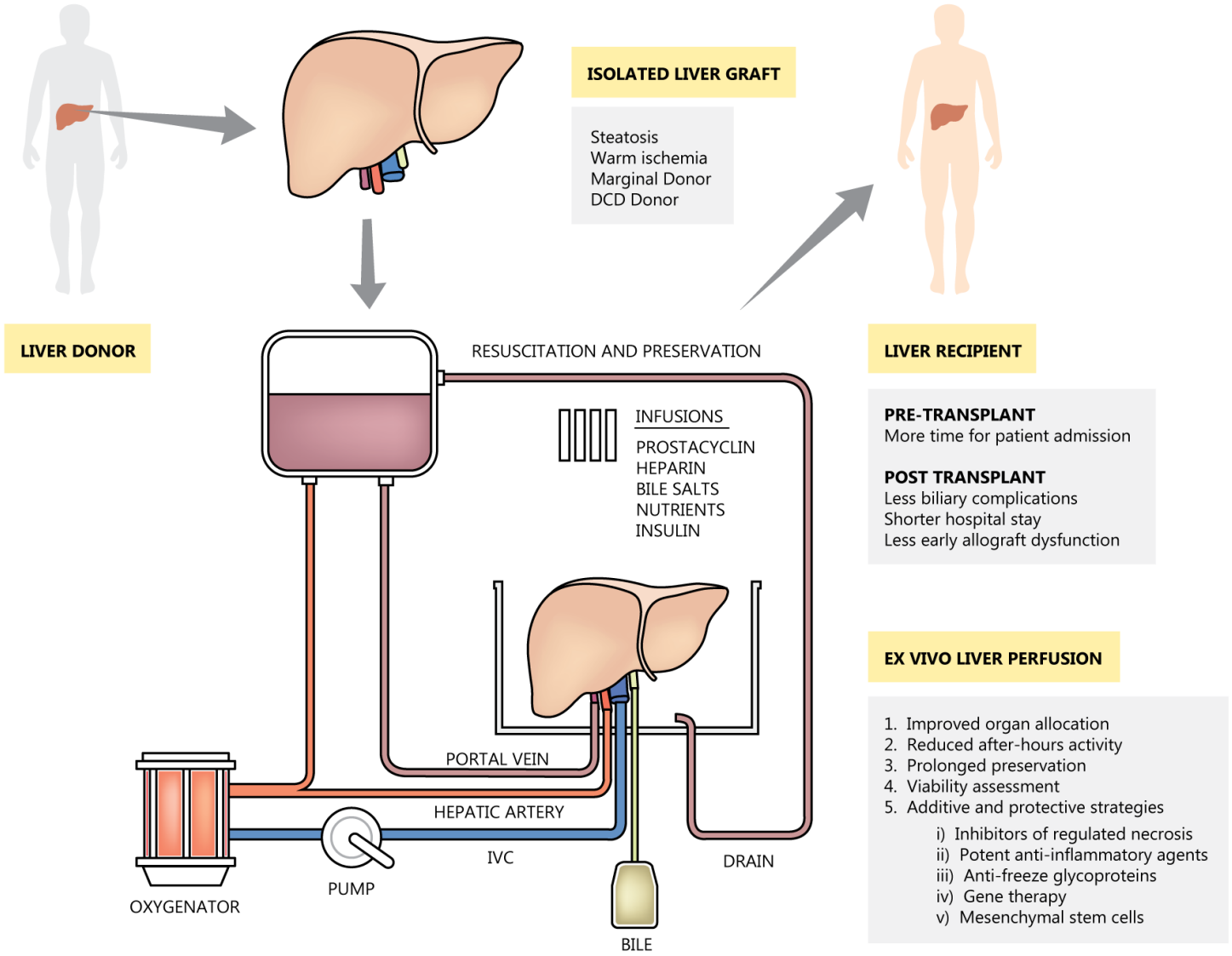


Figure 1-9 *Ex situ* liver perfusion: from donor to recipient.

The depicted perfusion machine schematic is one of a few currently applied in liver preservation and evaluation.

with recipients having a shorter hospital length of stay when compared with SCS graft recipients [14,19].

In two NMP clinical studies, Ravikumar et al. and Selzner et al. both demonstrated lower post-transplant peak AST values in recipients of NMP grafts, implying less post-transplant graft damage. No significant differences were seen in PNF or EAD incidence, length of ICU or hospital stay, 30-day mortality or 6 month recipient survival [16,18]. Post-transplant AST values have been shown to correlate with graft and patient survival [54,55]

1.18.3 Viability assessment

Perhaps the greatest potential of MP is in ex situ viability assessment of DCD and ECD grafts. Accepted markers of hepatic function in ex situ liver MP are still undefined, and predicting the functional status of a particular liver requires interpolation of multiple variables. Often these are based on predefined biochemical or perfusion limits, and the correlation with post-transplant graft function still remains unclear [56].

Several groups have attempted to define combinations of parameters in assessing graft viability, however none have been clinically validated to date **(Table 2)**. To be clinically relevant, predictive markers need to be easily accessible, reproducible, with results available in a short period of time. The optimal time for viability assessment is currently unknown, and likely depends on the temperature and MP strategy used.

Table 1-2. Predicting liver transplant function from *ex situ* viability parameters.

Author	Species	Suggested predictive graft viability parameters
Mergental et al., 2016 [49]	Human	Within 3 hours of perfusion: bile production or lactate less than 2.5 mmol/L, or two of the following: (1) perfusate pH greater than 7.3 (2) stable arterial flow > 150 mL and portal vein flow > 500 mL (3) homogenous graft perfusion with soft consistency and parenchyma
Bral et al., 2016 [17]	Human	Stable physiologic pH and normalizing lactate.
Guarrera et al., 2015 [19]	Human	AST and ALT levels measured during ex situ perfusion strongly correlate with post transplant peak AST and ALT
Liu et al., 2014 [84]	Porcine	pH, AST, l-FABP, ATP and redox-active iron, HAR.
Sutton et al., 2014 [85]	Human	Bile output >30 g after 6 hours of ex situ perfusion.
Boehnert et al., 2013 [36]	Porcine	Lactate dehydrogenase in bile, bilirubin, phospholipids in bile.
Monbaliu et al., 2012 [86]	Human	Lower levels of AST and LDH in more more viable livers.
Brockmann et al., 2009 [57]	Porcine	Bile output 11.4 ± 1.4 (mL/h), BE (mEq/L) 3.3 ± 1.7, AST (IU/L) 964 ± 302, ALT (IU/L) 62 ± 10, hyaluronic acid 108 (ng/ml), PV pressure 2.5 ± 1 (mmHg), PVR 35.4 ± 17.3.

ALT (alanine aminotransferase), AST (aspartate transaminase), ATP (adenosine triphosphate), BE (base excess), HAR (hepatic artery resistance), LDH (lactate dehydrogenase), l-FABP (L-type fatty acid binding protein), PV (portal vein), PVR (portal vein resistance)

In an assessment of porcine livers perfused under normothermic conditions, Brockman et al. outlined a practical list of hemodynamic and functional parameters which could serve as predictors of post-transplant viability. The list included: bile output, base excess, AST, ALT, hyaluronic acid, portal pressure (mmHg), and portal venous resistance, to be used in tandem during NMP at hour 16 [57]. Bruinsma et al. used SNMP to perfuse 7 discarded human livers for 3 hours at 21°C, at the end of which they observed improved graft oxygen uptake, decreased lactate levels, and improved adenosine triphosphate content. Bile production also increased, and the composition of bile improved [32]. Margental et al. used perfusate lactate/pH, arterial and venous flows, bile production, as well as homogeneity of perfusion and parenchymal texture criteria in a post SCS NMP strategy to assess and ultimately transplant five from six discarded liver allographs. At seven months follow up all patients were well, with normalized liver tests [49].

1.18.4 Additive and protective strategies targeting specific graft injury

The ex situ phase of liver preservation provides an opportunity to apply therapies “on circuit” that may not be active or effective during SCS. The therapeutic window for safety is broadened, as only the liver graft and not the patient is exposed to the treatment. Administration of therapeutic additives or cellular conditioning therapies could accelerate repair and provide protection, especially for ECD and DCD grafts.

During the preservation process, liver cells are exposed to numerous types of stress induced by non-physiologic stimuli, leading to the activation of various cellular damage pathways including danger-associated molecular patterns, accumulation of free radicals by oxidative stress and caspase activations, among others. These mechanisms promote the release of a

variety of pro-inflammatory cytokines, leading to a perpetuation of cellular damage and eventually cell death. Previous research has explored strategies to confer cell resistance to stress-induced damage. These strategies could be utilized to mitigate mechanisms of cell injury, preserving tissue viability and function ex situ. Some of the most promising strategies include: 1) anti-oxidants to protect from oxidative cell injury 2) potent anti-inflammatory agents used to decrease the inflammatory response occurring in the donor and the recipient upon reperfusion 3) caspase inhibitors to decrease the risk of apoptosis during preservation and reperfusion, and 4) the use of specific protocols to decrease the fat content in the liver tissue, as a novel strategy to rescue currently discarded organs. A major hurdle to current application of additive and protective strategies in MP is the lack of standardized perfusion parameters and strategies. A summary of potential compounds added to NMP is listed in **Table 3**.

Antioxidants have proven to enhance organ preservation and transplantation at various stages [58]. Oxidative stress is initiated by the excessive production of reactive oxygen species, which are potent inducers of pro-inflammatory stress responses often marked by pro-inflammatory cytokines (TNF- a, IL-1β, IL-6, and IFN-γ), and chemokine synthesis [59]. Published findings with glutathione-ethyl-ester, showing increased islet viability, cell protection for apoptosis and better engraftment [60]. Similarly, mimetics of superoxide dismutase have demonstrated to be beneficial in improving islet survival in culture by successfully scavenging a broad range of oxidants [61].

Inflammatory pathways are known to be activated during organ donation and preservation. IRI occurs as a consequence of prolonged tissue hypoxia with ATP depletion. This injury mechanism is mediated by cytokines, chemokines, adhesion molecules and other compounds,

Table 1-3. Potential additive strategies in ex situ NMP.

Oxidative Stress	**Anti-apoptotic agents**	**IDN-6556, F573, Z-VAD-FMK**
	Metaloproteinase Inhibitors	RXPO3, lactobionic acid
	Vasodilators	BQ123, Epoprosenol, Verapamil
	Anti-oxidants	glutathione-ethyl-ester, albumin, BMX-001
	Anti-inflammatory agents	acetylcysteine, carbon monoxide, sevofluorane, anti-aging glycopeptide, Etanercept
Cell Death Inhibitors	RIPK1 kinase inhibitor, Radical trapping anti-oxidant	Fer-1, Lip-1, Necrostatin-1
Liver Defatting	Lipolytics	Forskolin, GW7646, scoparone, hypericin, visfatin, GW501516, L-carnitine
Energy Depleted Mitochondria	Alkaloid derivative,	Berberine, niacinamide, S-15176
Gene Manipulation	Viral Vectors	miRNA-122
Immuno-modulation		Regulatory T cells, mesenchymal stem cells,

and activates the innate immune system's Toll-like receptors, as well as the complement and adaptive systems.The result is a profound inflammatory tissue reaction, which may compromise the graft function in the recipient [62]. This is the rational to incorporate anti-inflammatory agents to the organ preservation phase, where machine preservation offers a unique opportunity to maximize their effects. Some of the strategies used with NMP may include: alprostadil, n-acetylcysteine, carbon monoxide, or sevoflurane [63]. Potent anti-inflammatory effects have been observed when using Anti-aging Glycopeptide (AAGP) in the context of experimental islet transplantation. The addition of this agent to islet culture medium resulted in a significant decrease of pro-inflammatory cytokines (IL-1b, Il-6, KC/GRO and TNF-alpha) [64]. This compound has now escalated into clinical trials and would be a potential candidate for anti-inflammatory treatment during NMP.

Another strategy is the amelioration of apoptosis during liver preservation and transplantation. Multiple anti-apoptotic agents have been characterized for liver protection against IRI. Among them, caspase inhibitors are the most popular and widely used in experimental and clinical settings. Apoptosis is a form of programmed cell death necessary to regulate tissue homeostasis. This cell death mechanism is mediated by activation of caspases. These proteases exist within the cell as zymogens and can be divided into initiator (caspases 2, 8, 9 and 10) and effector caspases (3, 6 and 7) [65]. A potent pan-caspase inhibitor IDN-6556, has shown protection against apoptosis in various cell types, including liver cells [66,67], and has progressed to pilot clinical trials [68]. Other pan-caspase inhibitors (e.g. F573) are available and may offer more potent anti-apoptotic protection [69]. Using these compounds during NMP would potentially provide the potential to further improve liver grafts by avoiding some of the mechanisms leading to IRI.

1.18.5 Targeting graft steatosis

The prevalence of hepatic steatosis is gradually increasing in western countries as a consequence of the obesity epidemic. Macrovesicular infiltration greater than 30% poses an increased risk factor for IRI and PNF. This phenomenon is negatively impacting the availability of suitable liver donors, prompting for experimental studies to decrease the fat content of these grafts. *Ex situ* graft perfusion has been repeatedly suggested as a possible delivery method of defatting pharmacological compounds to macrosteatotic livers, particularly under normothermic conditions. Nagrath et al. demonstrated a dramatic decrease in graft steatosis by combining NMP and pharmacologic therapy in a rat model. After only 3 hours of perfusion, the authors were able to observe a 50% reduction in intracellular triglyceride content and reduction in lipid droplet size [70]. Alternatively, in a porcine model, Jamieson et al. applied 48 hours of ex situ NMP to mildly steatotic livers, which resulted in a 50% reduction of lipid droplet size without the use of any additives [71]. Liver steatosis is an inbalance between triglyceride (TG) production and utilization. The main objective of such defatting strategies is to rapidly decrease the proportion of steatotic hepatocytes, while maintaining a viable organ [72]. The ideal protocol should rapidly decrease TG synthesis and accelerate enzymatic lipolysis selectively to avoid damaging structural and functional lipids normally present in liver tissue. Further strategies are also needed to eliminate all related byproducts from a closed circuit and to objectively determine the duration of perfusion to achieve effective fat reduction.

Rational targeted application of these compounds, isolated or in combination may be beneficial to injured grafts, or may potentially be given before withdrawal of life support in the DCD setting if ethically permitted, as an additional strategy to expand the donor pool.

1.18.6 Genetic modification of liver grafts

Additional potent compounds are under investigation to genetically modify liver grafts, for example, to treat hepatitis positive livers. Goldaracena et al. treated pig livers during NMP with Miravirsen, a locked-nucleic acid oligonucleotide that sequesters microRNA-122 (miR-122), which is essential for hepatitis C virus replication. This strategy resulted in significant miR-122 sequestration, and miR-122 target gene depression was noted with NMP but not in the CS group. This was the first 'proof of concept' large animal study using NMP to modify liver grafts prior to transplantation [73]. In series of rejected donor human lungs, adenoviral vector IL-10 gene therapy applied ex situ served to improve function and lower markers of inflammation [74]. Using viral vectors in an ex situ circuit can serve to potentially immune-modulate liver grafts, possibly obviating the need for future immunosuppression altogether [75,76].

1.18.7 Graft Immunomodulation during ex situ perfusion

Ex situ perfusion offers potential for direct manipulation of expressed HLA antigens that could be stripped or blocked during perfusion. Removal of passenger immunogenic cells of donor origin could be readily accomplished during NMP using antibody, silencing RNA, or viral manipulative approaches. Alternatively, addition of immune-regulatory cells such as regulatory T cells, mesenchymal stem cells (MSCs) could be added and perfused, potentially conferring anti-inflammatory or tolerogenic properties to ameliorate graft injury [77]. In other organ systems, MSCs have already shown promise, with the delivery of MSCs to lungs ex situ restoring endothelial barrier permeability and alveolar fluid balance after injury [78]. Intravascular administration of MSCs during ex situ perfusion may be beneficial to restore injured livers [79]. Application of MSCs has not been documented in ex situ liver perfusion to-date.

1.19 Conclusions

Ex situ liver perfusion has moved from concept to clinic since the 1930s and through the early evolution of liver transplantation. Studies have now established ex situ to be both feasible and safe in the clinical setting, and reliable machine perfusion systems now offer a real alternative to the current standard practice of SCS in preservation solution. Despite these advances, many open questions remain surrounding optimal circulatory temperature and solutions, the perfusion timing strategy and duration of perfusion, as well as optimal additives and metabolic support for liver grafts. Establishing reliable predictive viability markers to confirm that a potential graft on ex situ perfusion should actually be transplanted is probably the highest current priority for the field as clinical trials move forward. The ultimate utility of MP technology will depend on safety, cost, practicality, and the much-anticipated outcomes of rigorously conducted randomized controlled clinical trials.

1.20 References

1. Kim WR, Lake JR, Smith JM, et al. OPTN/SRTR 2015 Annual Data Report: Liver. *Am J Transplant.* 2017;17 Suppl 1:174-251.
2. Furukawa H, Todo S, Imventarza O, et al. Effect of cold ischemia time on the early outcome of human hepatic allografts preserved with UW solution. *Transplantation.* 1991;51(5):1000-1004.
3. Adam R, Bismuth H, Diamond T, et al. Effect of extended cold ischaemia with UW solution on graft function after liver transplantation. *Lancet.* 1992;340(8832):1373-1376.
4. Op den Dries S, Sutton ME, Lisman T, Porte RJ. Protection of bile ducts in liver transplantation: looking beyond ischemia. *Transplantation.* 2011;92(4):373-379.
5. Merion RM, Goodrich NP, Feng S. How can we define expanded criteria for liver donors? *J Hepatol.* 2006;45(4):484-488.
6. Kalisvaart M, de Haan JE, Polak WG, et al. Comparison of Postoperative Outcomes Between Donation After Circulatory Death and Donation After Brain Death Liver Transplantation Using the Comprehensive Complication Index. *Ann Surg.* 2017.
7. Merion RM, Pelletier SJ, Goodrich N, Englesbe MJ, Delmonico FL. Donation after cardiac death as a strategy to increase deceased donor liver availability. *Ann Surg.* 2006;244(4):555-562.
8. Otero A, Gomez-Gutierrez M, Suarez F, et al. Liver transplantation from Maastricht category 2 non-heart-beating donors. *Transplantation.* 2003;76(7):1068-1073.
9. Dutkowski P, de Rougemont O, Clavien PA. Alexis Carrel: genius, innovator and ideologist. *Am J Transplant.* 2008;8(10):1998-2003.

10. Starzl TE, Groth CG, Brettschneider L, et al. Extended survival in 3 cases of orthotopic homotransplantation of the human liver. *Surgery.* 1968;63(4):549-563.
11. Brettschneider L, Daloze PM, Huguet C, et al. The Use of Combined Preservation Techniques for Extended Storage of Orthotopic Liver Homografts. *Surg Gynecol Obstet.* 1968;126(2):263-274.
12. Collins GM, Bravo-Shugarman M, Terasaki PI. Kidney preservation for transportation. Initial perfusion and 30 hours' ice storage. *Lancet.* 1969;2(7632):1219-1222.
13. Friend PJ, Imber C, St Peter S, Lopez I, Butler AJ, Rees MA. Normothermic perfusion of the isolated liver. *Transplantation proceedings.* 2001;33(7-8):3436-3438.
14. Guarrera JV, Henry SD, Samstein B, et al. Hypothermic machine preservation in human liver transplantation: the first clinical series. *Am J Transplant.* 2010;10(2):372-381.
15. Dutkowski P, Schlegel A, de Oliveira M, Mullhaupt B, Neff F, Clavien PA. HOPE for human liver grafts obtained from donors after cardiac death. *J Hepatol.* 2014;60(4):765-772.
16. Ravikumar R, Jassem W, Mergental H, et al. Liver transplantation after ex vivo normothermic machine preservation: a Phase 1 (first-in-man) clinical trial. *Am J Transplant.* 2016.
17. Bral M, Gala-Lopez B, Bigam D, et al. Preliminary Single-Center Canadian Experience of Human Normothermic Ex Vivo Liver Perfusion: Results of a Clinical Trial. *Am J Transplant.* 2016.
18. Selzner M, Goldaracena N, Echeverri J, et al. Normothermic ex vivo liver perfusion using steen solution as perfusate for human liver transplantation: First North American results. *Liver Transpl.* 2016;22(11):1501-1508.

19. Guarrera JV, Henry SD, Samstein B, et al. Hypothermic machine preservation facilitates successful transplantation of "orphan" extended criteria donor livers. *Am J Transplant.* 2015;15(1):161-169.

20. Minor T, Efferz P, Fox M, Wohlschlaeger J, Luer B. Controlled oxygenated rewarming of cold stored liver grafts by thermally graduated machine perfusion prior to reperfusion. *Am J Transplant.* 2013;13(6):1450-1460.

21. Nassar A, Liu Q, Farias K, et al. Impact of Temperature on Porcine Liver Machine Perfusion From Donors After Cardiac Death. *Artif Organs.* 2016;40(10):999-1008.

22. Hoyer DP, Paul A, Luer S, Reis H, Efferz P, Minor T. End-ischemic reconditioning of liver allografts: Controlling the rewarming. *Liver Transpl.* 2016;22(9):1223-1230.

23. Jain S, Lee CY, Baicu S, et al. Hepatic function in hypothermically stored porcine livers: comparison of hypothermic machine perfusion vs cold storage. *Transplantation proceedings.* 2005;37(1):340-341.

24. Guarrera JV, Estevez J, Boykin J, et al. Hypothermic machine perfusion of liver grafts for transplantation: technical development in human discard and miniature swine models. *Transplantation proceedings.* 2005;37(1):323-325.

25. Bessems M, Doorschodt BM, Dinant S, de Graaf W, van Gulik TM. Machine perfusion preservation of the pig liver using a new preservation solution, polysol. Transplantation proceedings. 2006;38(5):1238-1242.

26. Guarrera JV, Henry SD, Chen SW, et al. Hypothermic machine preservation attenuates ischemia/reperfusion markers after liver transplantation: preliminary results. *The Journal of surgical research.* 2011;167(2):e365-373.

27. Schlegel A, Dutkowski P. Role of hypothermic machine perfusion in liver transplantation. *Transpl Int.* 2015;28(6):677-689.

28. Fondevila C, Hessheimer AJ, Maathuis MH, et al. Hypothermic oxygenated machine perfusion in porcine donation after circulatory determination of death liver transplant. *Transplantation.* 2012;94(1):22-29.

29. Dutkowski P, Polak WG, Muiesan P, et al. First Comparison of Hypothermic Oxygenated PErfusion Versus Static Cold Storage of Human Donation After Cardiac Death Liver Transplants: An International-matched Case Analysis. *Ann Surg.* 2015;262(5):764-770; discussion 770-761.

30. Knaak JM, Spetzler VN, Goldaracena N, et al. Subnormothermic ex vivo liver perfusion reduces endothelial cell and bile duct injury after donation after cardiac death pig liver transplantation. *Liver Transpl.* 2014;20(11):1296-1305.

31. Spetzler VN, Goldaracena N, Echiverri J, et al. Subnormothermic ex vivo liver perfusion is a safe alternative to cold static storage for preserving standard criteria grafts. *Liver Transpl.* 2016;22(1):111-119.

32. Bruinsma BG, Yeh H, Ozer S, et al. Subnormothermic machine perfusion for ex vivo preservation and recovery of the human liver for transplantation. *Am J Transplant.* 2014;14(6):1400-1409.

33. Fondevila C, Hessheimer AJ, Maathuis MH, et al. Superior preservation of DCD livers with continuous normothermic perfusion. *Ann Surg.* 2011;254(6):1000-1007.

34. Butler AJ, Rees MA, Wight DG, et al. Successful extracorporeal porcine liver perfusion for 72 hr. *Transplantation.* 2002;73(8):1212-1218.

35. Schon MR, Kollmar O, Wolf S, et al. Liver transplantation after organ preservation with normothermic extracorporeal perfusion. *Ann Surg.* 2001;233(1):114-123.

36. Boehnert MU, Yeung JC, Bazerbachi F, et al. Normothermic acellular ex vivo liver perfusion reduces liver and bile duct injury of pig livers retrieved after cardiac death. *Am J Transplant.* 2013;13(6):1441-1449.

37. Imber CJ, St Peter SD, Lopez de Cenarruzabeitia I, et al. Advantages of normothermic perfusion over cold storage in liver preservation. *Transplantation.* 2002;73(5):701-709.

38. op den Dries S, Karimian N, Sutton ME, et al. Ex vivo normothermic machine perfusion and viability testing of discarded human donor livers. *Am J Transplant.* 2013;13(5):1327-1335.

39. Vogel T, Brockmann JG, Quaglia A, et al. The 24-hour normothermic machine perfusion of discarded human liver grafts. *Liver Transpl.* 2017;23(2):207-220.

40. Hoyer DP, Mathe Z, Gallinat A, et al. Controlled Oxygenated Rewarming of Cold Stored Livers Prior to Transplantation: First Clinical Application of a New Concept. *Transplantation.* 2016;100(1):147-152.

41. Watson CJE, Kosmoliaptsis V, Randle LV, et al. Normothermic Perfusion in the Assessment and Preservation of Declined Livers Before Transplantation: Hyperoxia and Vasoplegia-Important Lessons From the First 12 Cases. *Transplantation.* 2017;101(5):1084-1098.

42. Morito N, Obara H, Matsuno N, et al. Regulated Oxygenation of Rewarming Machine Perfusion for Porcine Donation After Cardiac Death Liver Transplantation. *Transplantation proceedings.* 2016;48(4):1244-1246.

43. Karangwa SA, Dutkowski P, Fontes P, et al. Machine Perfusion of Donor Livers for Transplantation: A Proposal for Standardized Nomenclature and Reporting Guidelines. *Am J Transplant.* 2016;16(10):2932-2942.

44. Reddy SP, Bhattacharjya S, Maniakin N, et al. Preservation of porcine non-heart-beating donor livers by sequential cold storage and warm perfusion. *Transplantation.* 2004;77(9):1328-1332.

45. Reddy S, Greenwood J, Maniakin N, et al. Non-heart-beating donor porcine livers: the adverse effect of cooling. *Liver Transpl.* 2005;11(1):35-38.

46. Watson CJ, Kosmoliaptsis V, Randle LV, et al. Preimplant Normothermic Liver Perfusion of a Suboptimal Liver Donated After Circulatory Death. *Am J Transplant.* 2016;16(1):353-357.

47. Gilbo N, Monbaliu D. Temperature and oxygenation during organ preservation: friends or foes? *Curr Opin Organ Transplant.* 2017;22(3):290-299.

48. De Carlis R, Di Sandro S, Lauterio A, et al. Successful donation after cardiac death liver transplants with prolonged warm ischemia time using normothermic regional perfusion. *Liver Transpl.* 2017;23(2):166-173.

49. Mergental H, Perera MT, Laing RW, et al. Transplantation of Declined Liver Allografts Following Normothermic Ex-Situ Evaluation. *Am J Transplant.* 2016;16(11):3235-3245.

50. Pienaar BH, Lindell SL, Van Gulik T, Southard JH, Belzer FO. Seventy-two-hour preservation of the canine liver by machine perfusion. *Transplantation.* 1990;49(2):258-260.

51. Garcia-Valdecasas JC, Tabet J, Valero R, et al. Liver conditioning after cardiac arrest: the use of normothermic recirculation in an experimental animal model. *Transpl Int.* 1998;11(6):424-432.

52. Xu H, Berendsen T, Kim K, et al. Excorporeal normothermic machine perfusion resuscitates pig DCD livers with extended warm ischemia. *The Journal of surgical research.* 2012;173(2):e83-88.

53. Op den Dries S, Sutton ME, Karimian N, et al. Hypothermic oxygenated machine perfusion prevents arteriolonecrosis of the peribiliary plexus in pig livers donated after circulatory death. *PLoS One.* 2014;9(2):e88521.

54. Robertson FP, Bessell PR, Diaz-Nieto R, et al. High serum Aspartate transaminase levels on day 3 postliver transplantation correlates with graft and patient survival and

would be a valid surrogate for outcome in liver transplantation clinical trials. *Transpl Int.* 2016;29(3):323-330.

55. Eisenbach C, Encke J, Merle U, et al. An early increase in gamma glutamyltranspeptidase and low aspartate aminotransferase peak values are associated with superior outcomes after orthotopic liver transplantation. *Transplantation proceedings.* 2009;41(5):1727-1730.

56. Verhoeven CJ, Farid WR, de Jonge J, Metselaar HJ, Kazemier G, van der Laan LJ. Biomarkers to assess graft quality during conventional and machine preservation in liver transplantation. *J Hepatol.* 2014;61(3):672-684.

57. Brockmann J, Reddy S, Coussios C, et al. Normothermic perfusion: a new paradigm for organ preservation. *Ann Surg.* 2009;250(1):1-6.

58. Shi S, Xue F. Current Antioxidant Treatments in Organ Transplantation. *Oxid Med Cell Longev.* 2016;2016:8678510.

59. Tse HM, Milton MJ, Piganelli JD. Mechanistic analysis of the immunomodulatory effects of a catalytic antioxidant on antigen-presenting cells: implication for their use in targeting oxidation-reduction reactions in innate immunity. *Free Radic Biol Med.* 2004;36(2):233-247.

60. do Amaral AS, Pawlick RL, Rodrigues E, et al. Glutathione ethyl ester supplementation during pancreatic islet isolation improves viability and transplant outcomes in a murine marginal islet mass model. *PLoS One.* 2013;8(2):e55288.

61. Bruni A, Pepper AR, Gala-Lopez B, et al. A novel redox-active metalloporphyrin reduces reactive oxygen species and inflammatory markers but does not improve marginal mass engraftment in a murine donation after circulatory death islet transplantation model. *Islets.* 2016;8(4):e1190058.

62. Lutz J, Thurmel K, Heemann U. Anti-inflammatory treatment strategies for ischemia/reperfusion injury in transplantation. *J Inflamm (Lond).* 2010;7:27.

63. Goldaracena N, Echeverri J, Spetzler VN, et al. Anti-inflammatory signaling during ex vivo liver perfusion improves the preservation of pig liver grafts before transplantation. *Liver Transpl.* 2016;22(11):1573-1583.

64. Gala-Lopez BL, Pepper AR, Pawlick RL, et al. Antiaging Glycopeptide Protects Human Islets Against Tacrolimus-Related Injury and Facilitates Engraftment in Mice. *Diabetes.* 2016;65(2):451-462.

65. Danial NN, Korsmeyer SJ. Cell death: critical control points. *Cell.* 2004;116(2):205-219.

66. Natori S, Higuchi H, Contreras P, Gores GJ. The caspase inhibitor IDN-6556 prevents caspase activation and apoptosis in sinusoidal endothelial cells during liver preservation injury. *Liver Transpl.* 2003;9(3):278-284.

67. McCall MD, Maciver AM, Kin T, et al. Caspase inhibitor IDN6556 facilitates marginal mass islet engraftment in a porcine islet autotransplant model. *Transplantation.* 2012;94(1):30-35.

68. Baskin-Bey ES, Washburn K, Feng S, et al. Clinical Trial of the Pan-Caspase Inhibitor, IDN-6556, in Human Liver Preservation Injury. *Am J Transplant.* 2007;7(1):218-225.

69. Pepper AR, Bruni A, Pawlick R, et al. Engraftment Site and Effectiveness of the Pan-Caspase Inhibitor F573 to Improve Engraftment in Mouse and Human Islet Transplantation in Mice. *Transplantation.* 2017.

70. Nagrath D, Xu H, Tanimura Y, et al. Metabolic preconditioning of donor organs: defatting fatty livers by normothermic perfusion ex vivo. *Metab Eng.* 2009;11(4-5):274-283.

71. Jamieson RW, Zilvetti M, Roy D, et al. Hepatic steatosis and normothermic perfusion-preliminary experiments in a porcine model. *Transplantation.* 2011;92(3):289-295.

72. Nativ NI, Maguire TJ, Yarmush G, et al. Liver defatting: an alternative approach to enable steatotic liver transplantation. *Am J Transplant.* 2012;12(12):3176-3183.

73. Goldaracena N, Spetzler VN, Echeverri J, et al. Inducing Hepatitis C Virus Resistance After Pig Liver Transplantation-A Proof of Concept of Liver Graft Modification Using Warm Ex Vivo Perfusion. *Am J Transplant.* 2016.

74. Cypel M, Liu M, Rubacha M, et al. Functional repair of human donor lungs by IL-10 gene therapy. *Sci Transl Med.* 2009;1(4):4ra9.

75. Lonze BE, Holzer HT, Knabel MK, et al. In vitro and ex vivo delivery of short hairpin RNAs for control of hepatitis C viral transcript expression. *Arch Surg.* 2012;147(4):384-387.

76. Yeung JC, Wagnetz D, Cypel M, et al. Ex vivo adenoviral vector gene delivery results in decreased vector-associated inflammation pre- and post-lung transplantation in the pig. *Mol Ther.* 2012;20(6):1204-1211.

77. Gotts JE, Matthay MA. Mesenchymal stem cells and acute lung injury. *Crit Care Clin.* 2011;27(3):719-733.

78. Lee JW, Fang X, Gupta N, Serikov V, Matthay MA. Allogeneic human mesenchymal stem cells for treatment of E. coli endotoxin-induced acute lung injury in the ex vivo perfused human lung. *Proc Natl Acad Sci U S A.* 2009;106(38):16357-16362.

79. Fang X, Neyrinck AP, Matthay MA, Lee JW. Allogeneic human mesenchymal stem cells restore epithelial protein permeability in cultured human alveolar type II cells by secretion of angiopoietin-1. *J Biol Chem.* 2010;285(34):26211-26222.

80. Angelico R, Perera MT, Ravikumar R, et al. Normothermic Machine Perfusion of Deceased Donor Liver Grafts Is Associated With Improved Postreperfusion Hemodynamics. *Transplant Direct.* 2016;2(9):e97.

81. de Rougemont O, Breitenstein S, Leskosek B, et al. One hour hypothermic oxygenated perfusion (HOPE) protects nonviable liver allografts donated after cardiac death. *Ann Surg.* 2009;250(5):674-683.

82. Uchiyama M, Matsuno N, Hama K, et al. Comparison between nonpulsatile and pulsatile machine perfusion preservation in liver transplantation from non-heart-beating donors. *Transplantation proceedings.* 2001;33(1-2):936-938.

83. Iwamoto H, Matsuno N, Narumi Y, et al. Beneficial effect of machine perfusion preservation on liver transplantation from non-heart-beating donors. *Transplantation proceedings.* 2000;32(7):1645-1646.

84. Liu Q, Nassar A, Farias K, et al. Comparing Normothermic Machine Perfusion Preservation With Different Perfusates on Porcine Livers From Donors After Circulatory Death. *Am J Transplant.* 2016;16(3):794-807.

85. Sutton ME, op den Dries S, Karimian N, et al. Criteria for viability assessment of discarded human donor livers during ex vivo normothermic machine perfusion. *PLoS One.* 2014;9(11):e110642.

86. Monbaliu D, Liu Q, Libbrecht L, et al. Preserving the morphology and evaluating the quality of liver grafts by hypothermic machine perfusion: a proof-of-concept study using discarded human livers. Liver Transpl. 2012;18(12):1495-1507.

2 Chapter 2.

Determination of minimal hemoglobin level necessary for normothermic porcine *ex situ* liver perfusion

Determination of Minimal Hemoglobin Level Necessary for Normothermic Porcine Ex Situ Liver Perfusion

Mariusz Bral, MD,[1,2] Boris Gala-Lopez, MD, PhD,[1,2] Aducio Thiesen, MD, PhD,[1] Sanaz Hatami, MD,[1,2] David L. Bigam, MD,[1] Darren M. Freed, MD, PhD,[1,2] and A.M. James Shapiro, MD, PhD[1,2]

Background. In current studies of ex situ liver perfusion there exists considerable variability in perfusate composition, including the type of oxygen carrier. Herein, we aim to clarify the minimal hemoglobin level necessary during normothermic porcine ex situ liver perfusion. **Methods.** Livers procured from 35 to 45 kg domestic pigs were connected to our experimental ex situ circuit (n = 10). In the treatment group, perfusate was sequentially diluted hourly to predetermined hemoglobin levels. At the end of each hemoglobin dilution, perfusate samples were analyzed for liver transaminases, lactate dehydrogenase (LD), total bilirubin, and lactate levels. Liver oxygen consumption was measured. In the control group, livers were perfused continually for a duration of 24 hours at target hemoglobin levels of 30 and 20 g/L. **Results.** Rising liver transaminases, significantly higher lactate ($P < 0.001$), and LD levels ($P < 0.001$) were noted at lower perfusate hemoglobin levels in the treatment group. Liver oxygen utilization ($P < 0.001$) and hepatic artery oxygen delivery ($P < 0.001$) were significantly lower at lower hemoglobin levels, whereas liver vessel resistance remained relatively constant. Histology demonstrated increasing parenchymal damage at lower hemoglobin levels. In control livers, higher perfusate transaminases, higher lactate, and LD levels were noted at a perfusion hemoglobin level of 20 g/L. **Conclusions.** Ex situ liver function decompensated during perfusion between a mean hemoglobin level of 30 to 20 g/L, as evidenced by notably rising lactate and LD levels. This study demonstrates optimal hemoglobin concentration during normothermic ex situ liver perfusion to ensure a fully metabolically functioning graft.

(*Transplantation* 2018;102: 1284–1292)

Ex situ liver perfusion has been repeatedly suggested as a way to resuscitate marginal and extended criteria liver grafts, thereby improving the quality and possibly the quantity of transplanted livers. With the worldwide shortage of organs available for transplantation, and the increased use of marginal and extended donor organs, the current approach of cold static preservation has declared its limitations.[1,2] As a result, in recent years, interest in achieving optimization in the field of ex situ organ perfusion has increased significantly. Among the different modalities, normothermic ex situ liver perfusion shows the most theoretical promise to facilitate dynamic assessment of organ viability before transplantation, with a suitable oxygen carrier to meet the metabolic demands of the liver.[3,4] Recent publication of a large multicenter randomized trial of ex situ normothermic liver perfusion reported significant reductions in graft injury, even after accounting for a significantly reduced rate of organ discard as well a longer mean preservation time as compared to static cold storage (SCS).[5] This landmark publication strongly demonstrates the utility of this technology in future clinical practice, as well as the prescient interest and need for further investigations to optimize its translational potential.

There currently exists remarkable variability in ex situ circuit design, ex situ perfusate composition, as well as surrogate viability measures used to evaluate liver function. Liver perfusion circuits differ predominantly in whether there are 1 or 2 perfusion pumps, whether perfusion is pulsatile or continuous, substrate additives, and the temperature of the circulating volume.[3,6-10] The perfusates within the circuits differ in the composition of the priming base, as well as the concentration and type of the oxygen carrier.[11-13]

In both experimental and clinical normothermic ex situ liver perfusion, most groups use an erythrocyte-based oxygen carrier; however, it has not been demonstrated what minimal

Received 11 November 2017. Revision received 1 May 2018.

Accepted 3 May 2018.

[1] *Department of Surgery, University of Alberta, Edmonton, Canada.*

[2] *Members of the Canadian National Transplant Research Project (CNTRP).*

The authors declare no conflicts of interest to disclose.

M.B. is the recipient of the American Society of Transplant Surgeons (ASTS) 2015 Scientist Scholarship. A.M.J.S. holds a Canada Research Chair in Transplantation Surgery and Regenerative Medicine, and a Senior Clinical Scholarship from Alberta Innovates Healthcare Solutions. Funding from the University Hospital Foundation at the University of Alberta is gratefully acknowledged. Funding from Astellas Pharma Canada is gratefully acknowledged. Funding from CNTRP (Project1) is gratefully acknowledged.

M.B. and B.G. performed the experimental study, and contributed to writing the manuscript. A.T. performed histological analysis. S.H. assisted in performing the study. D.B., D.F. and A.M.J.S. were responsible for experimental design and writing as well as editing the manuscript.

Correspondence: A.M. James Shapiro, MD, PhD, Clinical Islet Transplant Program, University of Alberta. 2000 College Plaza, 8215-112th St, Edmonton, Alberta, Canada T6G 2C8. (amjs@islet.ca).

ISSN: 0041-1337/18/10208-1284

DOI: 10.1097/TP.0000000000002272

Originally published after peer review in *Transplantation* 2018; 102(8) 1284-92.

Original Paper

Title: Determination of Minimal Hemoglobin Level Necessary for Normothermic Porcine *Ex situ* Liver Perfusion

Authors: Mariusz Bral, MD[1, 2], Boris Gala-Lopez MD PhD [1, 2], Aducio Thiesen MD Phd[1], Sanaz Hatami MD[1, 2], David L. Bigam MD[1], Darren M. Freed MD PhD [1, 2], and A.M. James Shapiro MD PhD[1,2]

Affiliations:

[1] Department of Surgery, University of Alberta, Edmonton, Canada

[2] Members of the Canadian National Transplant Research Project (CNTRP)

Corresponding Author:

A.M. James Shapiro, MD, PhD, Canada Research Chair in Transplantation Surgery and Regenerative Medicine, Professor, Director of Clinical Islet and Living Donor Liver Transplant Programs, Clinical Islet Transplant Program, University of Alberta. 2000 College Plaza, 8215-112th St, Edmonton T6G 2C8, Alberta, Canada. amjs@islet.ca

Telephone: +1-780-4077330, Fax: +1-780-4078259

2.1 Abstract

Background: In current studies of *ex situ* liver perfusion there exists considerable variability in perfusate composition, including the type of oxygen carrier. Herein we aim to clarify the minimal hemoglobin level necessary during normothermic porcine *ex situ* liver perfusion.

Methods: Livers procured from 35- 45 Kg domestic pigs were connected to our experimental *ex situ* circuit (n=10). In the treatment group, perfusate was sequentially diluted hourly to predetermined hemoglobin levels. At the end of each hemoglobin dilution, perfusate samples were analyzed for liver transaminases, lactate dehydrogenase, total bilirubin, and lactate levels. Liver oxygen consumption was measured. In the control group, livers were perfused continually for a duration of 24 hours at target hemoglobin levels of 30 and 20 g/L.

Results: Rising liver transaminases, significantly higher lactate ($p<0.001$) and lactate dehydrogenase levels ($p<0.001$) were noted at lower perfusate hemoglobin levels in the treatment group. Liver oxygen utilization ($p<0.001$) and hepatic artery oxygen delivery ($p< 0.001$) were significantly lower at lower hemoglobin levels, whereas liver vessel resistance remained relatively constant. Histology demonstrated increasing parenchymal damage at lower hemoglobin levels. In control livers, higher perfusate transaminases, higher lactate and lactate dehydrogenase levels were noted at a perfusion hemoglobin level of 20 g/L.

Conclusions: Ex situ liver function decompensated during perfusion between a mean hemoglobin level of 30-20 g/L, as evidenced by notably rising lactate and lactate dehydrogenase levels. This study demonstrates optimal hemoglobin concentration

during normothermic *ex situ* liver perfusion to ensure a fully metabolically functioning graft.

2.2 Introduction

Ex situ liver perfusion has been repeatedly suggested as a way to resuscitate marginal and extended criteria liver grafts, thereby improving the quality and possibly the quantity of transplanted livers. With the worldwide shortage of organs available for transplantation, and the increased use of marginal and extended donor organs, the current approach of cold static preservation has declared its limitations [1,2]. As a result, in recent years interest in achieving optimization in the field of *ex situ* organ perfusion has increased significantly. Among the different modalities, normothermic *ex situ* liver perfusion shows the most theoretical promise to facilitate dynamic assessment of organ viability prior to transplantation, with a suitable oxygen carrier to meet the metabolic demands of the liver[3,4]. Recent publication of a large multi-center randomized trial of *ex situ* normothermic liver perfusion reported significant reductions in graft injury, even after accounting for a significantly reduced rate of organ discard as well a longer mean preservation time as compared to static cold storage[5]. This landmark publication strongly demonstrates the utility of this technology in future clinical practice, as well as the prescient interest and need for further investigations to optimize its translational potential.

There currently exists remarkable variability in *ex situ* circuit design, *ex situ* perfusate composition, as well as surrogate viability measures used to evaluate liver function.

Liver perfusion circuits differ predominantly in whether there are one or two perfusion pumps, whether perfusion is pulsatile or continuous, substrate additives, and the temperature of the circulating volume [3,6-10]. The perfusates within the circuits differ in the composition of the priming base, as well as the concentration and type of the oxygen carrier [11-13].

In both experimental and clinical normothermic *ex situ* liver perfusion, most groups use an erythrocyte-based oxygen carrier, however it has not been demonstrated what minimal hemoglobin level is sufficient to meet the needs of a fully functioning liver graft. Perfusate hemoglobin concentrations are seldom reported, and are often well below species appropriate normal levels (**Table 1**).

More recently, novel acellular oxygen carriers have been investigated as alternatives to blood based carriers, with promising results. These products offer a number of theoretical advantages, including eliminating blood born infection risk, lower immunogenic reactivity, simplified logistics and a longer shelf life, the ultimate advantages of which remain to be determined [14-16]. Nevertheless, most groups currently use erythrocytes in normothermic machine perfusion (NMP) and we therefore sought to investigate what level of hemoglobin would be optimal to maintain graft viability.

The hemoglobin levels chosen in the experimental perfusions were on the lower spectrum of what would likely be physiologically feasible to sustain a liver over the duration of NMP. Blood products are a scarce resource, and the clinical implication of perfusions performed with lower hemoglobin levels, in the absence of alternatives, simplifies logistics and improves resource utilization.

Table 2-1. Selected normothermic ex situ liver perfusion studies.

Study	Year	Study Type/Device	Species	Perfusion Temp.	Perfusate Composition	Hemoglobin Concentration
Nasralla et al.[5]	2018	Clinical Transplant (OrganOx *metra*®)	Human	37 °C	1 L STEEN or Gelofusine + 3 units PRBC	Not reported
Watson et al.[4]	2017	Clinical Transplant (Liver Assist)	Human	37 °C	1 L STEEN or Gelofusine + 3 units PRBC	Median 6.1 (5.1 – 7.4) g/dL
Angelico et al.[30]	2016	Clinical Transplant (OrganOx *metra*®)	Human	37 °C	1 L 'colloid' + 3- units PRBC	Not reported
Mergental *et al*. [3]	2016	Clinical Transplant (OrganOx *metra*® and Liver Assist)	Human	37 °C	1000 mL 5% human albumin + 3 units PRBC	Not reported
Bral *et al.* [12]	2016	Clinical Transplant (OrganOx *metra*®)	Human	37 °C	500 mL Gelofusine + 3 units PRBC	104 ± 18 (g/L)
Selzner *et al*. [11]	2016	Clinical Transplant (OrganOx *metra*®)	Human	37 °C	500 mL STEEN + 3 units PRBC	Not reported
Ravikumar *et al*. [6]	2016	Clinical Transplant (OrganOx *metra*®)	Human	37 °C	500 mL Gelofusine + 3 units PRBC	Not reported
Watson *et al*. [31]	2016	Clinical Transplant (Liver Assist)	Human	37 °C	'Erythrocyte-based fluid'	Not reported
Perera *et al*. [29]	2016	Clinical Transplant (Liver Assist)	Human	37 °C	'Third-party red cell based fluid'	Not reported
Vogel *et al*. [32]	2017	Experimental Perfusion	Porcine	37 °C	1.5 L of pig blood	Not reported
Vogel *et al*. [33]	2016	Experimental Perfusion	Human	37 °C	500 mL Sterofundin + 3- 4 units PRBC	Not reported
Banan *et al*. [34]	2015	Experimental Perfusion	Porcine	38 °C	1.5 L saline + autologous blood (volume not reported)	Hematocrit 15 to 20
Nassar *et al*. [20]	2014	Experimental Perfusion	Porcine	38 °C	2.5 L heterologous blood + acellular solution	Not reported

					combinations ± PRBC	
Op den Dries *et al*. [26]	2013	Experimental Perfusion	Human	37 °C	750 mL 'Red blood cell concentrate' + 900 mL FFP + 100 mL human albumin	4.7 ± 0.1 (mmol/L)
Boehnert *et al*. [21]	2013	Experimental Transplant	Porcine	38 °C	3 L STEEN solution	Acellular
Xu *et al*. [35]	2011	Experimental Perfusion	Porcine	39 °C	1.5 L autologous blood + 0.5 L sterile porcine plasma	Not reported
Fondevila *et al*. [36]	2011	Experimental Transplant	Porcine	38 °C	Autologous blood (volume not reported)	Not reported
Brockmann *et al*. [18]	2009	Experimental Transplant	Porcine	38 °C	1.5 L autologous blood	Not reported
Butler *et al*. [17]	2002	Experimental Perfusion	Porcine	39 °C	1.5 L of heterologous blood	Not reported
Schoen *et al*. [19]	2001	Experimental Transplant	Porcine	38 °C	2 L whole blood + 1 L 'balanced electrolyte' solution	Not reported

2.3 Methods

2.3.1 Study design

The Institutional Animal Care Committee at the University of Alberta approved the experimental protocol. A total of 10 Landrance pigs were used for this experiment.

2.3.2 Donor liver procurement

All pigs were pre-medicated with Atropine (0.05 mg/Kg) (Rafter 8, Calgary, Canada) and Ketamine (20 mg/Kg) (Bimeda, Cambridge, Canada), following which orotracheal intubation was established, and general anesthesia was maintained with 2% isofluorane (Fresenius Kabi Canada Ltd, Richmond Hill, Canada). A midline laparotomy was performed, and livers were retrieved using a standard technique [17]. All livers were dissected until they were only connected by vascular elements. A median sternotomy was performed, and a two stage venous cannula was inserted into the right atrium. Intravenous heparin (Fresenius Kabi Canada Ltd, Richmond Hill, Canada), was administered (30, 000 units), and the infra-renal aorta was cannulated with a 20 French cannula. The animals were then exsanguinated via the cannula in the right atrium, and the blood was collected to prime the perfusion circuit. Aortic cross-clamp was established. The suprahepatic vena cava was divided near to the heart for venous venting, and the abdominal organs were then flushed with 2 liters of cold (4°C) Histidine-Tryptophan-Ketoglutarate (Custodiol HTK, Methapharm Inc., Brantford, ON, Canada) solution.

2.3.3 Ex situ perfusion circuit design

The locally-designed experimental *ex situ* perfusion circuit was assembled using the following components: a Medtronic Affinity NT oxygenator, two BPX-80 Bi-Medicus centrifugal pumps

(Medtronic, Minneapolis, MN), and a leukocyte arterial blood filter (LeukoGuard LG, PALL Medical, Port Washington, NY) **(Figure 1).** The centrifugal pumps were computer controlled to maintain the desired hepatic artery and portal vein pressures. Oxygen and carbon dioxide flows were titrated through the membrane oxygenator to maintain a partial pressure of arterial oxygen between 130 and 200 mmHg, and a partial pressure of carbon dioxide of 35 to 45 mmHg.

A sufficient quantity of whole blood was added to Krebs-Henseleit with albumin solution (glucose, sodium chloride, potassium chloride, calcium chloride, magnesium chloride, sodium bicarbonate, sodium phosphate monobasic, 8% bovine serum albumin) to achieve an *ex situ* circuit perfusate with a target mean hemoglobin level of 50 g/L. The circuit was primed with bolus additives including cefuroxime 750 mg (SteriMax Inc., Oakville, Canada), methylprednisolone sodium succinate 500 mg (Pfizer Canada Inc., Kingston, Canada), and sodium heparin 10,000 U (Pharmaceutical Partners Canada, Richmond Hill, Canada). Sodium bicarbonate 8.4% (Hospira, Montreal, Canada) was added as needed to maintain pH between 7.35 – 7.45. Continuous infusions were established of 2 IU/hour of regular insulin (Eli Lilly Canada Inc., Toronto, Canada), and 1,000 units/hour of sodium heparin.

2.3.4 Ex situ liver perfusion

Following procurement, livers were flushed with 1 L of 0.9% normal saline. The livers were then connected and perfused on our *ex situ* liver perfusion circuit, at a temperature of 39°C. Livers were perfused through both the hepatic artery and portal vein. Both of the cannulated vessels were under automated computer pressure control, with the hepatic artery perfused at a set-point of 70 mmHg and the portal vein perfused at a set-point of 2 mmHg. All perfusions were

initially commenced at a target mean hemoglobin level of 50 g/L, and allowed to proceed for 2 hours.

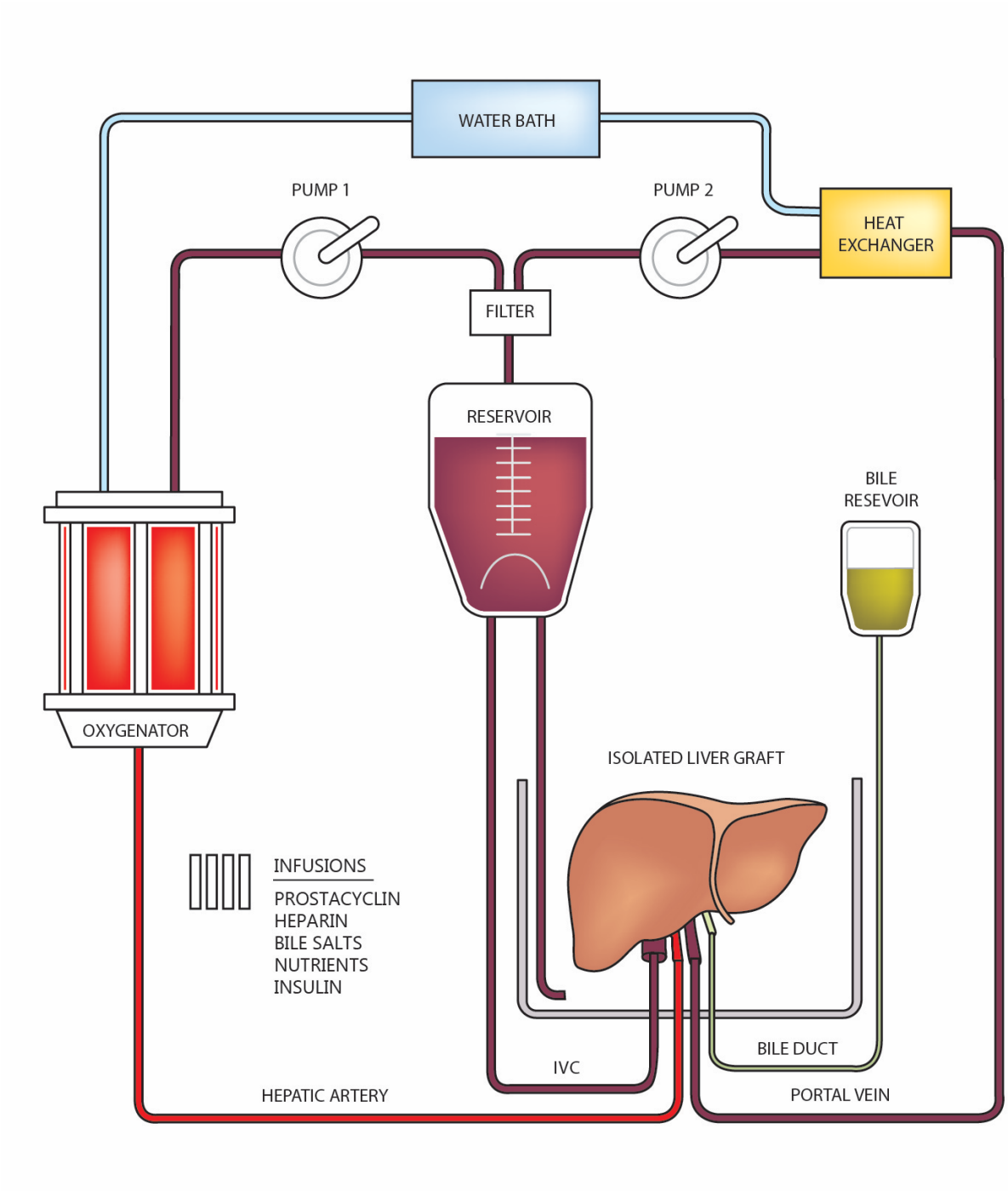


Figure 2-1. Schematic diagram of the *ex situ* circuit.

We then performed hourly serial dilutions of the perfusate with additional Krebs-Henseleit with albumin solution, to systematically perfuse the livers at decreasing levels of hemoglobin (Dilution 1, mean hemoglobin 30 g/L; dilution 2, mean hemoglobin 20 g/L; dilution 3, mean hemoglobin 10 g/L; dilution 4, mean hemoglobin 7.5 g/L; dilution 5, mean hemoglobin level 5 g/L). Each dilution interval was allowed to proceed for 1 hour, at which point perfusate samples were withdrawn, centrifuged, and supernatant was stored at minus -80°C until biochemical analysis was performed. Blood gasses were also analyzed on-site. At the end of each perfusion run (mean target hemoglobin level 5 g/L) tissue samples were taken for histological analysis. One perfused liver was used exclusively for further histological analysis, with samples taken at the end of each dilution point.

In the control group, perfusions were established as above, and were performed for durations of 24 hours, at target hemoglobin levels of 30 g/L and 20 g/L. Perfusate samples were collected, centrifuged, and supernatant was stored at minus -80°C until biochemical analysis was performed. Blood gasses were also analyzed on-site **(Figures 2A, 2B)**.

2.3.5 Perfusate composition analysis

Hemoglobin, electrolyte, pH, total bilirubin, lactate and partial pressures of oxygen and carbon dioxide were measured using the ABL Flex Analyzer (Radiometer Medical ApS, Bronshoj, Denmark). Perfusate samples were obtained from the hepatic artery circuit at the end of each dilution perfusion and sent for the analysis of aspartate transaminase (AST), alanine aminotransferase (ALT), total bilirubin, lactate dehydrogenase, and lactate using a BeckmanCoulter Unicel Dxc800 Syncron (Brea, California, USA).

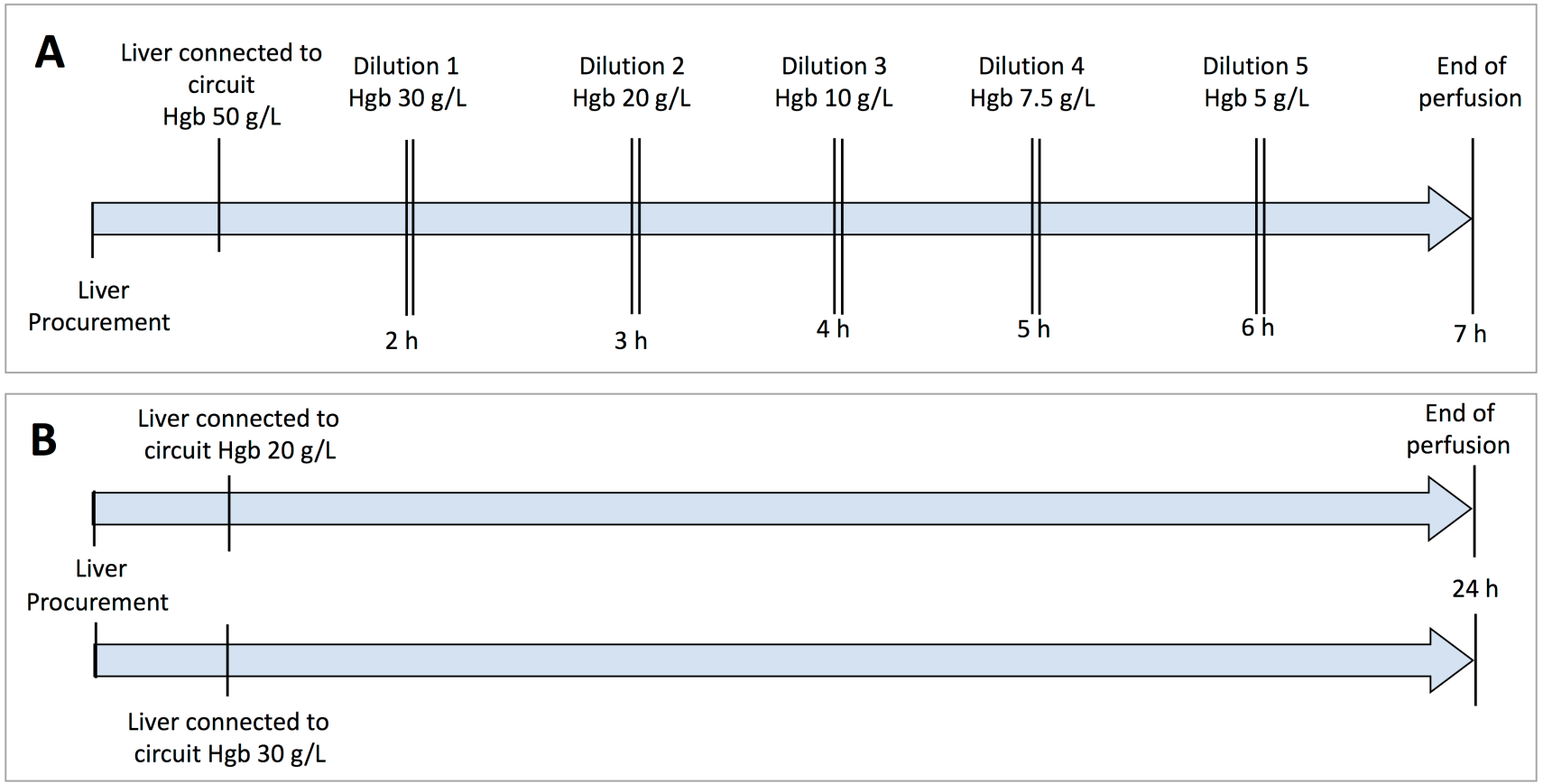


Figure 2-2. Flow diagrams of the experimental design.

2.3.6 Hepatic oxygen consumption and vascular resistance

Liver graft oxygen consumption was calculated using the Fick equation, based on arterial and venous blood gases and compared between different hemoglobin levels [17]. Hepatic artery and portal vein vascular resistance was also compared between each successive hemoglobin level. Vessel resistances were calculated by dividing the pressure by the flow indexed to 100 g of liver tissue.

2.3.7 Histology

Livers were fixed in 10% formalin. Liver tissue samples were analyzed at the end of each hemoglobin dilution, embedded in paraffin, stained with hematoxylin and eosin, and examined in a blinded fashion by an expert pathologist who assigned a semi-quantitative score to evaluate for hepatocyte injury and bile sequestration. Biopsy tissue was examined for necrosis (0- absent, 1- pericentral, 2- Zone 2 and 3, 3- panlobular); hemorrhage (score 0- absent, 1- focal, 2- zonal, 3- panlobular), cholestasis (score 0- absent, 1- present); and sinusoidal dilatation (score 0- none, 1- mild, 2- moderate, 3- severe), as previously reported [18].

2.3.8 Statistical Analysis

Data are represented as means ± standard error of the means (SEM). Normally distributed continuous variables were compared using a one-way ANOVA with Tukey's multiple comparisons. Overall comparison between hemoglobin groups was performed with a 95% confidence interval. A p-value of <0.05 was considered statistically significant and all the analysis was performed using Graphpad Prism (GraphPad Software Inc., La Jolla, CA, USA).

2.4 Results

Livers were subjected to comparable periods of cold ischemia before *ex situ* liver perfusion was initiated (34 min ± 3 min). *Ex situ* liver perfusions were established as described, with a mean initial hemoglobin of 50 ± 1.9 g/L. After two hours of perfusion, the serial perfusate dilutions were commenced (Dilution 1, mean hemoglobin 31 ± 1.2 g/L; dilution 2, mean hemoglobin 21 ± 1.0 g/L; dilution 3, mean hemoglobin 11 ± 0.6 g/L; dilution 4, mean hemoglobin 7 ± 0.5 g/L; dilution 5, mean hemoglobin level 5 ± 0.5 g/L).

The perfusate levels of liver transaminases, lactate dehydrogenase, and lactate were evaluated and compared at each hemoglobin level. Hepatic transaminases (AST and ALT) rose progressively with each hemoglobin dilution, higher at level of 5 ± 0.46 g/L, as compared to 50 ± 1.9 g/L, although this was not significant ($p=0.09$, $p=0.06$ respectively) **(Figure 3A, 3B).** With increasing serial hemoglobin dilution, higher levels of lactate were observed within the perfusate, first noticed at a hemoglobin level of 21 ± 1.0 g/L (0.4 ± 0.19 mM). This accumulation first becomes significant at a hemoglobin level of 11 ± 0.6 g/L (2.36 ± 0.87 mM) and continues throughout lower hemoglobin levels **(Figure 3C).** In parallel, levels of lactate dehydrogenase also increased sequentially ($p<0.001$), more noticeably at 21 ± 1.0 g/L (756 ± 294 U/L) of hemoglobin, reaching significance in hemoglobin dilutions less than 11 ± 0.6 g/L (1185 ± 486 U/L) **(Figure 3D).**

Vascular parameters also demonstrated changes over different hemoglobin levels. Hepatic oxygen consumption decreased sequentially throughout hemoglobin dilutions, with significance ($p<0.0001$) observed at hemoglobin levels diluted below 50 ± 1.9 g/L (3.14 ± 0.4

mL O_2/min/100 g) **(Figure 4A).** As expected, hepatic oxygen delivery was also significantly reduced as the concentration of hemoglobin decreased ($p<0.0001$)**,** with significance noted at levels below 50 ± 1.9 g/L (9.52 ± 1.49 mL O_2/min/100 g) **(Figure 4B).** Hepatic artery resistance remained relatively stable over the dilutions ($p=0.33$) **(Figure 4C**). Portal vein resistance did rise when hemoglobin reached 5 ± 0.46 g/L (1.25 ± 0.13 mmHg*min/L/gram), although this was not significant ($p=0.15$) **(Figure 4D).**

Histological analysis revealed that the development of hepatocyte injury was more clearly evident with each successive hemoglobin dilution. Livers perfused at increasingly lower hemoglobin levels demonstrated increased evidence of sinusoidal dilatation, and eventual necrosis **(Figures 5, 6).**

In the control group, levels of liver transaminases, lactate and lactate dehydrogenase were compared over the duration of the perfusions. Hepatic transaminases increased over time, higher in livers perfused at a hemoglobin level of 20 **(Figure 7A, 7B).** Higher levels of lactate were observed over time in the perfusate with a hemoglobin level of 20 g/L **(Figure 7C).** Levels of lactate dehydrogenase also increased progressively, more so at hemoglobin level of 20 g/L **(Figure 7D).**

In the control group, histological analysis again confirmed that hepatocyte injury was more evident in livers perfused at the lower hemoglobin level of 20 g/L as compared to 30 g/L, with increasing sinusoidal dilatation at the end of the 24 hour perfusion period **(Figures 8A, 8B).**

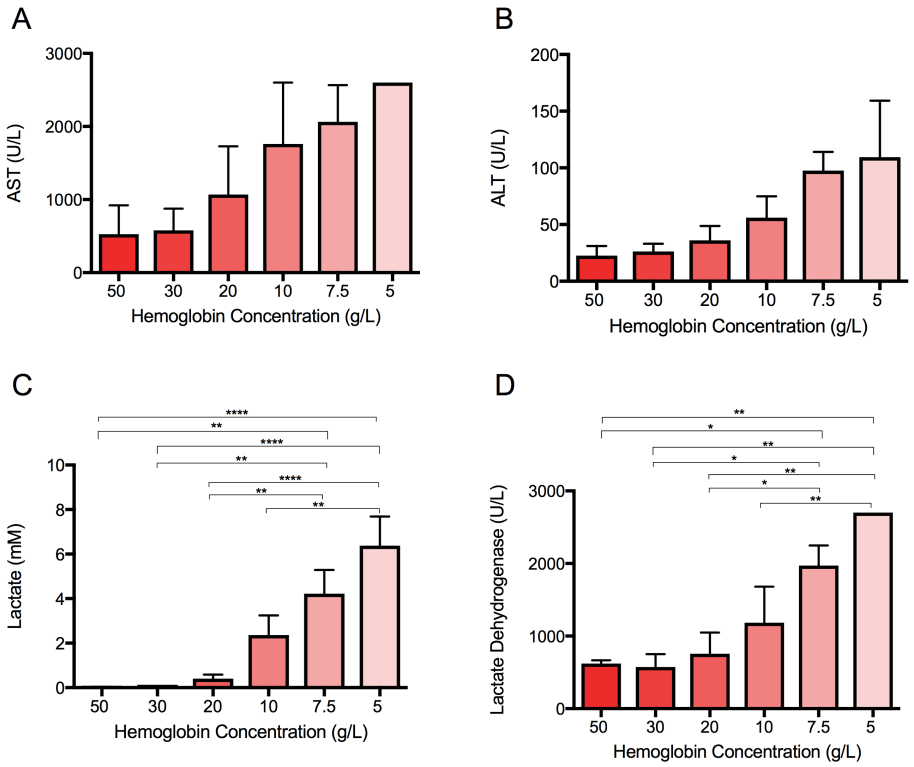


Figure 2-3. *Ex situ* circulating perfusate liver biochemistry after successive perfusate dilutions during NMP

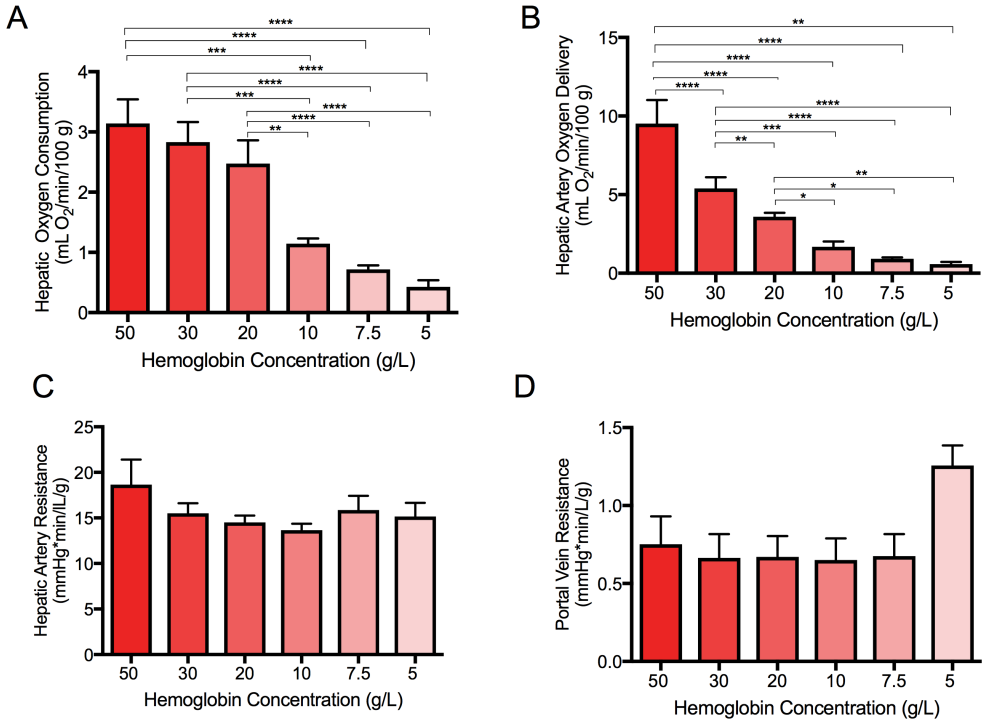


Figure 2-4. Normothermic *ex situ* liver vascular parameters after successive perfusate dilutions during NMP.

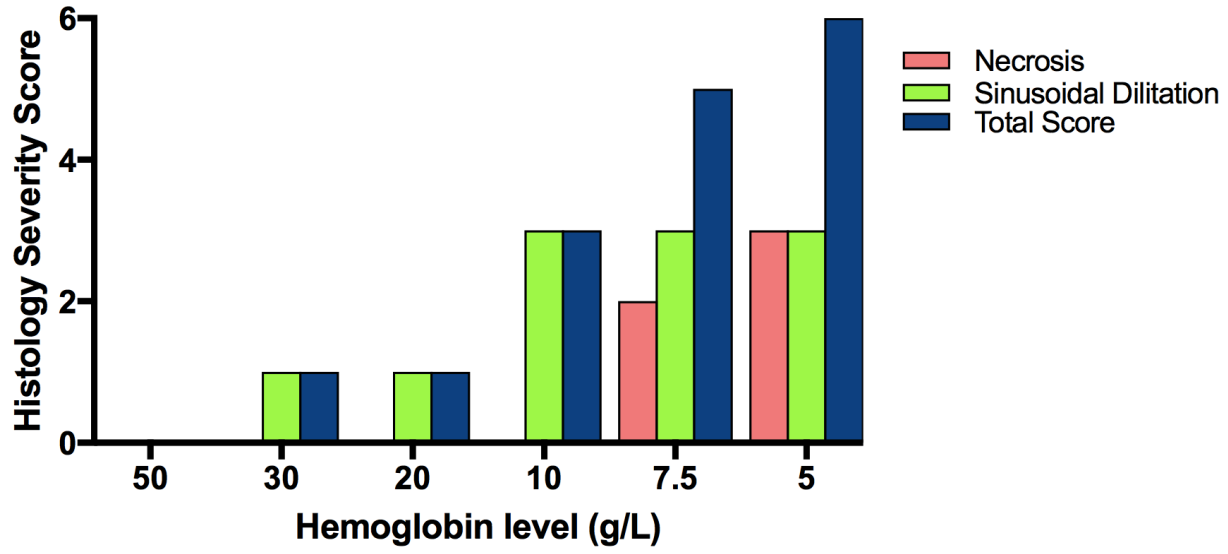


Figure 2-5. Histologic scoring after each successive hemoglogin dilution.

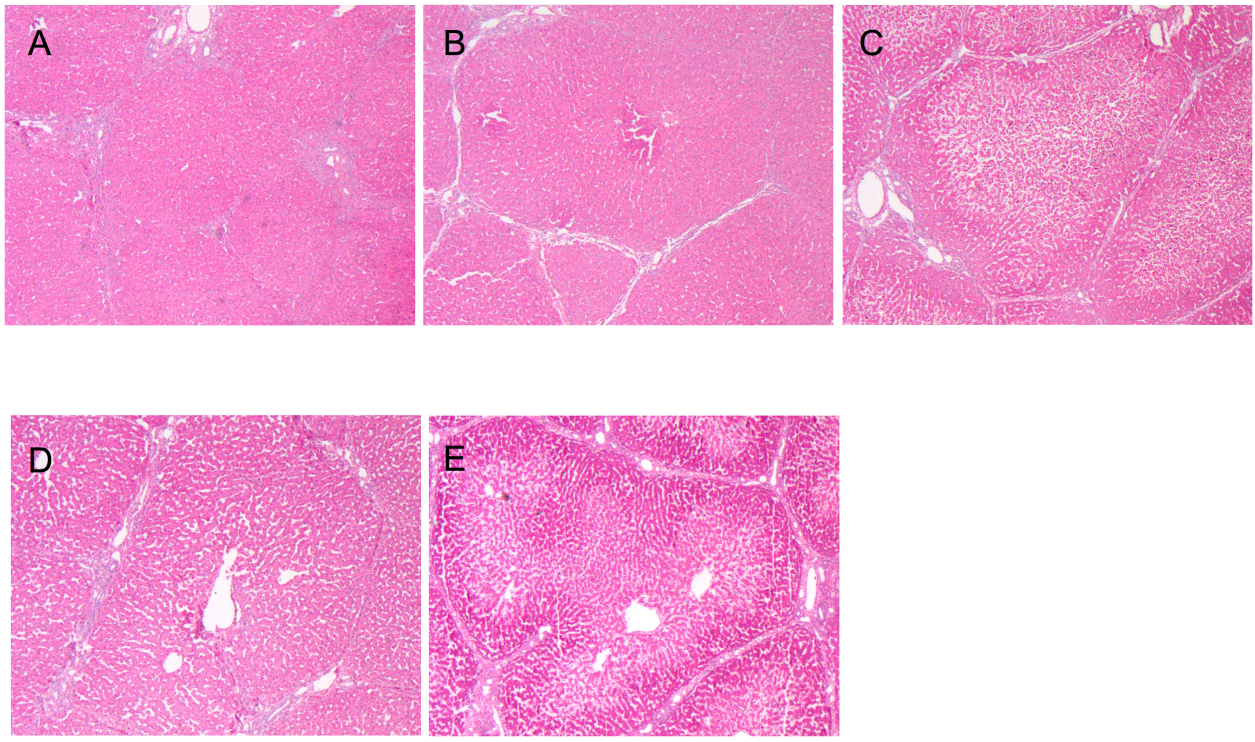


Figure 2-6. Representative histologic sections of liver parenchyma, stained with hematoxylin and eosin, taken after each hemoglobin dilution.

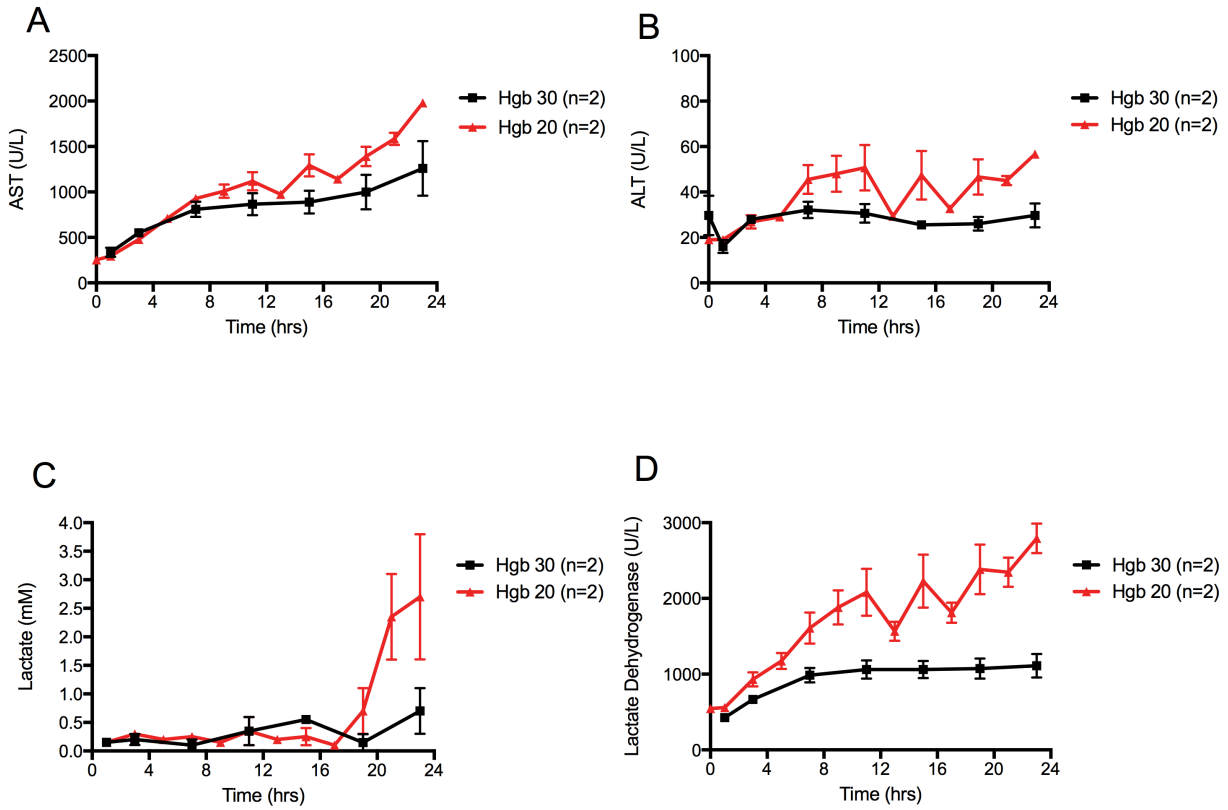


Figure 2-7. *Ex situ* perfusate biochemistry over 24 hours of NMP.

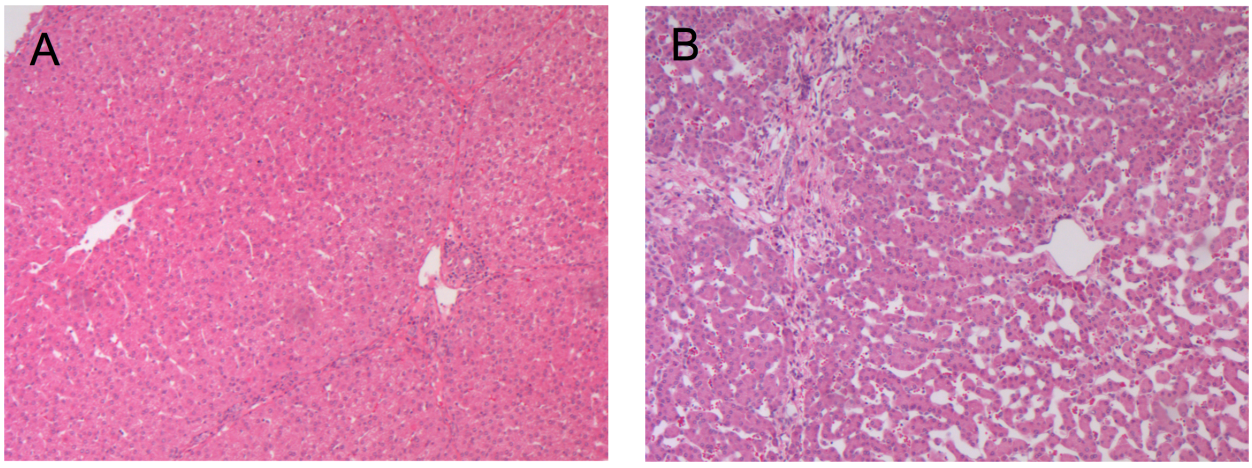


Figure 2-8. Representative histologic sections of liver parenchyma, stained with hematoxylin and eosin, taken from control livers at the end of 24 hours of perfusion.

2.5 Discussion

We demonstrate herein the effect of different perfusate hemoglobin concentrations in an experimental porcine normothermic *ex situ* liver perfusion model. Sequential hemoglobin dilution resulted in progressively higher circulating perfusate levels of markers of liver cellular injury, escalating pathological injury, and decreasing graft metabolic function.

Normothermic *ex situ* liver perfusion provides the potential to resuscitate marginal donor livers that might otherwise be discarded from the transplant process, and as such has potential to increase the donor pool. However, evaluation of such livers is imperative prior to transplantation, in order to minimize the possibility of primary non-function. The assessment of a liver on an *ex situ* circuit should occur at or near normothermic conditions, and the circuit perfusate requires an oxygen carrier in order to meet the metabolic demands of the liver.

In the experimental setting, most normothermic *ex situ* studies have used whole blood, or whole blood with colloid solution [17-20]. Recent improved development of alternative hemoglobin-based oxygen carriers has shown safety in NMP, with potential advantages over blood product oxygen carriers[16]. One isolated experimental porcine study successfully perfused livers under normothermic conditions without an oxygen carrier, demonstrating a protective benefit over static cold storage [21]. Investigators from Cleveland compared STEEN Solution™ alone or with erythrocytes, versus whole blood. In the whole blood and erythrocyte groups, levels of transaminases were lower, with livers demonstrating better bile production and more favourable histology[22]. These findings have also been noted in other organs[23].

All NMP clinical transplant studies have perfused livers with packed red blood cells (PRBC) and either STEEN Solution™ or Gelofusine [6,11,12]. To date, no study has demonstrated what level of hemoglobin is adequate to maintain the metabolic needs of a working liver under normothermic *ex situ* conditions **(Table 1)**.

In most *ex situ* perfusion studies, higher release of liver transaminases during perfusion is deemed to be an indicator of increased hepatocellular injury. Guarrera *et al*. determined perfusate levels of AST and ALT to strongly correlate with post transplant liver transaminase levels [24]. Monbaliu *et al*. used AST to determine transplantable grafts during hypothermic machine perfusion [25]. In another study, under normothermic conditions, liver transaminases during perfusion were deemed to be predictive of graft recipient survival in a porcine model [18]. In the treatment group, AST and ALT levels rose with each successive hemoglobin dilution, although this did not reach statistical significance. This may have been a result of accumulation of the liver enzymes in a fixed volume of perfusate over time, however, was also offset by the sequential dilution of the perfusate with fresh colloid. The absolute increase in transaminase levels was not determined, and the resultant rise over time may be in fact an underestimation of the real values. Further, liver transaminases are naturally metabolized by the liver, and rising perfusate transaminase levels may be a result of a metabolically failing graft. The higher transaminase increase in livers perfused at a hemoglobin level of 20 g/L in the control group may be a result of this as well. The significance of liver transaminases in closed circuit remains to be elucidated.

To date a number of groups have proposed that perfusate lactate is one of the more relevant surrogate markers of graft viability on an *ex situ* circuit[4,26,27]. In the treatment perfusions, lactate levels sequentially elevated with each successive hemoglobin dilution. Under normothermic *ex*

situ conditions, on-circuit lactate clearance is considered an indicator of good graft function [3]. Rising perfusate lactate levels in control livers perfused at a lower hemoglobin level may result from the combination of increased anerobic metabolism and inferior lactate clearance, both of which may have resulted from progressing metabolic failure.

Lactate dehydrogenase (LD) is a well defined marker of cellular injury, and has been reported previously by a number of groups in *ex situ* perfusion studies [25,28]. Both in the treatment and control groups, livers perfused at lower hemoglobin levels demonstrated higher levels of LD, likely a result of increasing cellular damage. The source of rising perfusate LD is not known, and may have resulted form either increasing liver cellular injury, or alternatively hemolysis. Although hemolysis is a well known complication of machine perfusion, the higher levels of LD in the control livers perfused at a 20 g/L support the assumption that this was of liver origin.

With progressive hemoglobin dilution there was significantly lower liver oxygen consumption at lower hemoglobin levels which paralleled oxygen delivery. Published data on oxygen consumption during *ex situ* perfusion demonstrates conflicting findings, with most studies reporting higher oxygen consumption as associated with better outcomes [21]. Utilizing NMP, Boehnert *et al.* noted that DCD grafts that were preserved using SCS for longer periods of time demonstrated a rapid drop in oxygen consumption, as compared to livers preserved by NMP, indicative of deteriorating metabolic activity [21].

Vascular resistance has previously been described as a possible marker for graft viability [18]. We found that hepatic artery resistance did not change significantly at different hemoglobin levels. In our study, portal vein resistance did not increase significantly until the lowest hemoglobin level, possibly indicating that this is a late indicator of a failing liver on NMP.

Based on these findings, it seems that a hemoglobin concentration of 31 ± 1.2 g/L is sufficient to preserve liver metabolism, whereas at a hemoglobin level of 21 ± 1.0 g/L, the oxygen supply to the organ barely meets the demand, with a resultant rise in lactate levels. We surmise that perfusions performed at a hemoglobin level higher than 31 ± 1.2 g/L is not detrimental to liver metabolism.

The development of liver parenchymal injury with each successive hemoglobin dilution, as evidenced by histology, may possibly be explained by the generation of reactive oxygen species leading to endothelial cell damage and increased microvascular permeability. As the perfusate was progressively more dilute, the buffering and antioxidant capacity of whole blood was likely increasingly overwhelmed. Liver damage is notably advanced on histology at hemoglobin levels below 20 g/L, suggesting that this threshold is too low to perform NMP safely. Using our circuit and perfusate, we sought to demonstrate the extreme physiological parameters that a liver will tolerate. In a large mammal experimental model, where multiple organs are often procured at the same time, this may provide a parameter for more optimal distribution of blood product between circuits.

There are a number of important limitations to our study. The serial dilution of perfusate within the experimental group makes it difficult to know with certainty whether observed effects are truly due to the hemoglobin dilution or due to the state of the liver at the start of the dilution, or the damaged state of the liver in general. The rationale behind the methodology was that in NMP, if a liver is functioning well, observed changes in perfusate biochemistry, such as decreasing lactate, occur in short periods of time[3,4,29].

Each hemoglobin level dilution was allowed to proceed for only one hour, and had this period been extended, some observations may have become more pronounced, or alternatively, the livers may have shown signs of failure at higher hemoglobin levels. The effect of the physiologic solution in contributing to progressive graft damage is unknown. Further only machine perfusion was performed, without a transplant recovery model, which likely would have strengthened the observations. Our conclusions are restricted to this porcine large animal model and the type of *ex situ* normothermic perfusion circuit and additives chosen. Altering any of these conditions could potentially alter the translatability of the findings.

Despite recent progress in developing alternative oxygen carriers, to date all clinical NMP transplant studies have been performed with human packed red blood cells as the oxygen carrier. Blood products continue to be a universally scarce resource, and the implications of performing MP at lower hemoglobin levels may result in more rational resource utilization, simplified logistics and cost savings.

Based on this study, we conclude that under normothermic *ex situ* perfusion conditions, a hemoglobin concentration of between 30-20 g/L should be maintained in order to assure optimal graft function. A value higher than this is not harmful and may be beneficial. Our experimental findings here suggest that this target may provide a reasonable reserve, and that the total *ex situ* NMP hemoglobin concentration does not need to be in the normal physiological range. Such a metabolically intact liver is necessary if any potential viability measures are to give some indication of post-transplant function.

2.6 References

1. Abt PL, Desai NM, Crawford MD, et al. Survival following liver transplantation from non-heart-beating donors. *Ann Surg.* 2004;239(1):87-92.
2. Brokelman W, Stel AL, Ploeg RJ. Risk factors for primary dysfunction after liver transplantation in the University of Wisconsin solution era. *Transplant Proc.* 1999;31(5):2087-2090.
3. Mergental H, Perera MT, Laing RW, et al. Transplantation of Declined Liver Allografts Following Normothermic Ex-Situ Evaluation. *Am J Transplant.* 2016;16(11):3235-3245.
4. Watson CJE, Kosmoliaptsis V, Randle LV, et al. Normothermic Perfusion in the Assessment and Preservation of Declined Livers Before Transplantation: Hyperoxia and Vasoplegia-Important Lessons From the First 12 Cases. *Transplantation.* 2017;101(5):1084-1098.
5. Nasralla D, Coussios CC, Mergental H, et al. A randomized trial of normothermic preservation in liver transplantation. *Nature.* 2018.
6. Ravikumar R, Jassem W, Mergental H, et al. Liver transplantation after ex vivo normothermic machine preservation: a Phase 1 (first-in-man) clinical trial. *Am J Transplant.* 2016.
7. Hoyer DP, Mathe Z, Gallinat A, et al. Controlled Oxygenated Rewarming of Cold Stored Livers Prior to Transplantation: First Clinical Application of a New Concept. *Transplantation.* 2016;100(1):147-152.
8. Echeverri J, Goldaracena N, Kaths JM, et al. Comparison of BQ123, Epoprostenol, and Verapamil as Vasodilators During Normothermic Ex Vivo Liver Machine Perfusion. *Transplantation.* 2018;102(4):601-608.

9. Rojas-Pena A, Bartlett RH. Ex Situ Organ Preservation: The Temperature Paradigm. *Transplantation.* 2018;102(4):554-556.

10. Nassar A, Quintini C. Vasodilation During Normothermic Machine Perfusion; Preventing the No-Reflow Phenomena. *Transplantation.* 2018;102(4):548-549.

11. Selzner M, Goldaracena N, Echeverri J, et al. Normothermic ex vivo liver perfusion using steen solution as perfusate for human liver transplantation: First North American results. *Liver Transpl.* 2016;22(11):1501-1508.

12. Bral M, Gala-Lopez B, Bigam D, et al. Preliminary Single-Center Canadian Experience of Human Normothermic Ex Vivo Liver Perfusion: Results of a Clinical Trial. *Am J Transplant.* 2016.

13. Fontes P, Lopez R, van der Plaats A, et al. Liver preservation with machine perfusion and a newly developed cell-free oxygen carrier solution under subnormothermic conditions. *Am J Transplant.* 2015;15(2):381-394.

14. Laing RW, Bhogal RH, Wallace L, et al. The Use of an Acellular Oxygen Carrier in a Human Liver Model of Normothermic Machine Perfusion. *Transplantation.* 2017;101(11):2746-2756.

15. Fontes PA. The Evolution of Oxygen Carrier Solutions for Machine Perfusion. *Transplantation.* 2017;101(11):2657-2658.

16. Matton APM, Burlage LC, van Rijn R, et al. Normothermic Machine Perfusion of Donor Livers Without the Need for Human Blood Products. *Liver Transpl.* 2017.

17. Butler AJ, Rees MA, Wight DG, et al. Successful extracorporeal porcine liver perfusion for 72 hr. *Transplantation.* 2002;73(8):1212-1218.

18. Brockmann J, Reddy S, Coussios C, et al. Normothermic perfusion: a new paradigm for organ preservation. *Ann Surg.* 2009;250(1):1-6.

19. Schon MR, Kollmar O, Wolf S, et al. Liver transplantation after organ preservation with normothermic extracorporeal perfusion. *Ann Surg.* 2001;233(1):114-123.

20. Nassar A, Liu Q, Farias K, et al. Ex vivo normothermic machine perfusion is safe, simple, and reliable: results from a large animal model. *Surg Innov.* 2015;22(1):61-69.

21. Boehnert MU, Yeung JC, Bazerbachi F, et al. Normothermic acellular ex vivo liver perfusion reduces liver and bile duct injury of pig livers retrieved after cardiac death. *Am J Transplant.* 2013;13(6):1441-1449.

22. Liu Q, Nassar A, Farias K, et al. Comparing Normothermic Machine Perfusion Preservation With Different Perfusates on Porcine Livers From Donors After Circulatory Death. *Am J Transplant.* 2016;16(3):794-807.

23. White CW, Hasanally D, Mundt P, et al. A whole blood-based perfusate provides superior preservation of myocardial function during ex vivo heart perfusion. *J Heart Lung Transplant.* 2015;34(1):113-121.

24. Guarrera JV, Henry SD, Samstein B, et al. Hypothermic machine preservation in human liver transplantation: the first clinical series. *Am J Transplant.* 2010;10(2):372-381.

25. Monbaliu D, Liu Q, Libbrecht L, et al. Preserving the morphology and evaluating the quality of liver grafts by hypothermic machine perfusion: a proof-of-concept study using discarded human livers. *Liver Transpl.* 2012;18(12):1495-1507.

26. op den Dries S, Karimian N, Sutton ME, et al. Ex vivo normothermic machine perfusion and viability testing of discarded human donor livers. *Am J Transplant.* 2013;13(5):1327-1335.

27. Watson CJ, Kosmoliaptsis V, Randle LV, et al. Preimplant Normothermic Liver Perfusion of a Suboptimal Liver Donated After Circulatory Death. *Am J Transplant.* 2016;16(1):353-357.

28. Liu Q, Nassar A, Farias K, et al. Sanguineous normothermic machine perfusion improves hemodynamics and biliary epithelial regeneration in donation after cardiac death porcine livers. *Liver Transpl.* 2014;20(8):987-999.

29. Perera T, Mergental H, Stephenson B, et al. First human liver transplantation using a marginal allograft resuscitated by normothermic machine perfusion. *Liver Transpl.* 2016;22(1):120-124.

30. Angelico R, Perera MT, Ravikumar R, et al. Normothermic Machine Perfusion of Deceased Donor Liver Grafts Is Associated With Improved Postreperfusion Hemodynamics. *Transplant Direct.* 2016;2(9):e97.

31. Watson CJ, Randle LV, Kosmoliaptsis V, Gibbs P, Allison M, Butler AJ. 26-hour Storage of a Declined Liver Before Successful Transplantation Using Ex Vivo Normothermic Perfusion. *Ann Surg.* 2016.

32. Vogel T, Brockmann JG, Pigott D, et al. Successful transplantation of porcine liver grafts following 48-hour normothermic preservation. *PLoS One.* 2017;12(11):e0188494.

33. Vogel T, Brockmann JG, Quaglia A, et al. 24-Hour Normothermic Machine Perfusion of Discarded Human Liver Grafts. *Liver Transpl.* 2016.

34. Banan B, Chung H, Xiao Z, et al. Normothermic extracorporeal liver perfusion for donation after cardiac death (DCD) livers. *Surgery.* 2015;158(6):1642-1650.

35. Xu H, Berendsen T, Kim K, et al. Excorporeal normothermic machine perfusion resuscitates pig DCD livers with extended warm ischemia. *J Surg Res.* 2012;173(2):e83-88.

36. Fondevila C, Hessheimer AJ, Maathuis MH, et al. Superior preservation of DCD livers with continuous normothermic perfusion. *Ann Surg.* 2011;254(6):1000-1007.

3 Chapter 3

Clearance of transaminases during normothermic *ex situ* liver perfusion

PLOS ONE

RESEARCH ARTICLE

Clearance of transaminases during normothermic *ex situ* liver perfusion

Mariusz Bral[1], Nader Aboelnazar[1☯], Sanaz Hatami[1☯], Aducio Thiesen[2], David L. Bigam[1], Darren H. Freed[1], A. M. James Shapiro[1]*

1 Department of Surgery, University of Alberta, Edmonton, Canada, **2** Department of Pathology, University of Alberta, Edmonton, Canada

☯ These authors contributed equally to this work.
* amjs@islet.ca

Citation: Bral M, Aboelnazar N, Hatami S, Thiesen A, Bigam DL, Freed DH, et al. (2019) Clearance of transaminases during normothermic *ex situ* liver perfusion. PLoS ONE 14(4): e0215619. https://doi.org/10.1371/journal.pone.0215619

Editor: Akshay Chauhan, University of Colorado, Anschutz Medical Campus, UNITED STATES

Received: February 4, 2019

Accepted: April 4, 2019

Published: April 24, 2019

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: MB is the recipient of the American Society of Transplant Surgeons (ASTS) 2015 Scientist Scholarship. AMJS holds a Tier-1 Canada Research Chair in Transplantation Surgery and Regenerative Medicine, and a Senior Clinical Scholarship from Alberta Innovates Healthcare Solutions. Funding from the Canadian Donation and Transplant Research Project (CDTRP) is gratefully acknowledged.

Abstract

Background

One of the most promising applications of liver normothermic machine perfusion (NMP) is the potential to directly assess graft viability and injury. In most NMP studies, perfusate transaminases are utilized as markers of graft injury. Our aim was to further elucidate the metabolism of transaminases by healthy porcine livers during NMP, specifically whether such livers could clear circuit perfusate transaminases.

Methods

A highly concentrated transaminase solution was prepared from homogenized liver, with an aspartate aminotransferase (AST) level of 107,427 U/L. Three livers in the treatment group were compared to three controls, during 48 hours of NMP. In the treatment group, the circuit perfusate was injected with the transaminase solution to artificially raise the AST level to a target of 7,500 U/L. Perfusate samples were taken at two-hour intervals and analyzed for biochemistry until NMP end. Graft oxygen consumption and vascular parameters were monitored.

Results

Compared to controls, treated perfusions demonstrated abrupt elevations in transaminase levels ($p>0.0001$) and lactate dehydrogenase (LDH) ($p>0.0001$), which decreased over time, but never to control baseline. Liver function, as demonstrated by lactate clearance and oxygen consumption was not different between groups. The treatment group demonstrated a higher portal vein resistance ($p = 0.0003$), however hepatic artery resistance was similar. Treated livers had higher bile production overall ($p<0.0001$).

Conclusions

Addition of high levels of transaminases and LDH to a healthy porcine liver during ex situ perfusion results in progressive clearance of these enzymes, suggesting preserved liver

Originally published after peer review in *PL0S One* 2019;14 (4)

Original Paper

Title: Clearance of transaminases during normothermic *ex situ* liver perfusion

Authors:

Mariusz Bral MD[1,3], Nader Aboelnazar MD[1&], Sanaz Hatami MD[1,3&], Aducio Thiesen MD PhD[2], David L. Bigam MD[1], Darren H. Freed[1,3], and A.M. James Shapiro MD PhD[1,3*]

Affiliations:

[1] Department of Surgery, University of Alberta, Edmonton, Canada

[2] Department of Pathology, University of Alberta, Edmonton, Canada

[3] Members of the Canadian Donation and Transplant Research Program (CDTRP)

*__Corresponding Author:__

A.M. James Shapiro, MD, PhD, Canada Research Chair in Transplantation Surgery and Regenerative Medicine, Professor, Director of Clinical Islet and Living Donor Liver Transplant Programs, Clinical Islet Transplant Program, University of Alberta. 2000 College Plaza, 8215-112th St, Edmonton T6G 2C8, Alberta, Canada. amjs@islet.ca

Telephone: +1-780-4077330, Fax: +1-780-4078259

[&]These authors also contributed equally to this work

3.1 Abstract

Background: One of the most promising applications of liver normothermic machine perfusion (NMP) is the potential to directly assess graft viability and injury. In most NMP studies, perfusate transaminases are utilized as markers of graft injury. Our aim was to further elucidate the metabolism of transaminases by healthy porcine livers during NMP, specifically whether such livers could clear circuit perfusate transaminases.

Methods: A highly concentrated transaminase solution was prepared from homogenized liver, with an aspartate aminotransferase (AST) level of 107,427 U/L. Three livers in the treatment group were compared to three controls, during 48 hours of NMP. In the treatment group, the circuit perfusate was injected with the transaminase solution to artificially raise the AST level to a target of 7,500 U/L. Perfusate samples were taken at two-hour intervals and analyzed for biochemistry until NMP end. Graft oxygen consumption and vascular parameters were monitored.

Results: Compared to controls, treated perfusions demonstrated abrupt elevations in transaminase levels ($p<0.0001$) and lactate dehydrogenase (LDH) ($p<0.0001$), which decreased over time, but never to control baseline. Liver function, as demonstrated by lactate clearance and oxygen consumption was not different between groups. The treatment group demonstrated a higher portal vein resistance ($p=0.0003$), however hepatic artery resistance was similar. Treated livers had higher bile production overall ($p<0.0001$).

Conclusions: Addition of high levels of transaminases and LDH to a healthy porcine liver during ex situ perfusion results in progressive clearance of these enzymes, suggesting preserved liver metabolism. Such tolerance tests may provide valuable indicators of prospective graft function.

3.2 Introduction

Due to a worldwide shortage of organs available for transplantation, there is increasing pressure to consider more marginal and 'extended criteria' liver grafts, in hopes of expanding the donor pool. It is well recognized that such grafts are not optimally preserved with current static cold storage (SCS), resulting in increased instances of both short and long term post-transplant complications. It is increasingly important that such grafts undergo some form of functional assessment before transplant to minimize any adverse outcomes.

Normothermic machine perfusion (NMP) has shown potential in resuscitating marginal liver grafts, and can improve the quality and quantity of transplanted livers [1]. Further, one the most promising applications of NMP is the possibility of dynamic, 'real-time' graft viability and injury assessment which optimally occurs under normothermic conditions to assess the functional capacity of an organ with physiologic metabolism. Many surrogate markers of graft viability and injury have been utilized, however none have been validated in the clinical setting. The ideal biomarker would be specific, easily processed, inexpensive, with a quick 'turn around' time. Almost ubiquitously across all clinical and experimental liver *ex situ* studies, perfusate transaminases are used as markers to inform of hepatocellular injury **(Table 1)**. Indeed, perfusate transaminases have been shown to correlate with post-transplant graft transaminase levels, and graft and recipient survival [2,3]. As such, transaminases are often used in combination with graft lactate clearance and bile production during NMP to determine the eligibility of a particular graft for implantation.

Table 3-1. Selected normothermic ex situ liver perfusion studies utilizing transaminases for 'on circuit' graft injury assessment.

Study	Year	Study Type/Device	Species	Sample Size (group)	Perfusate Assessment	Outcome Assessment	NMP Transaminase Correlation to Post-transplant Transaminase
Watson *et al.* [4]	2018	Clinical Transplant (Liver Assist)	Human	47	In graph form.	41/46 livers had ALT <6000 I/U at 2 hours of perfusion. One liver with ALT 9490 I/U resulted in PNF.	Yes. ALT after 2 hours of perfusion with peak ALT (days 1- 7) post-transplant (R=0.73, p= 0.0001)
Nasralla *et al.* [1]	2018	Clinical Transplant (OrganOx *metra*®)	Human	220	Not reported.	Peak AST 488.1 (U/L) NMP vs 964.9 (U/L) SCS (days 1-7).	Not reported
Watson *et al*. [5]	2017	Clinical Transplant (Liver Assist)	Human	12	In graph form.	In graph form.	Yes. ALT after 2 hours of perfusion with peak ALT (days 1- 7) post-transplant (R=0.56, p= 0.005)
Mergental *et al*. [6]	2016	Clinical Transplant (OrganOx *metra*® and Liver Assist)	Human	5	Not reported.	In graph form.	
Bral *et al.* [7]	2016	Clinical Transplant (OrganOx *metra*®)	Human	9	DCD livers much higher AST when compared to DBD ($p < 0.001$).	No difference in peak AST (day 1-7) levels between NMP and SCS grafts (p= 0.24).	Not Reported
Selzner *et al*. [8]	2016	Clinical Transplant (OrganOx *metra*®)	Human	10	Peak AST 1647 (U/L), peak ALT 444 (U/L).	NMP livers had lower peak AST and ALT (day 1-3) compared to SCS (not significant).	Not reported

Ravikumar *et al*. [9]	2016	Clinical Transplant (OrganOx *metra*®)	Human	20	Not reported.	Peak AST 417 (U/L) NMP vs 902 (U/L) SCS (days 1-7) (p= 0.34).	Not reported
Watson *et al*. [10]	2016	Clinical Transplant (Liver Assist)	Human	1	In graph form.	Peak ALT (days 1-7) 1198 (IU/L).	Not reported
Perera *et al*. [11]	2016	Clinical Transplant (Liver Assist)	Human	1	Not reported.	Peak ALT (days 1-7) 1215 (IU/L).	Not reported
Vogel *et al*. [12]	2017	Experimental Perfusion	Porcine	4	End perfusion (48 hrs) ALT 52.5 +/- 38.1 U/l	Day 5 ALT was 31.0 +/- 1.4 U/l.	Not reported
Vogel *et al*. [13]	2016	Experimental Perfusion	Human	13	In graph form.	Not transplanted.	Not applicable
Banan *et al*. [14]	2015	Experimental Perfusion	Porcine	12	NMP preserved DCD grafts had lower AST and ALT compared to SCS (p<0.01).	Not transplanted.	Not applicable
Nassar *et al*. [15]	2014	Experimental Perfusion	Porcine	20	NMP preserved livers had lower AST (p= 0.002) and ALT (p= 0.009) compared to SCS (p<0.01).	Not transplanted.	Not applicable
Op den Dries *et al*. [16]	2013	Experimental Perfusion	Human	4	In graph form. ALT stable throughout perfusion.	Not transplanted.	Not applicable
Boehnert *et al*. [17]	2013	Experimental Transplant	Porcine	6	NMP preserved grafts had six- fold lower ALT compared to SCS (p <0.001).	NMP preserved DCD grafts had lower mean AST compared to SCS.	Not reported
Xu *et al*. [18]	2011	Experimental Perfusion	Porcine	6	Livers with 60 min of warm ischemia had higher perfusate ALT levels (p< 0.05).	Not transplanted.	Not applicable

Fondevila *et al*. [19]	2011	Experimental Transplant	Porcine	6	NECMO perfused livers had lower AST 94 (38-148) compared to NMP livers 213 (119-413) U/L.	In graph form.	Not reported
Brockmann *et al*. [3]	2009	Experimental Transplant	Porcine	5	Significantly lower AST and ALT in livers preserved with NMP vs SCS (DCD model).	In graph form.	Yes. Successful livers AST 964+/-302 and ALT 62 +/- 10 vs AST 3198 +/-677, ALT 223+/-75 at 4 hours from NMP start
Reddy *et al*.[20]	2004	Experimental Perfusion	Porcine	5	Circuit transaminases lower in immediate NMP group compared to NMP + 4 hrs SCS	Not transplanted.	Not applicable.
Butler *et al*. [21]	2002	Experimental Perfusion	Porcine	5	ALT level 51.4 (U/L) after 72 hours of NMP.	Not transplanted.	Not applicable.
Schoen *et al*. [22]	2001	Experimental Transplant	Porcine	6	Not reported.	AST levels lower in livers preserved with NMP.	Not reported.

Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Donation after brain death (DBD), Donation after cardiac death (DCD), Normothermic machine perfusion (NMP), Normothermic extra-corporeal membrane oxygenation (ECMO), Primary non-function (PNF), Static cold storage (SCS).

Herein, we aim to further elucidate the metabolism of transaminases by healthy porcine livers during NMP, specifically whether healthy livers could clear transaminases from a NMP perfusate. Such information would potentially further shed light on whether transaminases could be used as a functional test to assess liver viability.

3.3 Methods

3.3.1 Study design overview

The Institutional Animal Care Committee at the University of Alberta approved the experimental protocol. A total of 7 Landrance pigs were used for these experiments. Three livers were allocated to the treatment group, and perfused on our locally designed *ex situ* circuit under normothermic conditions for 48 hours, and compared to three control livers perfused for the same duration. In the treatment group, after stable perfusion was established for 5 hours, the circuit perfusate was injected with a prepared high concentration transaminase solution to artificially elevate the circuit AST to a projected target level of 7,500 U/L **(Fig 1)**. Throughout all perfusions, perfusate samples were drawn at two-hour intervals, and frozen at -80 °C until biochemical analysis could be performed. Samples were analyzed for AST, alanine aminotransferase (ALT), LDH, and lactate. Blood gas analysis was also performed at two-hour intervals, and graft oxygen consumption and vessel resistance was monitored.

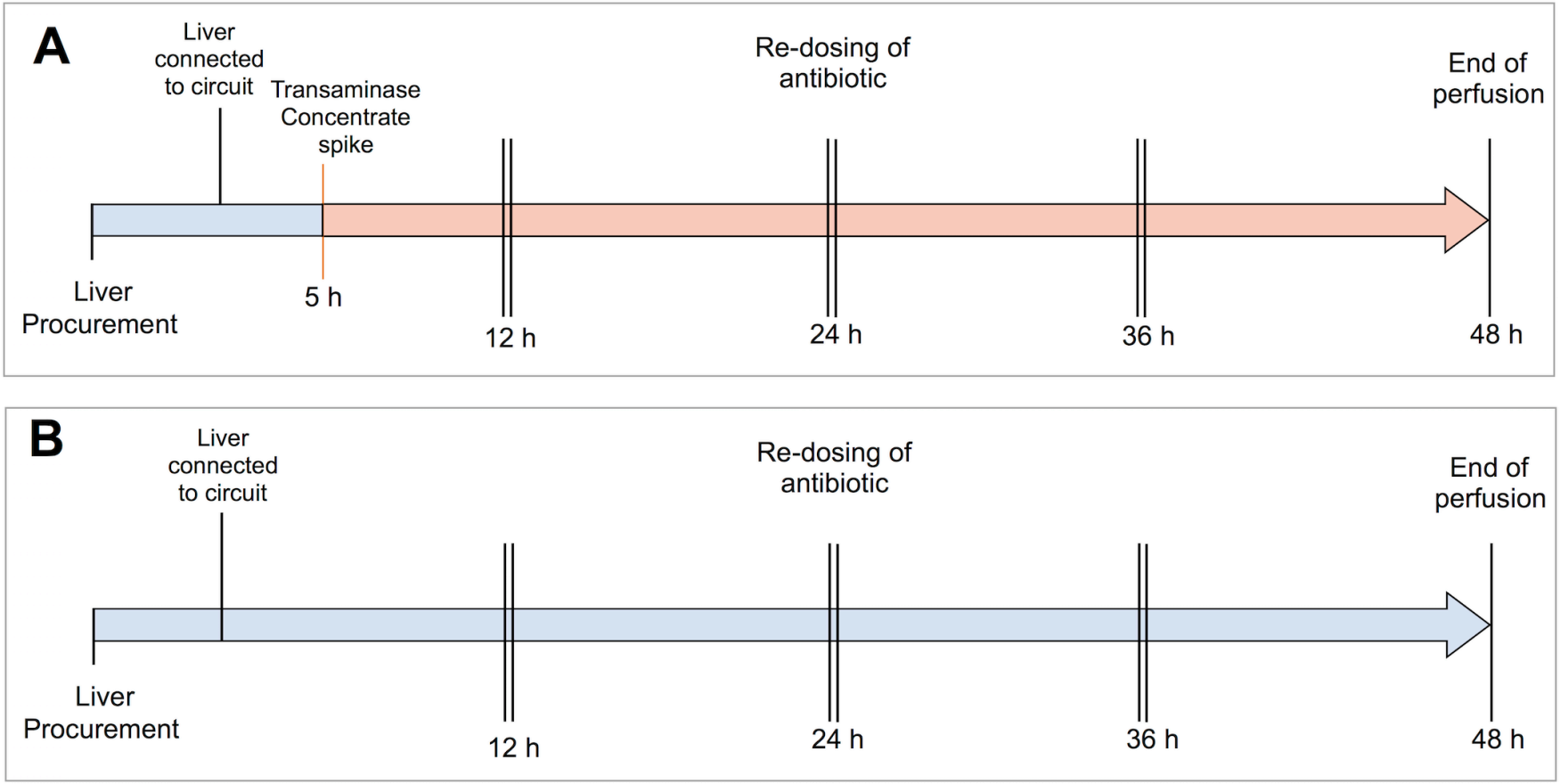


Figure 3-1. Flow diagrams of the experimental design.

(A) Schematic diagram of the ex situ liver perfusion treatment group, perfused for 48 hours. (B) Schematic diagram of control livers perfused for 48 hours

3.3.2 Donor liver procurement

Livers were procured from 35 - 45 Kg domestic pigs and connected to our experimental *ex situ* circuit. Donor pigs were premedicated with Atropine (0.05 mg/Kg) (Rafter 8, Calgary, Canada) and Ketamine (20 mg/Kg) (Bimeda, Cambridge, Canada), orotracheal intubation was performed, and general anesthesia was sustained with 2% isofluorane (Fresenius Kabi Canada Ltd, Richmond Hill, Canada). Through a midline laparotomy livers were retrieved as previously described [21,23]. All livers were dissected and vascular elements appropriately isolated. Through a median sternotomy, a two stage venous cannula was inserted into the right atrium. Intravenous heparin (30,000 (Fresenius Kabi Canada Ltd, Richmond Hill, Canada), was administered, and the infra-renal aorta was cannulated with a 20 French cannula in preparation for cold flush. Exsanguination of the animals was then performed using the right atrial cannula, and the collected blood was used to prime the perfusion circuit. The aorta was cross-clamped, the suprahepatic vena cava was divided in the chest for venous venting, and the abdominal viscera were flushed with 2 liters of cold (4°C) Histidine-Tryptophan-Ketoglutarate (Custodiol HTK, Methapharm Inc., Brantford, ON, Canada).

3.3.3 Preparation of liver transaminase concentrate

A modified isolation protocol was used to prepare a highly concentrated transaminase solution. A single porcine liver (917 g) was mechanically homogenized, following which the liquefied tissue was frozen at -80 °C. This tissue homogenate was then thawed, and aliquoted into 30 mL samples and then subjected to a further 2 minutes of homogenization using a PowerGen125 (Thermo Fisher Scientific, NY). Samples were kept on ice at all times. The liquefied liver samples were then sonicated using a Virtis

Virsonic 100 Ultrasonic Cell Disrupter (VirTis, NY), two times, for a duration of 30 seconds each. Samples were then centrifuged at 16, 000 rpm for 15 min (Sorvall™ Legend™ XTR, Thermo Fisher Scientific, MA). The supernatant was collected, and refrozen in 50 mL aliquots at -80 °C. A sample of this was processed for AST levels. The sample was then refrozen at -80 °C, thawed again, and resent to the lab to ensure enzyme stability. The reported AST concentration was reported as 107,427 U/L. This concentrated transaminase supernatant was used as an additive in the treatment group to raise the AST level to a target of 7,500 U/L.

3.3.4 Ex situ perfusion circuit design

The locally-designed experimental *ex situ* perfusion circuit was assembled using the following components: a Medtronic Affinity NT oxygenator, two BPX-80 Bi-Medicus centrifugal pumps (Medtronic, Minneapolis, MN), and a leukocyte arterial blood filter (LeukoGuard LG, PALL Medical, Port Washington, NY) **(Fig 2)**. The centrifugal pumps were computer controlled to maintain the desired hepatic artery and portal vein pressures. Oxygen and carbon dioxide flows were titrated through the membrane oxygenator to maintain a partial pressure of arterial oxygen between 130 and 200 mmHg, and a partial pressure of carbon dioxide of 35 to 45 mmHg.

A volume of 1000 mL of whole blood was added to 750 mL of Krebs-Henseleit with albumin solution (Glucose, sodium chloride, potassium chloride, calcium chloride, magnesium chloride, sodium bicarbonate, sodium phosphate monobasic, 8 % bovine serum albumin). The circuit was primed with bolus additives including calcium

gluconate 10 mls 10% solution (Fresenius Kabi, Richmond Hill, Canada), cefuroxime 750mg (SteriMax Inc., Oakville, Canada), methylprednisolone sodium succinate 500 mg

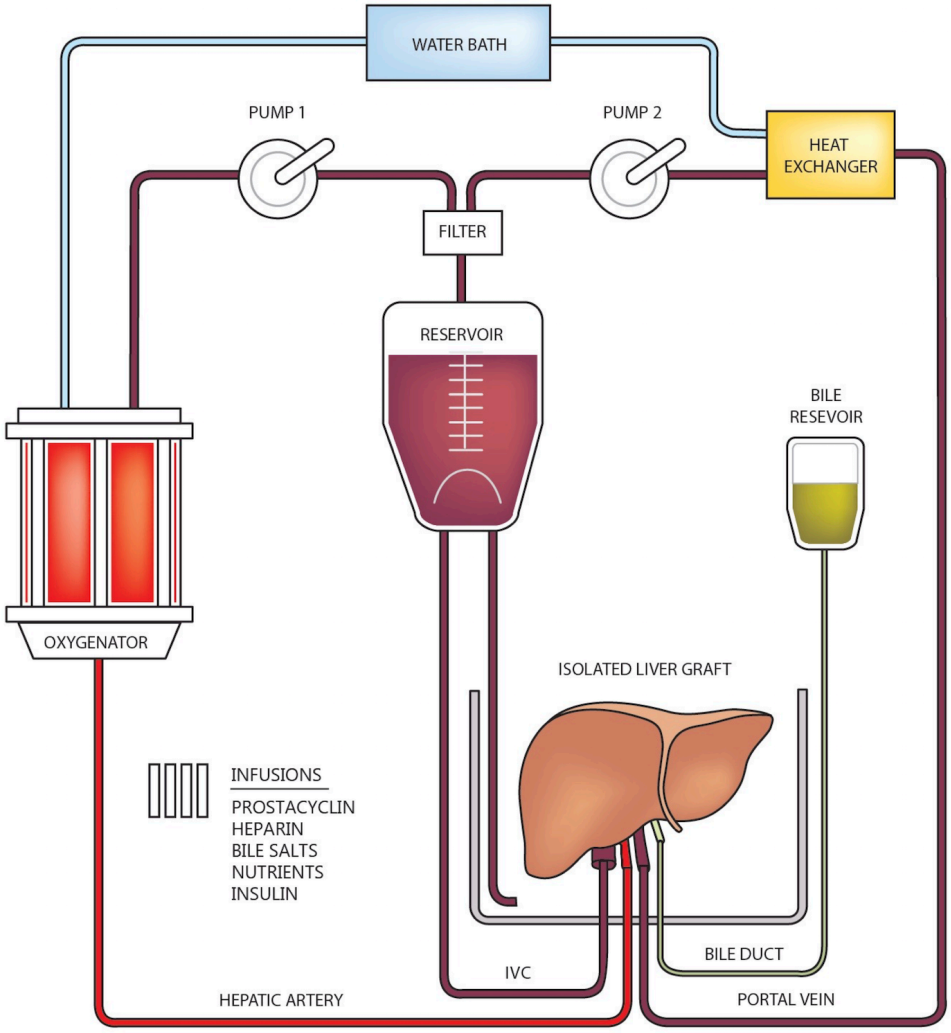


Figure 3-2. Schematic diagram of the experimental ex situ circuit.

In this study prostacyclin, bile salts and nutrients were omitted from the infusions. Reprinted from 'Mariusz Bral, Boris Gala-Lopez, Aducio Thiesen, Sanaz Hatami, David L. Bigam, Darren M. Freed, AM James Shapiro, Determination of Minimal Hemoglobin Level Necessary for Normothermic Porcine Ex Situ Liver Perfusion, *Transplantation.* 2018;102(8):1284-1292, with permission from Transplantation.

(Pfizer Canada Inc., Kingston, Canada), and sodium heparin 10,000 U (Pharmaceutical Partners Canada, Richmond Hill, Canada). Sodium bicarbonate (8.4%) was added (Hospira, Montreal, Canada) as needed to maintain pH between 7.35 – 7.45. Cefuroxime 750mg (SteriMax Inc., Oakville, Canada) and was re-bolused at 24 hours of perfusion. Continuous infusions were established of 2 IU/hour of regular insulin (Eli Lilly Canada Inc., Toronto, Canada), and 1,000 units/hour of sodium heparin.

3.3.5 Ex situ liver perfusion

Following procurement, livers were flushed with 1 L of 0.9% normal saline. Livers were connected to our *ex situ* perfusion circuit at a temperature of 39 °C, the normal pig core body temperature. Livers were perfused through both the hepatic artery and portal vein. Both of the perfused vessels were under automated computer pressure control, with the hepatic artery perfused at a target of 70 mmHg and the portal vein perfused at a target pressure of 2 mmHg. All perfusions were performed for durations of 48 hours.

In the designated treatment group (n= 3), perfusions were allowed to proceed for 5 hours, at which point the circuit was injected through the portal vein component with a calculated volume of the prepared high concentration transaminase supernatant to raise the AST level up to a target of 7,500 U/L. Following this intervention, liver perfusion was continued to 48 hours. In the control liver group (n=3), perfusions were established and allowed to proceed for 48 hours, without any intervention. At 2 hour intervals, perfusate samples were withdrawn, centrifuged, and the supernatant was stored at minus 80 °C until biochemical analysis was performed. Blood gasses were

also analyzed every 2 hours. Tissue samples were taken at the end of each perfusion for blinded histological analysis.

3.3.6 Perfusate composition analysis

Hemoglobin, electrolyte, pH, lactate and partial pressures of oxygen and carbon dioxide were measured using the ABL Flex Analyzer (Radiometer Medical ApS, Bronshoj, Denmark). Perfusate samples were obtained from the hepatic artery circuit and analyzed for levels of AST, ALT, LDH using a Beckman Coulter Unicel Dxc800 Syncron (Brea, California, USA), in our hospital clinical laboratory.

3.3.7 Hepatic oxygen consumption and vascular resistance

Liver graft oxygen consumption was calculated using the Fick equation and compared at two-hour intervals. Hepatic artery and portal vein vascular resistance was calculated by dividing the vessel pressure by the flow indexed to 100 grams of liver tissue.

3.3.8 Histology

Liver tissue samples were obtained at the end of each perfusion, and were fixed in 10% formalin. Tissue was embedded in paraffin, stained with hematoxylin and eosin, and examined in a blinded fashion by an independent expert pathologist, blinded to the experimental group, who assigned a semi-quantitative score to evaluate for hepatocyte injury and bile sequestration. Biopsy tissue was examined for necrosis (0- absent, 1- pericentral, 2- Zone 2 and 3, 3- panlobular); hemorrhage (score 0- absent, 1- focal, 2- zonal, 3- panlobular), cholestasis (score 0- absent, 1- present); and sinusoidal dilatation (score 0- none, 1- mild, 2- moderate, 3- severe), as previously reported [3].

3.3.9 Statistical analysis

Data are represented as means ± standard error of the means (SEM). Differences between continuous variables were compared using a repeated measures ANOVA or the Mann Whitney U-test. Overall comparison between groups was performed with a 95% confidence interval. A p-value of <0.05 was considered statistically significant and all the analysis was performed using Graphpad Prism (GraphPad Software Inc., La Jolla, CA, USA)

3.4 Results

The *ex situ* circuit was primed concomitantly with donor procurement surgery and NMP was established in all cases without difficulty. Mean starting hemoglobin concentration of the perfusates was 62.8 ± 8.7 g/L. There were no technical complications during the machine perfusions for any of the livers. The starting enzyme values in the prepared concentrated supernatant were as follows: AST 107,427 U/L, ALT 2,788 U/L and LDH 28,050 U/L.

Transaminase levels in the re-circulating ex situ perfusate initially began to increase in all cases. Perfusate transaminase levels sharply elevated in the treatment group at the intervention time point (5 hours), and were significantly higher for both AST ($p<0.0001$) and ALT ($p<0.0001$) **(Fig 3A and B).** The transaminase levels decreased over the duration of perfusion in the treatment group, but never to the same level as in the control group. In the control group, transaminase levels initially increased and then stabilized out after hour 8 of perfusion. LDH levels also increased sharply at the intervention point in the treatment group ($p<0.0001$) **(Fig 3C)** and also decreased over

time, to the level of the controls. In the control group, LDH increased, and then leveled at approximately perfusion hour 8. Lactate levels increased in all perfusions, with no statistical significance between groups (p= 0.12) **(Fig 3D)**.

Hepatic vascular parameters demonstrated changes between groups. Hepatic artery resistance (HAR) increased in the treatment group at the time of the intervention, but then decreased to the baseline of the controls with no statistical difference (p=0.14) **(Fig 4A)**. Portal vein resistance (PVR), also increased at the time of the intervention, returned to control levels, and then demonstrated a steady rise until the end of perfusion duration (p=0.0003) **(Fig 4B)**. Liver graft oxygen consumption fluctuated over the duration of perfusions, but was not statistically different between groups, decreasing over the perfusion duration (p=0.41) **(Fig 4C).** Total bile output in both groups steadily accumulated in both groups, but was statistically greater in the intervention group (p<0.0001) **(Fig 4D).**

Comparison of end of perfusion liver histology demonstrated evidence of hepatocyte injury in all liver biopsies, including hemorrhage, necrosis and sinusoidal dilatation. The severity however, was not different between groups (p>0.99) **(Figure 5).**

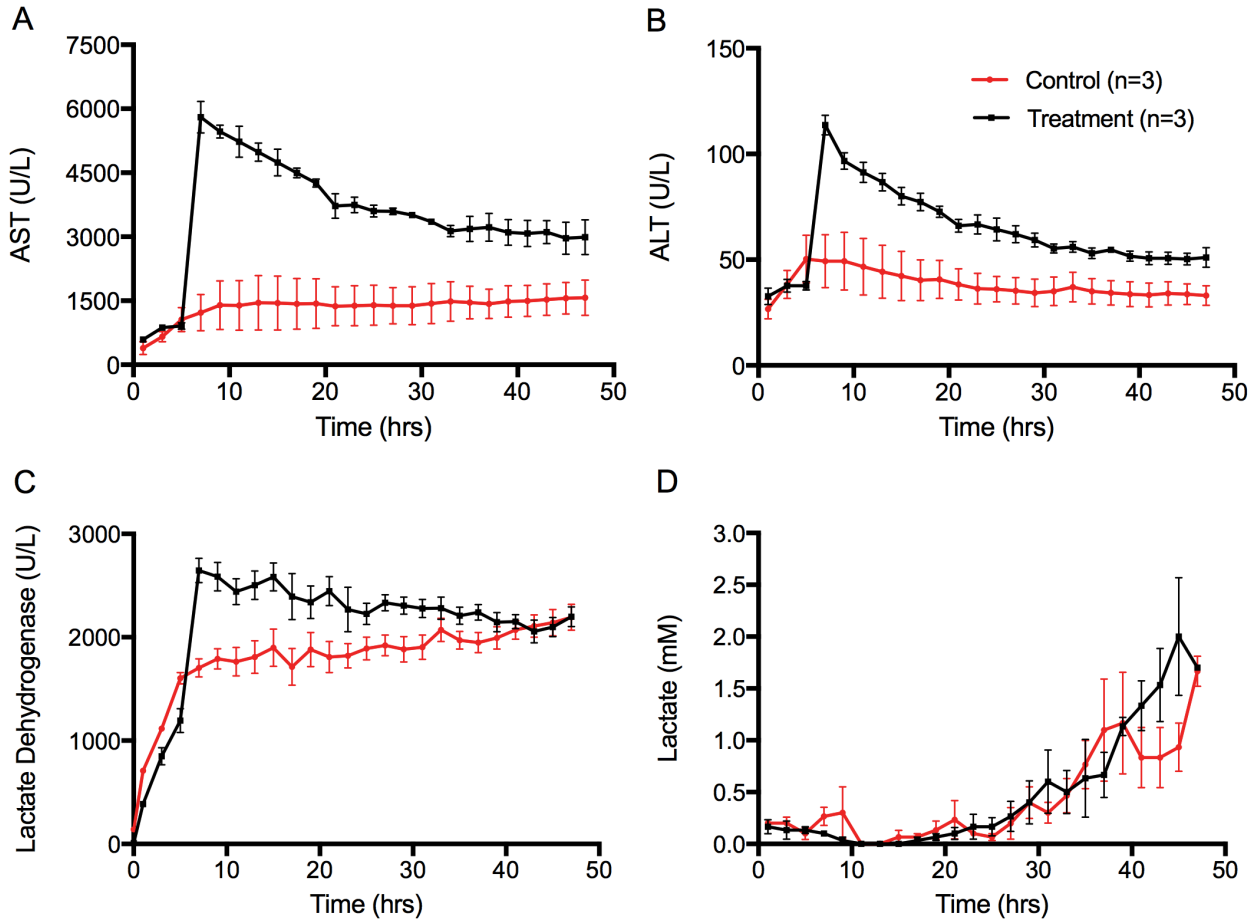


Figure 3-3. 'On circuit' perfusate biochemistry during NMP.

(A) Circulating AST perfusate levels during NMP, ($p<0.0001$). (B) Circulating ALT perfusate levels ($p<0.0001$). (C) *Ex situ* circulating perfusate lactate dehydrogenase levels at each hemoglobin level ($p<0.0001$). (D) Lactate levels at each time point($p=0.24$). Data points show means and SEM, 95% confidence interval. Aspartate aminotransferase (AST), Alanine aminotransferase (ALT).

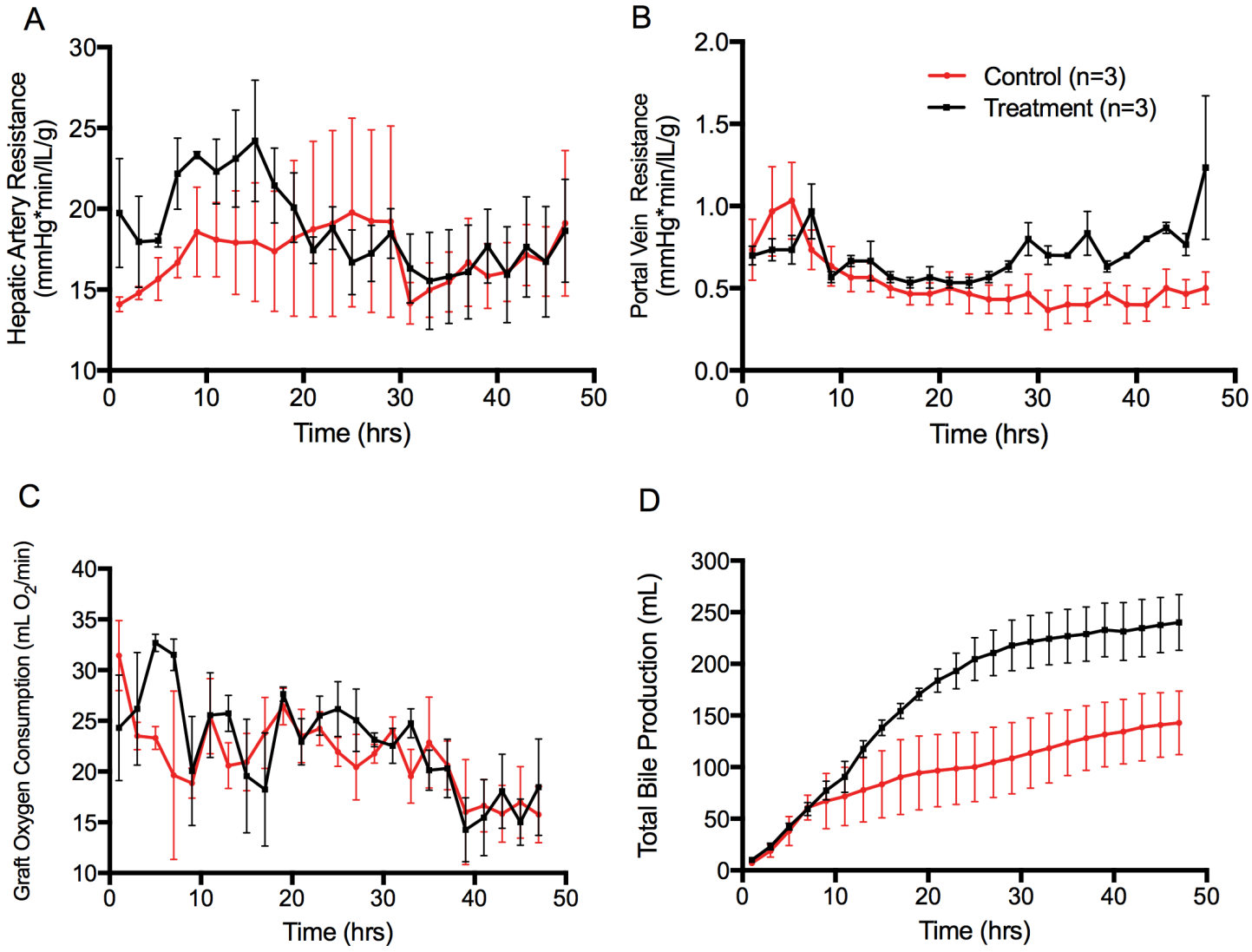


Figure 3-4. Perfusion vascuar parameters, oxygen consumption and pile output.

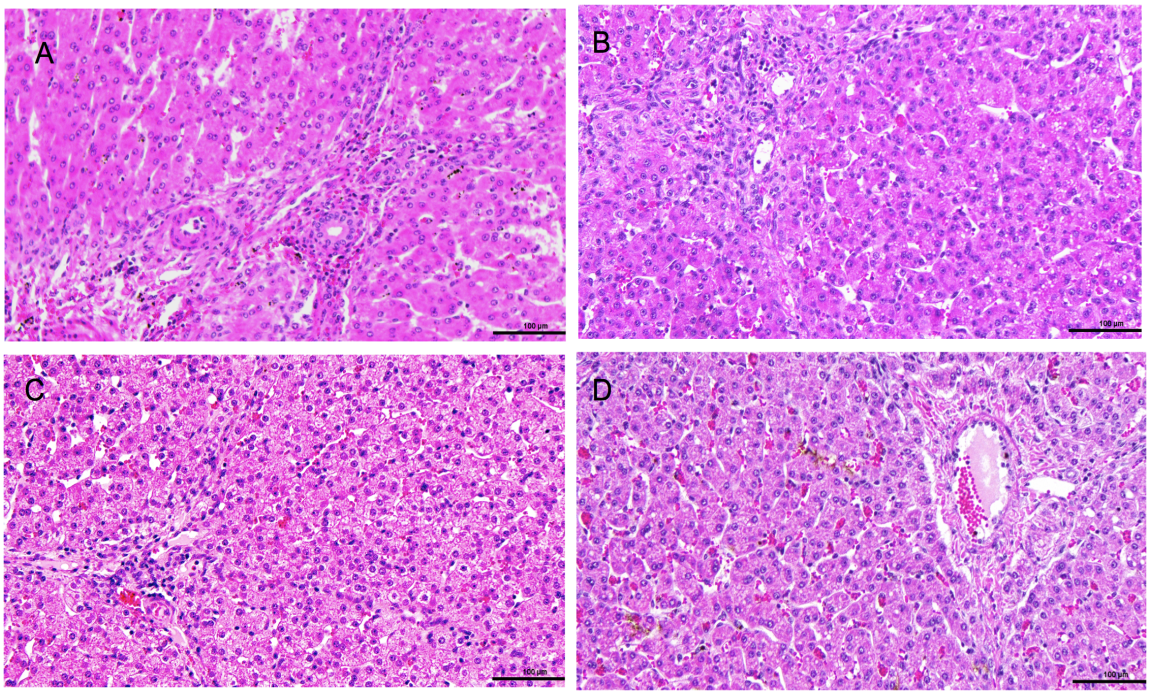


Figure 3-5. Representative sections of liver parenchyma, stained with hematoxylin and eosin, taken at termination of 48 hours of perfusion.

(A) Representative section of tissue from a liver in the intervention group. (B) Representative section of tissue from a liver in the intervention group. (C) Representative section of tissue from a liver in the control group. (D) Representative tissue from a liver in the control group.

3.5 Discussion

Liver *ex situ* NMP has previously demonstrated the potential to resuscitate donor livers that would otherwise be discarded from the transplant process [1]. Functional evaluation of such livers is necessary prior to implantation, in order to minimize the possibility of the catastophic outcome of primary non-function (PNF). *Ex situ* NMP offers a potentially ideal temperature modality, as it allows for viability assessment occur in the setting of physiologic metabolism.

Transaminase levels are ubiquitously used as a marker of graft hepatocellular injury, and indeed, as such have been used as an endpoint in most clinical liver *ex situ* studies [1,7-9]. Further, transaminases in liver procurement flush solution and in machine perfusate have been shown to correlate with post-transplant graft function [4]. In previous studies, machine perfusate transaminase levels would be reported, but to-date, not one group has elucidated the kinetics of how these enzymes are potentially cleared in the context of an isolated, perfused liver circuit. The goal of the present study was to clarify this as an important means to interpret *ex situ* quantification and kinetics of transaminase degradation.

In this study, we found that liver transaminases increased in the control group at a slow rate, as previously described [12]. In the treatment group transaminase levels elevated promptly as anticipated at the intervention time-point, and then slowly decrease after. It is possible that a degree of hepatocellular injury resulting from the intervention could also have contributed to the observed enzyme elevation. The *in vivo* natural half-live of AST is 17 ± 5 hours, and the half life of ALT is 47 ± 10 hours, as reported previously [24].

Since the rate of transaminase decrease in the treatment group did not correspond to these half-lives, we surmise that the decrease in enzyme levels in the treatment group is in part the result of endogenous breakdown of these enzymes in the liver. It has been previously shown that transaminases are taken up by sinusoidal cells and cleared by Küpffer cells in the liver [25,26]. The transaminase levels in the treatment group never decreased to the level of the control group, and we speculate that at a certain point, enzymatic transaminase catabolism reaches a steady state, with enzyme degradation equivalent to that of release from damaged cells. In this scenario, it is likely that at the end of the perfusion duration, some of the exogenous transaminases in the intervention group remained in the circulating perfusate. An unknown possibility whether the observed decrease in transaminase levels was a result of enzyme binding to components of the perfusion circuit. We cannot fully predict the outcome if these perfusions had been allowed to proceed for a longer duration.

LDH is a well-known marker of cellular injury, and has been suggested as an indicator of the quality of a liver graft, and ultimately its transplant potential [27]. In the control group, LHD increased and then reached a plateau, as previously demonstrated [27]. In the experimental group, LDH elevated significantly at the intervention time point, likely from an exogenous bolus of LDH present in the transaminase supernatant. Again, cannot say with certainty that any degree of hepatocellular injury resulting directly from the intervention did not contribute to the observed enzyme elevation.

Machine perfusate lactate levels are consistently reported as one of the most important markers of graft injury during perfusion. Indeed, any group that has suggested a composite series of viability markers for *ex situ* graft assessment has invariably

included decreasing lactate as a dominant variable [4,6,16,28]. Recently, it has been suggested that perhaps it is not just the absolute decrease of lactate levels, but the fall in lactate per weight of liver tissue, which may be of most utility. This is in view of the fact that in a recent clinical NMP series livers that had cleared lactate still went on to develop PNF [4].

In this study, all livers had a very minimal rise in lactate, ostensibly indicating minimal liver damage, in keeping with previously reported longer-term perfusion experiments [12,21,29]. Both control and treatment groups demonstrated similar lactate curves, indicating that the intervention did not compromise liver perfusion or lead to significant functional injury. Although perfusate lactate level is commonly used as a marker of liver damage, it is reported to be cleared by zone 1 of the hepatic lobule [30]. This zone is closest to the portal venules and arterioles, and as such is well perfused and oxygenated. Lactate levels would therefore likely only rise if a pan-lobular injury was present or if an entire liver segment was not perfused, as in the case of a damaged accessory artery. Nevertheless, lactate currently continues to be used by most groups in assessing *ex situ* viability, and indeed, we have discarded livers based on poor lactate clearance.

Data surrounding oxygen consumption by liver grafts undergoing *ex situ* perfusion is conflicting, with different groups reporting higher consumption associated with poor liver function, and others the converse. In the present study both control and treatment groups exhibited similar oxygen consumption curves, likely indicating similar liver metabolic demands and activity.

Vascular resistance has previously been described as a possible marker for graft viability, with the suggestion that a higher PVR was correlated to a graft with poorer function [3]. In this study, we found that HAR did not change significantly in either the intervention group or in the control group. PVR increased at the intervention time point in the treatment group, decreased to the level of controls, and then increased again from approximately 24 hours of perfusion. This was in contrast to the control livers, which demonstrated a consistently level PVR. This may possibly be explained in part by the fact that the concentrated transaminase solution, which was quite viscous in consistency and was injected into the portal vein at the intervention time-point, may have embolized distant venules within the liver parenchyma. Nevertheless, PVR did not rise to a level that would be considered deleterious to a graft, at least from previous reports [3].

To-date, bile production during *ex situ* liver perfusion was considered to be a potential surrogate marker of liver viability, with multiple groups suggesting this utility [3,6,16,28,31]. In theory, production of bile requires the integrity of multiple cellular and metabolic components, and as such would rationally implicate a well-functioning graft. This seemed to hold true in experimental large mammal *ex situ* experiments, with higher bile output correlating with improved post-transplant function. In clinical series however, this was not necessarily the case, with some livers that had produced bile proceeding to develop PNF post-transplant, and others that had produced no bile functioning well [4]. Recently, Matton *et al* reported that the absolute volume of bile may not be as critical as the bile quality, with recent findings demonstrating bile pH, glucose, and bicarbonate levels to correlate with ischemic cholangiopathy [32]. We observed that the total bile output in the treatment group was significantly higher than in the controls. This is not

readily explainable as the formation and secretion of bile is a complex process dependent on various hormonal, electrolyte, neural, and humoral factors [33]. It had been previously shown that after 10 hours of experimental *ex situ* perfusion, bile production would slow down if the perfusate was not supplemented with exogenous bile salts. An infusion of taurocholate would maintain bile production at physiological levels (8 mL/h +/_ 0.75) throughout a 20 hour perfusion [34]. At the time of this study, we did not have access to commercial grade bile salts, and so did not supplement our perfusions. Despite this, all livers steadily produced bile, albeit not at the suggested physiological levels, likely due to a paucity of bile salts.

The presence of hepatocyte injury at the end of our perfusions, evident on histology, is likely due to progressive, cumulative on-circuit graft damage. This may be a result of the formation of reactive oxygen species, bile salt depletion, progressive mechanical perfusion injury, or damage as a direct result of the performed intervention.

There are several limitations to our study. The highly concentrated transaminase supernatant used to elevate circuit transaminase levels contains many cytoplasmic constituents and cell fragments, and we ultimately do not know how these interacted with each other and the metabolism of the livers during NMP. By not performing transplant experiments, we cannot ultimately predict how these livers would have functioned *in vivo*, or the resultant post-transplant biochemistry.

Herein we have demonstrated that concentrated high levels of exogenous transaminases injected into a NMP ex situ circuit during perfusion of a healthy porcine liver are cleared from the perfusate, indicating preserved liver metabolism of

transaminases. The functional status of the livers was not evidently affected by the increase in transaminase levels, as evidenced by similar lactate levels, and oxygen consumption between groups. Clearance of endogenous or exogenous transaminases during NMP may be a graft tolerance test that indicates good graft function and viability, with decreasing levels indicating good graft function.

3.6 References

1. Nasralla D, Coussios CC, Mergental H, et al. A randomized trial of normothermic preservation in liver transplantation. *Nature.* 2018.
2. Guarrera JV, Henry SD, Samstein B, et al. Hypothermic machine preservation in human liver transplantation: the first clinical series. *Am J Transplant.* 2010;10(2):372-381.
3. Brockmann J, Reddy S, Coussios C, et al. Normothermic perfusion: a new paradigm for organ preservation. *Ann Surg.* 2009;250(1):1-6.
4. Watson CJE, Kosmoliaptsis V, Pley C, et al. Observations on the ex situ perfusion of livers for transplantation. *Am J Transplant.* 2018;18(8):2005-2020.
5. Watson CJE, Kosmoliaptsis V, Randle LV, et al. Normothermic Perfusion in the Assessment and Preservation of Declined Livers Before Transplantation: Hyperoxia and Vasoplegia-Important Lessons From the First 12 Cases. *Transplantation.* 2017;101(5):1084-1098.
6. Mergental H, Perera MT, Laing RW, et al. Transplantation of Declined Liver Allografts Following Normothermic Ex-Situ Evaluation. *Am J Transplant.* 2016;16(11):3235-3245.
7. Bral M, Gala-Lopez B, Bigam D, et al. Preliminary Single-Center Canadian Experience of Human Normothermic Ex Vivo Liver Perfusion: Results of a Clinical Trial. *Am J Transplant.* 2016.
8. Selzner M, Goldaracena N, Echeverri J, et al. Normothermic ex vivo liver perfusion using steen solution as perfusate for human liver transplantation: First North American results. *Liver Transpl.* 2016;22(11):1501-1508.

9. Ravikumar R, Jassem W, Mergental H, et al. Liver transplantation after ex vivo normothermic machine preservation: a Phase 1 (first-in-man) clinical trial. *Am J Transplant.* 2016.

10. Watson CJ, Randle LV, Kosmoliaptsis V, Gibbs P, Allison M, Butler AJ. 26-hour Storage of a Declined Liver Before Successful Transplantation Using Ex Vivo Normothermic Perfusion. *Ann Surg.* 2016.

11. Perera T, Mergental H, Stephenson B, et al. First human liver transplantation using a marginal allograft resuscitated by normothermic machine perfusion. *Liver Transpl.* 2016;22(1):120-124.

12. Vogel T, Brockmann JG, Pigott D, et al. Successful transplantation of porcine liver grafts following 48-hour normothermic preservation. *PLoS One.* 2017;12(11):e0188494.

13. Vogel T, Brockmann JG, Quaglia A, et al. 24-Hour Normothermic Machine Perfusion of Discarded Human Liver Grafts. *Liver Transpl.* 2016.

14. Banan B, Chung H, Xiao Z, et al. Normothermic extracorporeal liver perfusion for donation after cardiac death (DCD) livers. *Surgery.* 2015;158(6):1642-1650.

15. Nassar A, Liu Q, Farias K, et al. Ex vivo normothermic machine perfusion is safe, simple, and reliable: results from a large animal model. *Surg Innov.* 2015;22(1):61-69.

16. op den Dries S, Karimian N, Sutton ME, et al. Ex vivo normothermic machine perfusion and viability testing of discarded human donor livers. *Am J Transplant.* 2013;13(5):1327-1335.

17. Boehnert MU, Yeung JC, Bazerbachi F, et al. Normothermic acellular ex vivo liver perfusion reduces liver and bile duct injury of pig livers retrieved after cardiac death. *Am J Transplant.* 2013;13(6):1441-1449.

18. Xu H, Berendsen T, Kim K, et al. Excorporeal normothermic machine perfusion resuscitates pig DCD livers with extended warm ischemia. *J Surg Res.* 2012;173(2):e83-88.

19. Fondevila C, Hessheimer AJ, Maathuis MH, et al. Superior preservation of DCD livers with continuous normothermic perfusion. *Ann Surg.* 2011;254(6):1000-1007.

20. Reddy SP, Bhattacharjya S, Maniakin N, et al. Preservation of porcine non-heart-beating donor livers by sequential cold storage and warm perfusion. *Transplantation.* 2004;77(9):1328-1332.

21. Butler AJ, Rees MA, Wight DG, et al. Successful extracorporeal porcine liver perfusion for 72 hr. *Transplantation.* 2002;73(8):1212-1218.

22. Schon MR, Kollmar O, Wolf S, et al. Liver transplantation after organ preservation with normothermic extracorporeal perfusion. *Ann Surg.* 2001;233(1):114-123.

23. Bral M, Gala-Lopez B, Thiesen A, et al. Determination of Minimal Hemoglobin Level Necessary for Normothermic Porcine Ex Situ Liver Perfusion. *Transplantation.* 2018;102(8):1284-1292.

24. Kim WR, Flamm SL, Di Bisceglie AM, Bodenheimer HC, Public Policy Committee of the American Association for the Study of Liver D. Serum activity of alanine aminotransferase (ALT) as an indicator of health and disease. *Hepatology.* 2008;47(4):1363-1370.

25. Radi ZA, Koza-Taylor PH, Bell RR, et al. Increased serum enzyme levels associated with kupffer cell reduction with no signs of hepatic or skeletal muscle injury. *Am J Pathol.* 2011;179(1):240-247.

26. Kamimoto Y, Horiuchi S, Tanase S, Morino Y. Plasma clearance of intravenously injected aspartate aminotransferase isozymes: evidence for preferential uptake by sinusoidal liver cells. *Hepatology.* 1985;5(3):367-375.

27. Monbaliu D, Liu Q, Libbrecht L, et al. Preserving the morphology and evaluating the quality of liver grafts by hypothermic machine perfusion: a proof-of-concept study using discarded human livers. *Liver Transpl.* 2012;18(12):1495-1507.

28. Mergental H, Stephenson BTF, Laing RW, et al. Development of Clinical Criteria for Functional Assessment to Predict Primary Nonfunction of High-Risk Livers Using Normothermic Machine Perfusion. *Liver Transpl.* 2018;24(10):1453-1469.

29. Liu Q, Nassar A, Buccini L, et al. *Ex situ* 86-hour liver perfusion: Pushing the boundary of organ preservation. *Liver Transpl.* 2018;24(4):557-561.

30. Watson CJE, Jochmans I. From "Gut Feeling" to Objectivity: Machine Preservation of the Liver as a Tool to Assess Organ Viability. *Curr Transplant Rep.* 2018;5(1):72-81.

31. Sutton ME, op den Dries S, Karimian N, et al. Criteria for viability assessment of discarded human donor livers during ex vivo normothermic machine perfusion. *PLoS One.* 2014;9(11):e110642.

32. Matton APM, de Vries Y, Burlage LC, et al. Biliary Bicarbonate, pH and Glucose Are Suitable Biomarkers of Biliary Viability During Ex Situ Normothermic Machine Perfusion of Human Donor Livers. *Transplantation.* 2018.

33. Boyer JL. Bile formation and secretion. *Compr Physiol.* 2013;3(3):1035-1078.

34. Imber CJ, St Peter SD, de Cenarruzabeitia IL, et al. Optimisation of bile production during normothermic preservation of porcine livers. *Am J Transplant.* 2002;2(7):593-599.

4 Chapter 4.

Preliminary single centre Canadian experience of human normothermic *ex vivo* liver perfusion: Results of a clinical trial

American Journal of Transplantation 2017; 17: 1071–1080
Wiley Periodicals Inc.

doi: 10.1111/ajt.14049

Preliminary Single-Center Canadian Experience of Human Normothermic *Ex Vivo* Liver Perfusion: Results of a Clinical Trial

M. Bral[1,2], B. Gala-Lopez[1,2], D. Bigam[1], N. Kneteman[1,2], A. Malcolm[1,2], S. Livingstone[1], A. Andres[1], J. Emamaullee[1], L. Russell[3], C. Coussios[4], L. J. West[1,2], P. J. Friend[5] and A. M. J. Shapiro[1,2,*]

[1]*Department of Surgery, University of Alberta, Edmonton, Canada*
[2]*Members of the Canadian National Transplant Research Project (CNTRP), Edmonton, Canada*
[3]*OrganOx, Oxford, UK*
[4]*Institute of Biomedical Engineering, University of Oxford, Oxford, UK*
[5]*Nuffield Department of Surgical Sciences, University of Oxford, Oxford, UK*
**Corresponding author: A. M. J. Shapiro, amjs@islet.ca*

After extensive experimentation, outcomes of a first clinical normothermic machine perfusion (NMP) liver trial in the United Kingdom demonstrated feasibility and clear safety, with improved liver function compared with standard static cold storage (SCS). We present a preliminary single-center North American experience using identical NMP technology. Ten donor liver grafts were procured, four (40%) from donation after circulatory death (DCD), of which nine were transplanted. One liver did not proceed because of a technical failure with portal cannulation and was discarded. Transplanted NMP grafts were matched 1:3 with transplanted SCS livers. Median NMP was 11.5 h (range 3.3–22.5 h) with one DCD liver perfused for 22.5 h. All transplanted livers functioned, and serum transaminases, bilirubin, international normalized ratio, and lactate levels corrected in NMP recipients similarly to controls. Graft survival at 30 days (primary outcome) was not statistically different between groups on an intent-to-treat basis (p = 0.25). Intensive care and hospital stays were significantly more prolonged in the NMP group. This preliminary experience demonstrates feasibility as well as potential technical risks of NMP in a North American setting and highlights a need for larger, randomized studies.

Abbreviations: AKI, acute kidney injury; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; CIT, cold ischemia time; DBD, donation after brain death; DCD, donation after circulatory death; DRI, donor risk index; EAD, early allograft dysfunction; ECD, extended criteria donor; GDA, gastroduodenal artery; HA, hepatic artery; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HTK, histidine–tryptophan–ketoglutarate; ICU, intensive care unit; INR, international normalized ratio; MELD, Model for End-Stage Liver Disease; MRCP, Magnetic resonance cholangiopancreatography; NASH, nonalcoholic steatohepatitis; NMP, normothermic machine perfusion; OLTx, Orthotopic liver transplantation; PNF, primary nonfunction; POD, postoperative day; SCS, static cold storage; SD, standard deviation; SMA, superior mesenteric artery; T-Bilirubin, total bilirubin; UW, University of Wisconsin; WIT, warm ischemia time

Received 27 March 2016, revised 28 August 2016 and accepted for publication 30 August 2016

Introduction

With a critical shortage of donor liver organs across transplant centers, programs have increasingly resorted to using organs from extended criteria donors (ECDs) and donation after circulatory death (DCD) or from living liver donation to expand the limited donor pool. Standard static cold storage (SCS) fails to provide optimal preservation of DCD and ECD grafts, often resulting in early allograft dysfunction (EAD) and potential for long-term complications (1–3). It has long been recognized that prolonged cold storage of marginal and DCD livers further exacerbates cellular damage and markedly increases rates of ischemia–reperfusion, EAD, primary nonfunction (PNF), and ischemic cholangiopathy (4). As an alternative preservation strategy, *ex vivo* normothermic machine perfusion (NMP) proffers the potential of (i) extended-duration liver preservation; (ii) optimized liver function; (iii) ability to measure dynamic viability during the *ex vivo* phase, which is not possible with SCS; and (iv) the potential to resuscitate compromised livers through delivery of targeted additives (5,6).

Alternative approaches to machine perfusion have included hypothermic (7,8), subnormothermic (9–11), and normothermic perfusion (12–14). Initial clinical *ex vivo* liver perfusions involved cold oxygenated *ex vivo* perfusion of livers with positive outcomes (15,16), followed more

Original article published after peer review in *American Journal of Transplantation* 2017;17(4):1071-80.

Original Paper

Title: Preliminary Single Centre Canadian Experience of Human Normothermic *Ex Vivo* Liver Perfusion: Results of a Clinical Trial

Authors:

Mariusz Bral, MD[1, 5], Boris Gala-Lopez MD[1, 5], David Bigam MD[1], Norman Kneteman MD[1,5], Andrew Malcolm PhD[1, 5], Scott Livingstone MD[1], Axel Andres MD[1], Juliet Emamaullee MD PhD[1], Leslie Russell PhD[4], Constantin Coussios PhD[2], Lori J West MD PhD[1,5], Peter J Friend MD [3], and A.M. James Shapiro MD PhD[1,5]

Affiliations:

[1] Department of Surgery, University of Alberta, Edmonton, Canada

[2] Institute of Biomedical Engineering, University of Oxford, UK

[3] Nuffield Department of Surgical Sciences, University of Oxford, UK

[4] OrganOx UK

[5] Members of the Canadian National Transplant Research Project (CNTRP)

Word Count: 3,972.

Corresponding Author:

A.M. James Shapiro, MD, PhD, Canada Research Chair in Transplantation Surgery and Regenerative Medicine, Professor, Director of Clinical Islet and Living Donor Liver Transplant Programs, Clinical Islet Transplant Program, University of Alberta. 2000 College Plaza, 8215-112th St, Edmonton T6G 2C8, Alberta, Canada. amjs@islet.ca

Telephone: +1-780-4077330, Fax: +1-780-4078259

4.1 Abstract

After extensive experimentation, outcomes of a first clinical normothermic machine perfusion (NMP) liver UK trial demonstrated feasibility and clear safety, with improved liver function when compared to standard static cold storage (SCS). We herein present a preliminary single centre North-American experience using identical NMP technology. Ten donor liver grafts were procured, 4 (40%) from donation after circulatory death (DCD) donors of which 9 were transplanted. One liver did not proceed due to a technical failure with portal cannulation, and discarded. Transplanted NMP grafts were matched 1:3 with transplanted SCS livers. Median NMP was 11.5 hours (range: 3.3 - 22.5) with one DCD liver perfused for 22.5 hours. All transplanted livers functioned, and serum transaminases, bilirubin, INR land lactate levels corrected in NMP recipients similarly to controls. Thirty-day graft survival (primary outcome) was 90% in the NMP group vs. 100% in the SCS group, on an intent-to-treat basis (p=0.08). Six-month graft survival was 8/10 (80%) in NMP and 30/30 (100%) in SCS livers (p=0.01). Intensive care and hospital stays were significantly more prolonged in the NMP group. This preliminary experience demonstrates feasibility of NMP, but raises concerns with technology-related graft loss, highlighting a need for larger, randomized studies.

4.2 Introduction

With a critical shortage of donor liver organs across transplant centres, programs have increasingly resorted to using extended criteria donors (ECD) and donation after circulatory death (DCD) organs, or living liver donation, to expand the limited donor pool. Standard static cold storage (SCS) fails to provide optimal preservation of DCD and ECD grafts, often resulting in early allograft dysfunction (EAD) and potential for long-term complications [1-3]. It has long been recognized that prolonged cold storage of marginal and DCD livers further exacerbates cellular damage and markedly increases rates of ischemia-reperfusion, EAD, primary non-function (PNF) and ischemic cholangiopathy [4]. As an alternative preservation strategy*, ex vivo* normothermic machine perfusion (NMP) proffers the potential of: 1) extended-duration liver preservation; 2) optimized liver function; 3) ability to measure dynamic viability during the *ex vivo* phase, which is not possible with SCS; and, 4) the potential to resuscitate compromised livers through delivery of targeted additives [5,6].

Alternative approaches to machine perfusion have included hypothermic [7,8], subnormothermic [9-11], and normothermic perfusion [12-14]. Initial clinical *ex vivo* liver perfusions involved cold oxygenated *ex vivo* perfusion of livers with positive outcomes [15,16], followed more recently by warmer perfusate approaches [17-19]. Large animal studies demonstrated consistently that *ex vivo* NMP technology can protect injured livers through elimination of prolonged SCS [14,20-22] with clinical translation of these studies following in rapid succession. Recently, novel portable normothermic *ex vivo* technology has been developed, and a first in-human pilot trial using portable NMP technology (OrganOx *metra®)* was completed in the UK, demonstrating feasibility and

safety [23]. Ravikumar *et al.* showed that NMP livers had lower median peak aspartate transaminase (AST) levels compared to matched SCS controls, suggesting enhanced hepatic protection.

In collaboration with the University of Oxford and OrganOx UK, we commenced our own investigator-initiated first-in-North-American pilot clinical trial of liver NMP in Edmonton, Canada, and herein report on our preliminary experience with the first 10 cases.

4.3 Methods

4.3.1 Study Design

From February to December 2015, a Phase 1, non-randomized pilot study was performed at the University of Alberta to evaluate outcomes of clinical livers perfused with NMP. The study was approved by the Health Research Ethics Board at the University of Alberta and by Health Canada (IRB # 00043239, ID: Pro00043239). All potential NMP recipients were consented at the time of initial assessment, ahead of transplantation, and affirmed at the time of admission for transplantation. The primary objective was to assess safety of NMP in continuous liver preservation, with the primary outcome measure being graft survival at day 30 post-transplant. Secondary outcomes included: 1) Patient survival at day 30; 2) Peak serum transaminase AST in the first 7 days; 3) EAD incidence in the first 7 days [24]; 4) Liver biochemistry in serum on days 1-7, 10, and 30; 5) Major complications defined by a Clavien-Dindo score ≥ 3 [25]; 6) Patient and graft survival at 6 months and; 7) Biliary complications at 6 months. Outcomes were compared 1:3 with matched control liver transplant recipients with conventional

SCS grafts. Control subjects were selected from a pool of 150 adult deceased-donor liver transplants at the University of Alberta over the previous 36 months based on closest matching for: 1) Recipient Model for End-Stage Liver Disease (MELD) score, 2) Donor Risk Index, 3) Donor age, 4) Recipient age and 5) Graft type (DBD vs DCD).

Deceased donors (≥40 Kg in weight) were included in both groups. All DCD livers were procured with < 30 minutes of warm ischemia. Living donors, and livers intended for split transplant were excluded. Recipient subjects with end-stage chronic, non-fulminant liver disease were included. Patients undergoing re-transplantation, or undergoing transplantation of other organs were excluded. OrganOx UK provided on-site training in Edmonton, and members of the surgical team also participated in clinical NMPs carried out in the UK as part of an ongoing randomized controlled trial (ISRCTN39731134). Allocation of livers to the NMP group was primarily determined by availability of the perfusion team coupled with prior recipient consent (**Supplementary Figure S1**).

4.3.2 Normothermic Machine Perfusion

Livers allocated to the intervention group were preserved using the NMP system (*metra*®, OrganOx. Oxford, UK), as recently described (**Supplementary Figure S2**) [23]. Briefly, the OrganOx *metra*® was calibrated and primed once a suitable liver graft was deemed acceptable for transplantation. All organ procurements were completed locally within Edmonton city hospitals, and the *metra*® initiated at the donor centre. Machine perfusate consisted of 500 mL of Gelofusine (B Braun, Melsungen, Germany), and 3

units of type O-packed red blood cells. All perfusate additives were otherwise identical to what was described by Ravikumar *et al.* [23]. Sodium bicarbonate 30 mL 8.4% (Hospira, Montreal, Canada) was added as needed to maintain pH between 7.35 – 7.45. A full description of the OrganOx *metra*® perfusate composition is listed under **Supplementary Appendix S1.**

All livers were procured in standard fashion, flushed *in situ* with Histidine-Tryptophan-Ketoglutarate solution (Custodiol HTK, Methapharm Inc., Brantford, ON, Canada), prepared and cannulated on a 'back-table' at the donor hospital. Prior to perfusion, liver grafts #1-4 were primed only with HTK on the back table. Livers #5-9 were flushed additionally with 1 L Gelofusine and 500 mL 5% human albumin (Plasbumin-5, Grifols Therapeutics Inc., Mississauga, ON, Canada) prior to NMP, with the intention to wash out carry-over of intra-hepatic HTK to the perfusion circuit. A notable difference between the Canadian and UK study was the use of HTK vs. University of Winsconsin (UW) solution for donor vascular flush.

4.3.3 Metabolic Parameters

Liver grafts were monitored during NMP with interval determination of blood gases (pH, lactate), alanine aminotransferase (ALT), AST and total bilirubin, at perfusion start and every 2 hours thereafter. Blood glucose was manually entered every 4 hours, and the Nutriflex infusion adjusted accordingly. Liver perfusion quality was documented by variation in perfusate pH, lactate concentration, perfusion vascular stability, and by hourly bile production. Once recipient hepatectomy was completed, the NMP was discontinued. In order to comply with accepted clinical practice, all liver grafts were

further flushed with cold HTK solution immediately before being brought into the surgical field.

4.3.4 Intraoperative Management

Surgical implantation techniques were identical between NMP and SCS groups. All livers were transplanted with standard caval replacement, without bypass or temporary portocaval shunt. The incidence of reperfusion syndrome, defined by a decrease in mean arterial pressure of >30% from baseline lasting >1 minute during the first 5 minutes after reperfusion, was documented only in the NMP group [26].

4.3.5 Post-transplant Care

Post-transplant care in both NMP and SCS groups was performed following standard protocols including tacrolimus-based immunosuppression where appropriate, and sirolimus where pre-existing renal dysfunction was present [27]. Of the 5/9 NMP liver grafts that were deemed to have EAD, only one case was due to a factor other than peak AST, specifically, elevated bilirubin on day 7. All subjects that received DCD liver grafts underwent magnetic resonance cholangiopancreatography (MRCP) at 6 months to rule out the presence of non-anastomotic biliary strictures secondary to ischemic cholangiopathy [28].

4.3.6 Statistical Analysis

Data are represented as median and ranges, as well as means ± standard deviations (SD) when necessary. The Mann-Whitney U-test, and 2-way ANOVA with Bonferroni's multiple comparisons were used to analyze differences between continuous variables.

Chi^2 test was used to compare proportions between groups for categorical outcomes. Overall comparison between NMP and SCS group was performed with a 95% confidence interval. A p-value <0.05 was considered significant and all the analysis was performed using Stata GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

4.4 Results

4.4.1 Donor Characteristics

A total of 10 liver grafts were procured, 9 of which were successfully perfused using NMP and transplanted. Four grafts (40%) were from DCD donors (Maastricht category III) and 6 (60%) were from DBD donors. One liver from a 60 year old DCD donor was procured and cannulated for NMP, but promptly discarded due to an occult portal venous twist that retracted into the liver hilum, preventing perfusion (**Video S1**). The median donor age was 56 years (range 14 - 71) in the NMP group vs. 52 years (range 20 - 77) in SCS controls (p=0.91) (**Tables 1 and 2**). Back-table reconstruction of aberrant hepatic arterial vasculature was completed where present (3/10 (30%)) before perfusion to ensure that all liver segments received adequate arterial inflow once warmed. Temporary iliac arterial extension grafts were added in the last 3 cases to minimize arterial desiccation surrounding the cannula tip (**Table 1**, **Supplementary Figure S3)**. The median Donor Risk Index (DRI) score was 1.6 (range 0.92- 2.66) in the NMP group vs. 1.6 (range 0.95 – 2.71) in SCS controls (p=0.82). A summary comparison of donor characteristics between NMP and SCS control grafts are provided in **Table 2**, and demonstrated no significant differences between variables.

Table 4-1. Donor and recipient characteristics for NMP liver grafts

No.	Age	Donor Cause of Death	Type	WIT (DCD)	CIT (min)	Notes/ Anatomical variations	NMP Time (hh:mm)	Recipient Age	Indication For Transplant	MELD	Post-transplant Complications
1	14y	Asphyxiation	DCD	20min	208	Replaced right HA. Right HA onto GDA	12:15	69y	HBV + HCC	9	EAD, laparotomy POD #2 for intra-abdominal bleed
2	19y	Head trauma	DBD	-	192	-	6:51	43y	Autoimmune	13	-
3	38y	Head trauma	DBD	-	95	-	11:31	50y	Autoimmune	29	EAD
4	35y	Asphyxiation	DCD	16min	108	-	3:16	62y	HCV + alcohol	16	EAD
5	60y	Brain stem stroke	DCD	26min	130	Liver discarded due to portal vein torsion on *metra*® + age/warm ischemia/operating room team availability	-	-	-	-	-
6	56y	Intracranial haemorrhage	DBD	-	150	Start of back-table albumin flush	15:52	50y	NASH	12	-
7	71y	Intracranial haemorrhage	DBD	-	184	-	8:14	54y	NASH	11	-
8	61y	Subdural hematoma	DBD	-	293	Left accessory HA, right replaced HA. Right HA onto GDA. Temporary iliac extension	7:41	53y	HCV + HCC	11	EAD, AKI requiring hemodialysis, death secondary to untreated HCV.
9	56y	Pulmonary fibrosis	DCD	23min	284	Complete occlusion of celiac origin with replaced right HA. Donor left HA onto right gastric origin, single SMA inflow. Temporary iliac extension	22:27	62y	HCV	26	EAD
10	59y	Intracranial haemorrhage	DBD	-	129	Temporary iliac extension	12:49	46y	NASH	32	-

Table 4-2. Summary of Donor and Graft Characteristics.

	NMP (n=10)	SCS (n=30)	p Value
Donor age (median- range)	56 years (14 – 71)	52 years (20 – 77)	0.91
Donor Risk Index (median – range)	1.6 (0.92 – 2.66)	1.6 (0.95 – 2.71)	0.82
Donor BMI (median – range)	27.5 (19.6 – 36.7)	25.6 (18.8 – 34.1)	0.34
Cold Ischemia Time (min) (median – range)	167 (95 - 293)	233 (64 - 890)	0.09
DCD Donor Proportion	4/10 (40%)	8/30 (27%)	0.43

4.4.2 Normothermic Machine Perfusion

The OrganOx *metra*® was primed concomitantly with donor procurement surgery; NMP was established in all but one case. Mean hemoglobin concentration of the perfusate was 104 ± 18 g/L and mean hematocrit was 32 ± 5%. Of 9 livers perfused successfully, there were no further technical complications during NMP. Median NMP time was 11.5 hours (range 3.3 - 22.5). NMP duration was determined by the recipient surgeon based upon logistics surrounding the transplant **(Table1)**. Graft 9, procured from a DCD donor, underwent NMP for 22.5 hours prior to implantation, and recovered normal function after a one-month period of gradually improving cholestasis.

Hepatic transaminases (AST and ALT) in the re-circulating NMP *ex vivo* perfusate circuit rose progressively in all cases. Perfusate aspartate transaminase (AST) was notably higher for DCD livers compared to DBD ($p<0.001$). During the longest perfusion (22.5 hours), AST levels on circuit peaked at 15,009 IU (**Figure 1A**), but the terminal perfusion lactate was 1.0 mmol/L; this graft functioned appropriately in the recipient. Most perfusions required occasional supplemental sodium bicarbonate to maintain physiologic pH, but all grafts cleared lactate rapidly while on circuit **(Figure 1B**) ($p=0.20$). During NMP, total bilirubin levels increased in all cases **(Figure 1C**), with higher levels in DCD livers ($p=0.002$). Total bile output for all perfused livers occurred at a median rate of 6.2 mL/hour (range 1.9 - 32.2). Bile output was lower for NMP DCD livers, with a median of 4.0 mL/hour (range 1.9 – 16.7) vs. NMP DBD livers with a median of 10.0 mL/hour (range 1.9 – 32.2) ($p<0.001$) (**Figure 1D**). All NMP grafts demonstrated stable portal vein and hepatic artery flow rates. Histological samples were not obtained routinely, and would have been helpful.

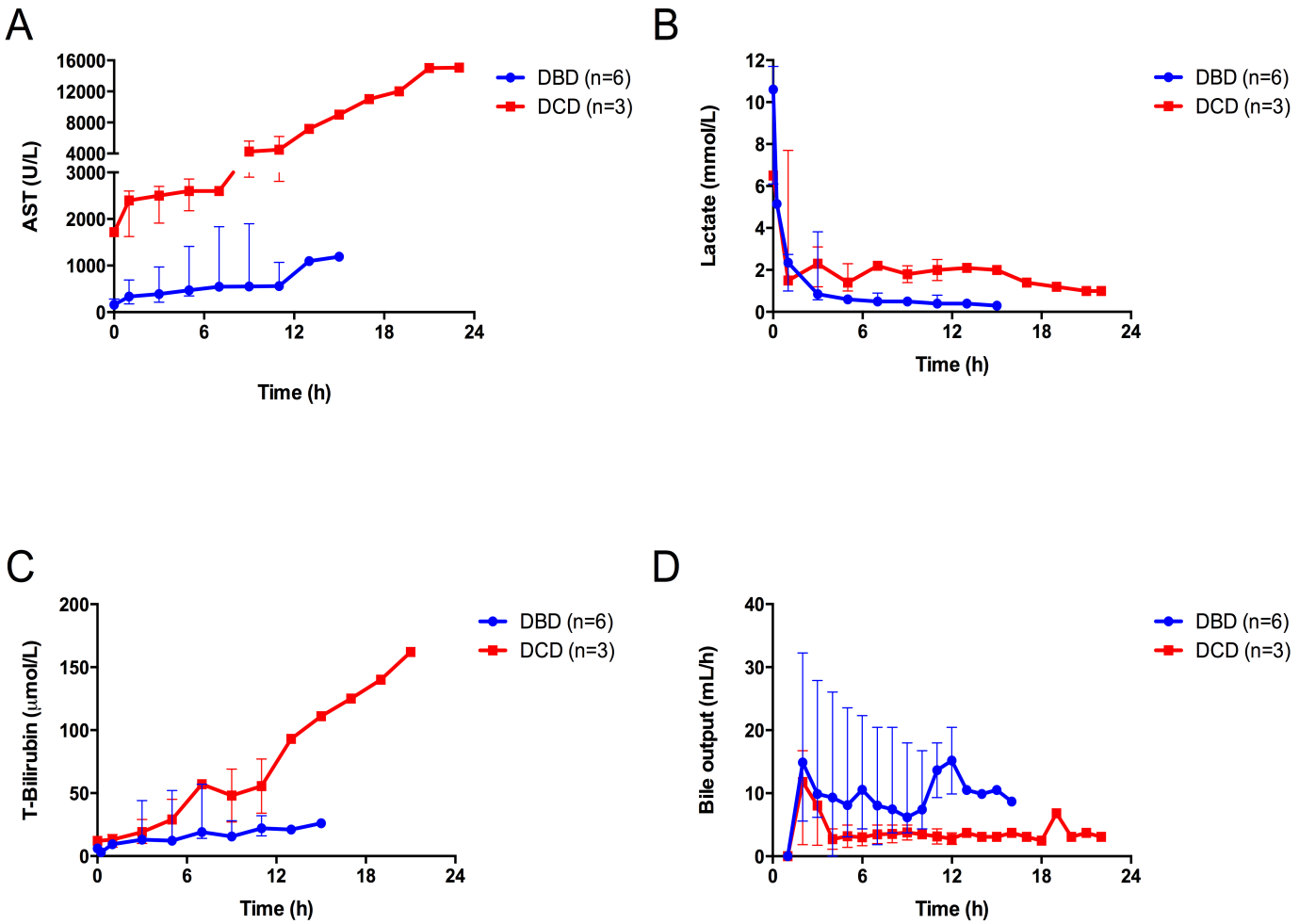


Figure 4-1. Ex vivo circulating perfusate liver biochemistry over time during NMP.

A. Circulating AST perfusate levels during NMP, separated by donor type (DBD vs. DCD) ($p<0.001$; 95% confidence interval (CI) 1760 - 10636); B. Circulating perfusate lactate levels during NMP, separated by donor type (DBD vs. DCD) ($p=0.20$; CI -0.91 - 1.6); C. *Ex vivo* circulating perfusate total bilirubin levels of liver grafts during NMP, separated by donor type DBD vs. DCD) ($p=0.002$; CI 10 - 114) D. Bile output for all livers during NMP, separated by donor type DBD vs. DCD) ($p<0.001$; CI -8.3 to – 6.0). Data points show medians and ranges, 95% confidence interval.

4.4.3 Recipients

In total, 9 subjects underwent transplantation with livers perfused by NMP. Transplant indications and pre-operative Model of End Stage Liver Disease (MELD) are shown in **Table 1**. Median recipient MELD was 13 (range 9 - 32) in the NMP group vs. 19 (range 7 - 34) in the SCS group (p= 0.37). Median recipient age was 53 years (range 28 - 67) in the NMP group vs. 59 years (range 43 - 69) in the SCS group (p=0.28) (**Table 3**). There were no significant differences between recipient characteristics in the NMP vs control SCS groups **(Table 3)**.

On an intent-to-treat basis, the primary outcome measure of 30-day graft survival was 90% in the NMP group and 100% in the SCS group (9/10 NMP and 30/30 SCS controls) (p=0.08). The 30-day transplanted patient survival was equivalent (100%) in both groups (**Table 3**). Six-month graft survival, a secondary outcome measure, was 8/10 (80%) in the NMP group and 30/30 (100%) in the SCS group (p=0.01) (**Table 3**). No PNF was observed in either NMP or SCS groups [29]. Incidence of EAD in NMP livers was 5/9 (55.5%) compared to 8/27 (29.6%) for SCS controls (p=0.16). EAD was caused principally by elevated transaminases in the initial 24 hours, and resolved promptly without other markers of graft dysfunction (**Figure 2**)**.** There were no cases of post-reperfusion syndrome in NMP grafts. NMP graft recipients had substantially longer median ICU stay: 16 days (range 2 - 65) vs. 4 days (range 1 - 29) in SCS controls (p=0.004) (**Table 3**). Median hospital stay in the NMP group was also much longer: 45 days (range 13 -114) vs. 25 days (range 9 - 89) in SCS controls (p=0.01) (**Table 3**).

Table 4-3. Outcome comparisons between NMP and SCS control liver transplant recipients.

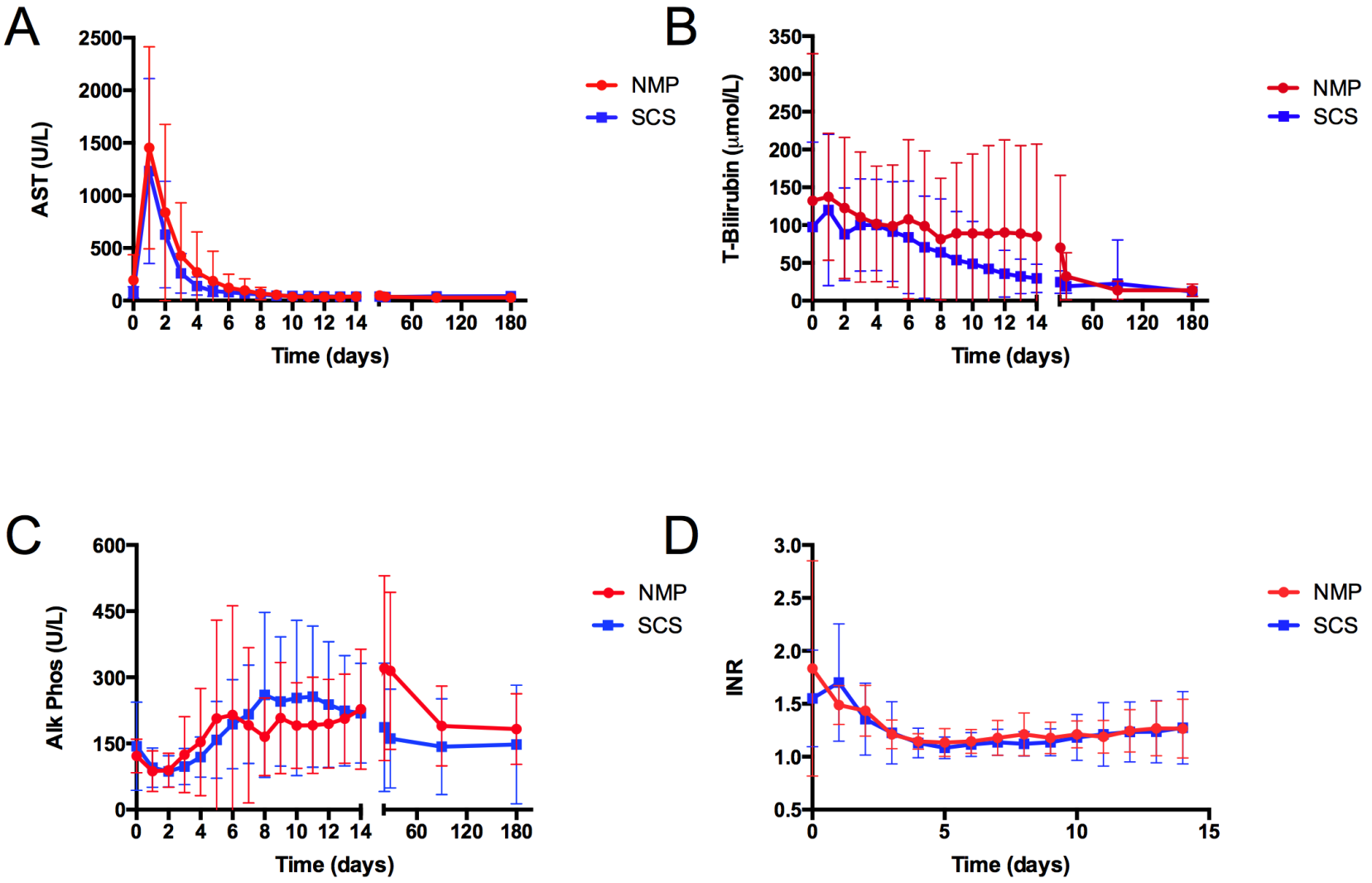
Outcomes	NMP	SCS	P Value
Intent to Treat Graft Survival	10	30	
30-day graft survival (primary outcome), intent to treat	9/10 (90%)	(30/30) (100%)	0.25
6-month graft survival, intent to treat	8/10 (80%)	(30/30) (100%)	0.06
Recipient outcomes for transplanted grafts, per protocol	9	27	
Total graft preservation time (median – range)	786 min (304 – 1631)	235 (64 – 890)	<0.001
Recipient age (median – range)	53 years (28 – 67)	59 years (43 – 69)	0.28
Recipient MELD (median – range)	13 (9 – 32)	19 (7 – 34)	0.37
Peak AST (U/l) days 1 -7 (median – range)	1252 (383 – >2600)	839 (153 – >2600)	0.52
Bilirubin day 7 (median – range)	79 (17 – 344)	53 (8 – 340)	0.35
INR day 7 (median – range)	1.1 (1.1 – 1.6)	1.1 (0.9 – 1.5)	0.44
PNF	0/9 (0%)	0/27 (0%)	-
EAD	5/9 (55.5%)	8/27 (29.6%)	0.23
DBD	2/6 (33%)	3/20 (15%)	0.56
DCD	3/3 (100%)	5/7 (71%)	0.30
ICU stay (median – range)	16 days (2 – 65)	4 days (1 – 29)	0.004
Hospital stay (median – range)	45 days (13 – 114)	25 days (9 – 89)	0.01
Major complications (Clavien-Dindo ≥ 3)	2/9 (22%)	10/27 (37%)	0.69
6-month biliary complications	0/8 (0%)	4/27 (14.8%)	0.55
30-day patient survival, per protocol	9/9 (100%)	27/27 (100%)	-
6-month patient survival, per protocol	8/9 (89%)	27/27 (100%)	0.25

4.4.4 Post-transplant Liver Function

There was no statistical difference between peak AST levels within the first 7 days in NMP vs. SCS preserved grafts (p=0.52, **Table 3**). Overall, there was no statistical difference between post-transplant AST trends in both groups (p=0.24, **Figure 2A**). There was no difference in bilirubin levels between groups on day 7 (p=0.35, **Table 3**) and globally (p=0.17, **Figure 2B**). Post-transplant alkaline phosphatase (ALP) levels were similar in recipients of NMP and SCS grafts (p=0.82) (**Figure 2C**). Comparison of coagulation parameters demonstrated stable, normal uncorrected INR synthetic function in both groups (p=0.63) (**Figure 2D**). Arterial lactate was not significantly different between groups, and normalized within one day (p=0.07) (**Figure 2E**).

4.4.5 *Complications*

Post-transplant, there was no hepatic arterial or portal vein thrombosis in either group, as determined by serial ultrasound Doppler interrogation. Comparing Clavien-Dindo scores ≥ 3, there was no significant difference in major complication rate in the initial 30 days between groups (2/9 (22%) NMP vs. 10/27 (37%) SCS controls, (p=0.41) (**Table 3**)**.** In the NMP group, complications included one re-operation for intra-abdominal bleeding, and one patient with renal insufficiency requiring transient hemodialysis. One NMP graft (Subject 8) developed early aggressive cholestatic hepatitis secondary to uncontrolled recurrent hepatitis C infection, and was unable to access preemptive sofosbuvir/ledipasvir, and died at 3 months post transplant. We cannot completely exclude the possibility that these complications were absolutely unrelated to NMP. Therefore 6-month patient survival for transplanted grafts was 8/9 (89%) in NMP and 27/27 (100%) in SCS controls (p=0.08, **Table 3**)..


A
AST (U/L)
2500
2000
1500
1000
500
0
0 2 4 6 8 10 12 14 60 120 180
Time (days)
B
NMP
SCS
T-Bilirubin (μmol/L)
350
300
250
200
150
100
50
0
0 2 4 6 8 10 12 14 60 120 180
Time (days)
NMP
SCS
C
Alk Phos (U/L)
600
450
300
150
0
0 2 4 6 8 10 12 14 60 120 180
Time (days)
D
NMP
SCS
INR
3.0
2.5
2.0
1.5
1.0
0.5
0 5 10 15
Time (days)
NMP
SCS

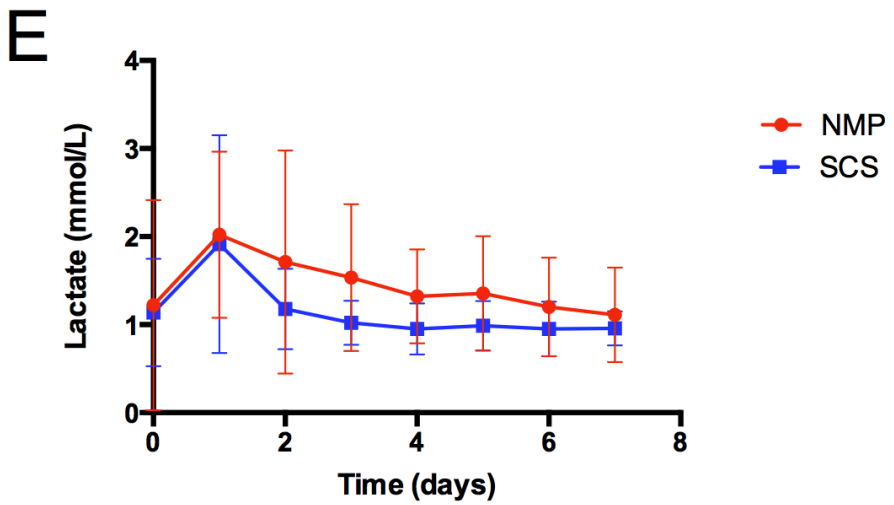


Figure 4-2. Post-transplant AST levels, markers of cholestasis, and post-transplant serum metabolic profiles.

A. Post-operative AST levels of graft recipients having received NMP and SCS livers. There was no statistical significance between recipients of NMP vs. SCS control livers (p=0.24). Data points show mean and standard deviation (SD), 95% confidence interval. B. Post-operative total bilirubin levels of graft recipients having received NMP and SCS livers (p=0.17). C. ALP levels in graft recipients having received NMP and SCS livers, (p=0.82). Data points show mean and standard deviation (SD), 95% confidence interval. D. Post-operative uncorrected INR levels of graft recipients having received NMP and SCS livers (p=0.63); E. Post-operative lactate levels of graft recipients having received NMP and SCS livers, (p=0.07). Data points show mean and standard deviation (SD), 95% confidence interval.

Biliary reconstructions in NMP grafts were duct-to-duct in 5/9 (56%) cases and roux-en-Y hepaticojejunostomy in the remainder. All 8 surviving subjects reached 6 months of follow-up, with no biliary complications in NMP livers compared to 4/27 (14.8%) in SCS controls (p=0.25), as assessed by liver function, ultrasound and magnetic resonance cholangiopancreatography (MRCP) (**Table 3**)**.** There was no ischemic cholangiopathy or anastomotic structuring by MRCP in any recipients of NMP group.

4.5 Discussion

Recently, Friend and colleagues partnered with a spin-off company associated with the University of Oxford, OrganOx UK, to fully automate, miniaturize and make portable an NMP liver system termed *metra*®. Their clinical experience in 20 initial livers undergoing NMP on *metra*® and subsequently transplanted at King's College Hospital London and University Hospital Birmingham demonstrated NMP technology to be safe and feasible [23]. When outcomes were compared with recipients of control SCS grafts, AST in recipients of NMP livers was significantly lower [23]. In a recent, similar study using identical technology, Selzner *et al*. from the University of Toronto perfused 12 liver grafts on NMP, using Steen solution in place of Gelofusine to prime, with transplanted graft outcomes comparable to SCS controls. No differences in post-operative graft function, duration of ICU stay, or post transplant hospital stay were observed when compared to SCS. NMP grafts had lower AST and ALT levels on days 1-3, without reaching statistical significance [30].

Using the OrganOx *metra*® technology with Gelofusine as the priming perfusate, we were the first in North America to successfully perfuse and clinically transplant a liver

with NMP on February 19, 2015. Our findings are concerning, however, as one liver intended for NMP perfusion was discarded, which potentially could have been transplanted. Furthermore, in contrast to Ravikumar *et al.,* and Selzner *et al.*, we did not observe lower early recipient transaminases in NMP perfused grafts [23,30]. ICU and total hospital stay was also more prolonged for recipients of NMP livers in our study.

Based on the experience of Ravikumar *et al.* and Selzner *et al.*, we had anticipated that NMP grafts would experience less transaminitis and improved early function. In our study, the median peak AST in the first 7 days in NMP grafts was higher (1,252 IU, range 383->2,600) than that observed in the Oxford/UK pilot study (417 IU, range 84 – 4,681), although these were not significantly higher than our SCS controls.

Despite the similarities between these comparative studies, there are several notable differences, which may partly explain our findings. Despite using a higher proportion of DCD livers (40%), we frequently prolonged the NMP period for logistical convenience, and cannot exclude the possibility that this could have exacerbated graft injury and may have been detrimental to early graft function (**Supplementary Figure S4**). Further, our cold ischemic period before initiation of NMP (**Tables 1 and 2**) was prolonged, mostly due to a higher proportion of complex back-table vascular reconstructions, potentially limiting the protective benefit of NMP [31], The median cold ischemia time of our control livers is notably shorter (3.9 h, range 1.07 – 14.8 h) than that reported in the Ravikumar study (8.9 h, range 4.2 - 11.4 h), which may also explain the variation in our findings. We cannot discount the effect of the use of different initial cold vascular flush preservation solution (HTK in Canada versus University of Wisconsin (UW) solution in the UK). While theoretically this should not contribute to graft injury, the potential of

carry-over of HTK to the warm NMP perfusate may have been additive to injury, which we attempted to correct in our more recent cases without obvious detectable effect. While all of this may have contributed to compromised early graft function, all transplanted livers demonstrated equivalent survival at 30 days compared to SCS controls.

Markers of cholestasis were non-significantly elevated within the initial 14 days post-transplant in recipients of NMP grafts. Despite this, no NMP grafts had late biliary complications detected by MRCP (0/8 at 6 months). In the Oxford/UK series, biliary strictures occurred in 4/20 cases (20%) by 6 months in NMP grafts, possibly reflecting a lower predilection for roux-en-Y hepaticojejunostomy in that series. We used roux biliary reconstructions in 44% of cases, largely due to donor-recipient duct size mismatch, which may have mitigated this risk.

ICU and total hospital stays were substantially prolonged in the NMP group compared to the SCS controls in the present study, which is concerning. While we largely attribute this to patient and concurrent disease-related factors, we cannot exclude the possibility that application of NMP technology contributed in some manner to these findings. Cost savings in terms of both ICU and total hospital stays will be an important measure in the future adoption and reimbursement of NMP technology, and requires a randomized controlled trial to further clarify the cost-benefit.

The loss of a potentially transplantable liver graft occurred in our fifth perfusion. A DCD liver from a 60 year old donor was discarded as a result of a failed technical attempt at successful NMP, due to an unrecognized twist of the donor portal vein at the level of

the hilar plate (**Video S1**). We rapidly confirmed that the portal cannula was located in the mid-portal vein, that the portal tubing was neither kinked nor clamped, completely disconnected and reconnected the liver twice, but still failed to recognize the occult twist. Reconnecting and adjusting the portal cannula orientation was insufficient to release this high twist which occurred due to the weight of the portal tubing. We later noted high tension in the portal vein by palpation. These observations in retrospect are striking, and unfortunate. We and others are now sensitized to this potential error, and the *metra*® manual has been amended specifically to alert to this pattern. This event highlights potential pitfalls in applying novel technologies as well as the learning curve involved with their early adoption.

The current study provides an analysis of perfusate biochemistry of human livers during NMP, including perfusate transaminases, lactate, total bilirubin and bile production. We found that circulating transaminase leak increased progressively over time, as did circulating bilirubin, with markedly elevated levels in NMP perfusates from DCD grafts. Similarly, bile output of DCD livers was less than DBD grafts. All NMP grafts demonstrated stable portal vein and hepatic artery flow rates. All of these livers were transplanted successfully, and functioned adequately in recipients, evidenced by post-operative normalizing lactate and INR. This data extends but differs from previous observations from large animal studies [14,22], and from perfusion studies of discarded human livers [32,33], where both 'on circuit' transaminases and bile output were deemed to be surrogate markers of graft viability. Our study was not designed or powered to shed light on this issue, and remains an unmet critical determinant for future assessment of graft viability in marginal livers.

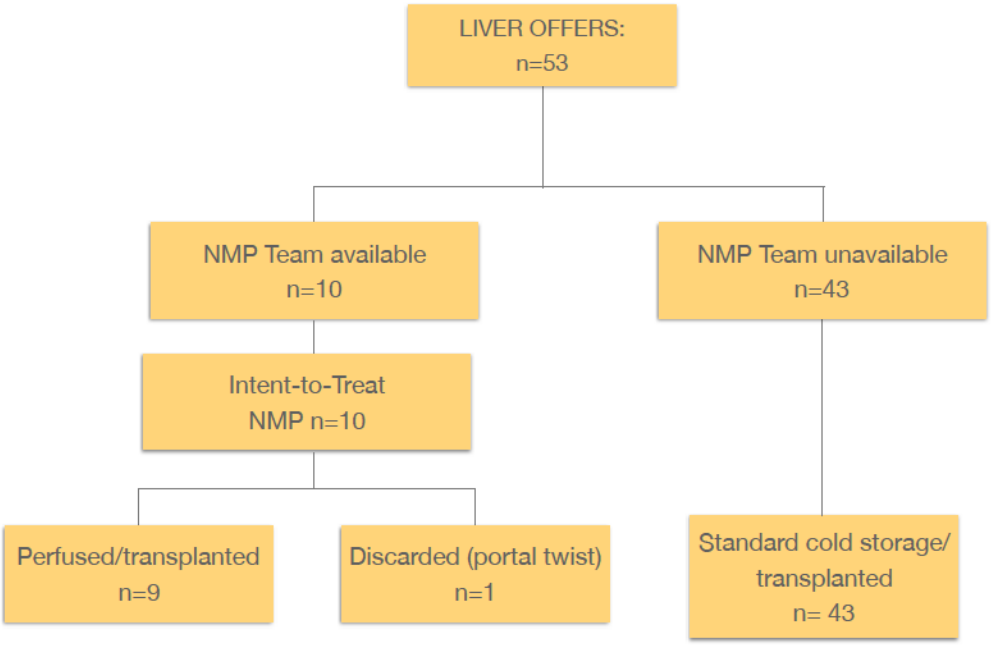
Limitations of the present study include non-randomized design, small cohort size, limited matching between NMP and SCS groups, and relatively short (6 month) follow-up. We acknowledge imbalance in selection and quality of liver donor organs between NMP and SCS groups, and while imperfect, the control group serves as comparative data for context and perspective. We further acknowledge potential for inclusion bias in the NMP group, with selection of subjects with considerable comorbidities and other cofounders that could have detracted from the robustness of this comparative study. The cold ischemic times taken to complete back-table preparation, cannulation and complex arterial reconstructions were considerable (median 2 hours 47 minutes), and likely offset the potential benefit of NMP technology in the present study. We anticipate that with greater experience, these times will become markedly curtailed.

A large prospective UK/Europe Phase 3 randomized controlled trial comparing NMP on *metra*® with SCS is completed but not yet published (ISRCTN 14355416). This trial will provide a more complete and robust comparison of outcomes.

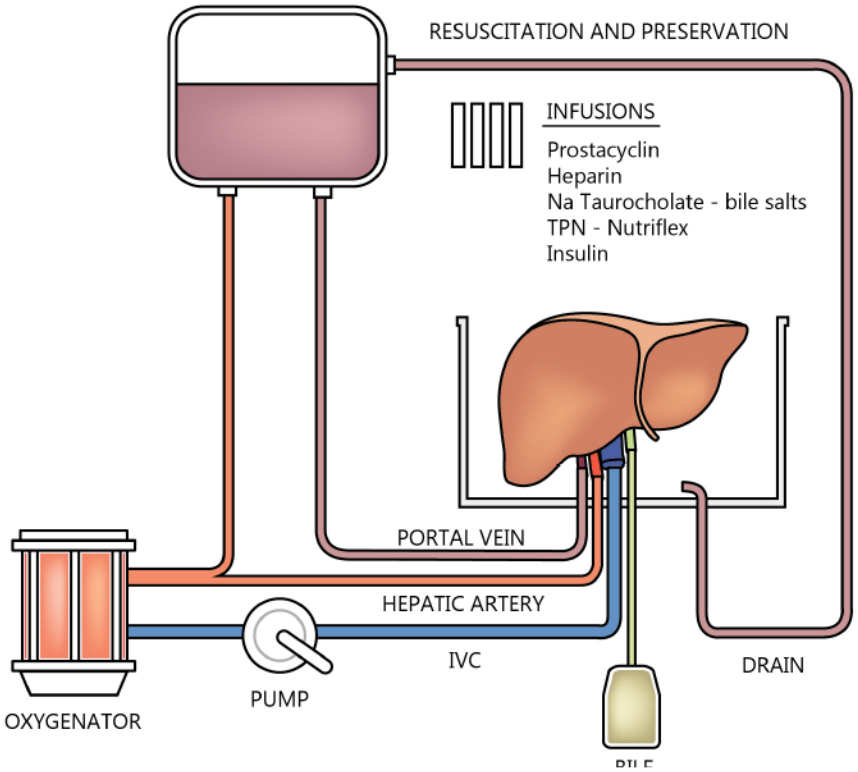
It further remains to be established whether NMP technology will expand the limited liver donor pool in a safe manner, or salvage livers that are routinely discarded presently. Based on the current study, we are unable to state that NMP technology improved either quantity or quality of liver organs at this juncture.

On an intent-to-treat basis, we observed significantly lower 6-month graft survival in NMP-perfused livers, but no significant differences in patient survival, peak AST, bilirubin or lactate between NMP and SCS liver grafts. Furthermore, the current study raises concern with the loss of a potentially transplantable liver graft as a direct result of

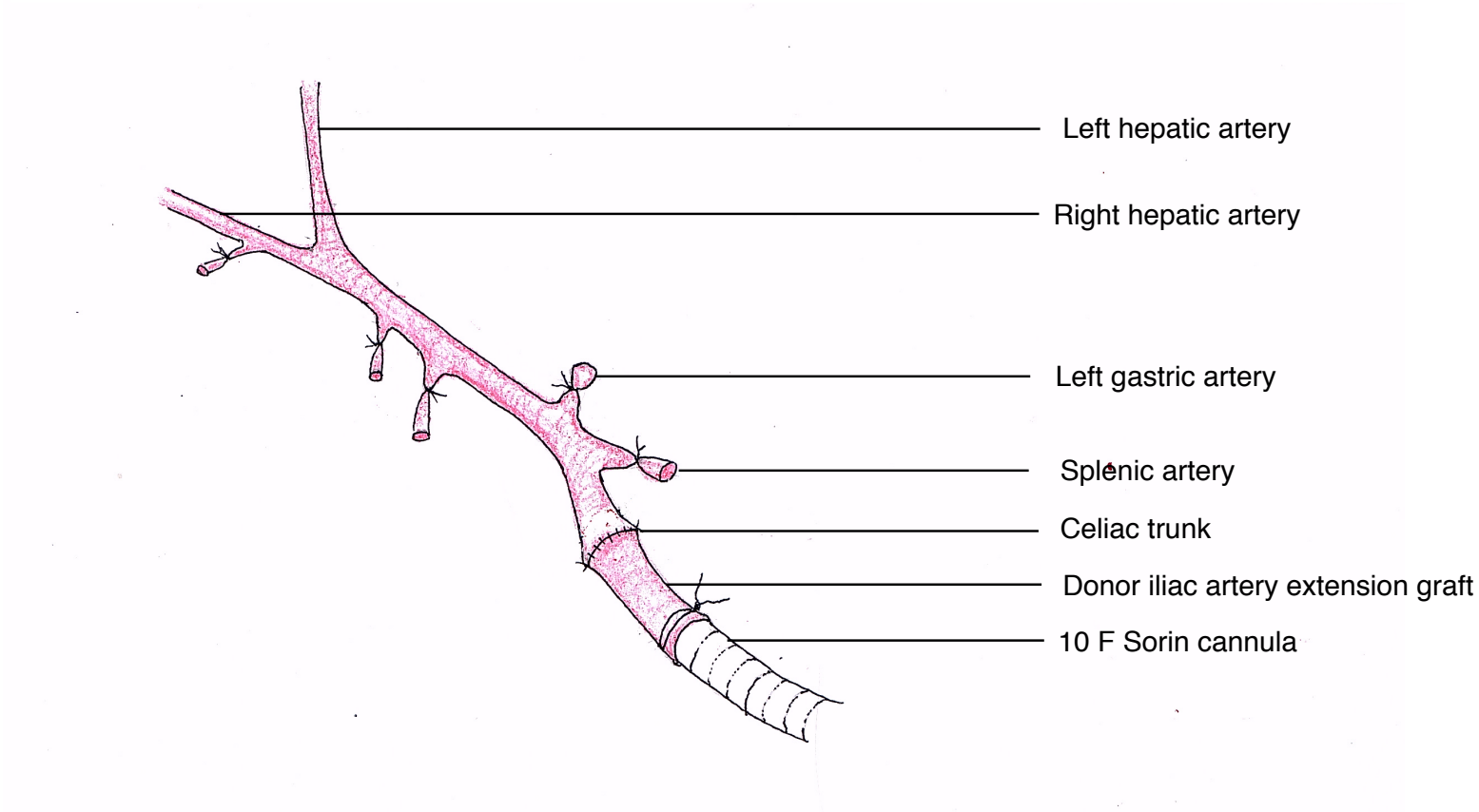
the NMP technology and operator error. We cannot discount the likelihood that as others adopt this technology, additional preventable graft losses will be sustained. We cannot exclude the possibility that our decisions to prolong certain NMP perfusions did not offset some of the potential benefit of this technology. Our prolonged ICU and hospital stays remain concerning and unexplained. Development of reliable *ex vivo* predictive potency scores remains a priority to guide surgical teams when more extreme marginal grafts are preserved with NMP. Overall, this study highlights plausible challenges of clinical NMP, and provides important groundwork for future trials that will explore the feasibility of this technology in expanding the current limited donor pool.



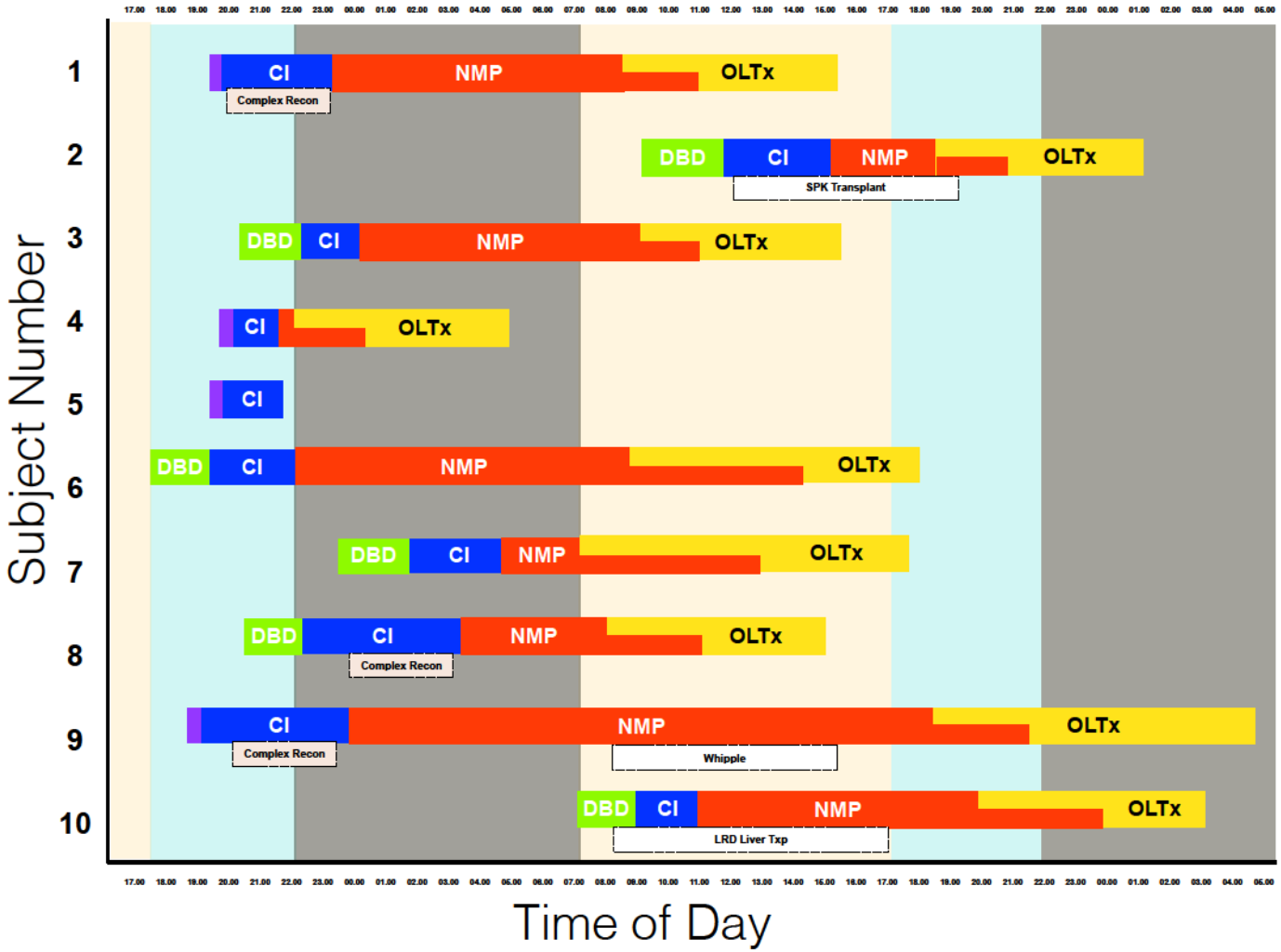
Supporting Figure S 2. Consort diagram demonstrating liver allocation.



Supporting Figure S 1. Schematic diagram of the OrganOx metra circuit.



Supporting Figure S 3. Schematic diagram of iliac artery extension graft.



Supporting Figure S 4. Timing logistics of normothermic machine perfusion (NMP) grafts

Legend: Purple bar – DCD withdrawal of life-support; Green Bar (DBD) – donation after brain death surgical retrieval period; Blue bar – Cold Ischemic (CI) period for liver preparation, complex microvascular reconstruction (denoted Complex Recon); Red bar – duration of normothermic perfusion (NMP); Yellow bar – timing of liver transplantation (OLTx); White box – competing transplant surgical activity by the recipient team.

4.6 References

1. Cursio R, Gugenheim J. Ischemia-Reperfusion Injury and Ischemic-Type Biliary Lesions following Liver Transplantation. *J Transplant.* 2012;2012:164329.

2. Grewal HP, Willingham DL, Nguyen J, et al. Liver transplantation using controlled donation after cardiac death donors: an analysis of a large single-center experience. *Liver Transpl.* 2009;15(9):1028-1035.

3. de Vera ME, Lopez-Solis R, Dvorchik I, et al. Liver transplantation using donation after cardiac death donors: long-term follow-up from a single center. *Am J Transplant.* 2009;9(4):773-781.

4. Op den Dries S, Sutton ME, Lisman T, Porte RJ. Protection of bile ducts in liver transplantation: looking beyond ischemia. *Transplantation.* 2011;92(4):373-379.

5. Vogel T, Brockmann JG, Coussios C, Friend PJ. The role of normothermic extracorporeal perfusion in minimizing ischemia reperfusion injury. *Transplant Rev (Orlando).* 2012;26(2):156-162.

6. Ravikumar R, Leuvenink H, Friend PJ. Normothermic liver preservation: a new paradigm? *Transpl Int.* 2015;28(6):690-699.

7. Fondevila C, Hessheimer AJ, Maathuis MH, et al. Hypothermic oxygenated machine perfusion in porcine donation after circulatory determination of death liver transplant. *Transplantation.* 2012;94(1):22-29.

8. Schlegel A, Rougemont O, Graf R, Clavien PA, Dutkowski P. Protective mechanisms of end-ischemic cold machine perfusion in DCD liver grafts. *J Hepatol.* 2013;58(2):278-286.

9. Knaak JM, Spetzler VN, Goldaracena N, et al. Subnormothermic ex vivo liver perfusion reduces endothelial cell and bile duct injury after donation after cardiac death pig liver transplantation. *Liver Transpl.* 2014;20(11):1296-1305.

10. Bruinsma BG, Yeh H, Ozer S, et al. Subnormothermic machine perfusion for ex vivo preservation and recovery of the human liver for transplantation. *Am J Transplant.* 2014;14(6):1400-1409.

11. Spetzler VN, Goldaracena N, Echiverri J, et al. Subnormothermic ex vivo liver perfusion is a safe alternative to cold static storage for preserving standard criteria grafts. *Liver Transpl.* 2016;22(1):111-119.

12. Butler AJ, Rees MA, Wight DG, et al. Successful extracorporeal porcine liver perfusion for 72 hr. *Transplantation.* 2002;73(8):1212-1218.

13. Fondevila C, Hessheimer AJ, Maathuis MH, et al. Superior preservation of DCD livers with continuous normothermic perfusion. *Ann Surg.* 2011;254(6):1000-1007.

14. Boehnert MU, Yeung JC, Bazerbachi F, et al. Normothermic acellular ex vivo liver perfusion reduces liver and bile duct injury of pig livers retrieved after cardiac death. *Am J Transplant.* 2013;13(6):1441-1449.

15. Guarrera JV, Henry SD, Samstein B, et al. Hypothermic machine preservation in human liver transplantation: the first clinical series. *Am J Transplant.* 2010;10(2):372-381.

16. Dutkowski P, Schlegel A, de Oliveira M, Mullhaupt B, Neff F, Clavien PA. HOPE for human liver grafts obtained from donors after cardiac death. *J Hepatol.* 2014;60(4):765-772.

17. Hoyer DP, Mathe Z, Gallinat A, et al. Controlled Oxygenated Rewarming of Cold Stored Livers Prior to Transplantation: First Clinical Application of a New Concept. *Transplantation.* 2016;100(1):147-152.

18. Perera T, Mergental H, Stephenson B, et al. First human liver transplantation using a marginal allograft resuscitated by normothermic machine perfusion. *Liver Transpl.* 2016;22(1):120-124.

19. Watson CJ, Kosmoliaptsis V, Randle LV, et al. Preimplant Normothermic Liver Perfusion of a Suboptimal Liver Donated After Circulatory Death. *Am J Transplant.* 2016;16(1):353-357.

20. Schon MR, Kollmar O, Wolf S, et al. Liver transplantation after organ preservation with normothermic extracorporeal perfusion. *Ann Surg.* 2001;233(1):114-123.

21. Liu Q, Nassar A, Farias K, et al. Sanguineous normothermic machine perfusion improves hemodynamics and biliary epithelial regeneration in donation after cardiac death porcine livers. *Liver Transpl.* 2014;20(8):987-999.

22. Brockmann J, Reddy S, Coussios C, et al. Normothermic perfusion: a new paradigm for organ preservation. *Ann Surg.* 2009;250(1):1-6.

23. Ravikumar R, Jassem W, Mergental H, et al. Liver transplantation after ex vivo normothermic machine preservation: a Phase 1 (first-in-man) clinical trial. *Am J Transplant.* 2016.

24. Olthoff KM, Kulik L, Samstein B, et al. Validation of a current definition of early allograft dysfunction in liver transplant recipients and analysis of risk factors. *Liver Transpl.* 2010;16(8):943-949.

25. Dindo D, Demartines N, Clavien PA. Classification of surgical complications: a new proposal with evaluation in a cohort of 6336 patients and results of a survey. *Ann Surg.* 2004;240(2):205-213.

26. Chung IS, Kim HY, Shin YH, et al. Incidence and predictors of post-reperfusion syndrome in living donor liver transplantation. *Clin Transplant.* 2012;26(4):539-543.

27. Toso C, Merani S, Bigam DL, Shapiro AM, Kneteman NM. Sirolimus-based immunosuppression is associated with increased survival after liver transplantation for hepatocellular carcinoma. *Hepatology.* 2010;51(4):1237-1243.

28. Axelrod DA, Dzebisashvilli N, Lentine KL, et al. National assessment of early biliary complications after liver transplantation: economic implications. *Transplantation.* 2014;98(11):1226-1235.

29. Kornasiewicz O, Bojarczuk K, Bugajski M, Golab J, Krawczyk M. Application of a proteomic approach to identify proteins associated with primary graft non-function after liver transplantation. *Int J Mol Med.* 2012;30(4):755-764.

30. Selzner M, Goldaracena N, Echeverri J, et al. Normothermic Ex Vivo Liver Perfusion Using Steen Solution as Perfusate for Human Liver Transplantation-First North American Results. *Liver Transpl.* 2016.

31. Reddy SP, Bhattacharjya S, Maniakin N, et al. Preservation of porcine non-heart-beating donor livers by sequential cold storage and warm perfusion. *Transplantation.* 2004;77(9):1328-1332.

32. Sutton ME, op den Dries S, Karimian N, et al. Criteria for viability assessment of discarded human donor livers during ex vivo normothermic machine perfusion. *PLoS One.* 2014;9(11):e110642.

33. Verhoeven CJ, Farid WR, de Jonge J, Metselaar HJ, Kazemier G, van der Laan LJ. Biomarkers to assess graft quality during conventional and machine preservation in liver transplantation. *J Hepatol.* 2014;61(3):672-684.

5 Chapter 5.

A ‘Back-to-base’ experience of human normothermic *ex situ* liver perfusion: Does the chill kill?

A Back-to-Base Experience of Human Normothermic Ex Situ Liver Perfusion: Does the Chill Kill?

Mariusz Bral,[1*] Khaled Dajani,[1*] Dayne Leon Izquierdo,[1] David Bigam,[1] Norman Kneteman,[1] Carlo D. L. Ceresa,[2] Peter J. Friend,[2,3] and A. M. James Shapiro[1]

[1]Department of Surgery, University of Alberta, Edmonton, Canada; [2]Nuffield Department of Surgical Sciences, University of Oxford, Oxford, United Kingdom; and [3]OrganOx Ltd., Oxford, United Kingdom

Normothermic machine perfusion (NMP) has been shown to protect livers from injury between procurement and transplantation in a randomized controlled trial, where the machine was transported to and from the donor center. The aim of this study was to determine whether an alternative, more practical back-to-base approach after initial static cold storage would compromise beneficial outcomes. Between February 2015 and June 2018, a nonrandomized pilot study was performed at a single site. Outcomes of back-to-base livers (n = 26) were compared with those of grafts procured locally that underwent immediate NMP (n = 17). The primary outcome measure (safety) was defined as 30-day patient and graft survival. A total of 46 liver grafts were perfused with NMP, of which 3 were discarded based on poor ex situ perfusion function. The 30-day patient and graft survival in the back-to-base and local NMP groups were both 100% (primary outcome: safety). Despite significantly prolonged mean cold ischemia time (6 versus 3.2 hours; $P = 0.001$), the back-to-base livers demonstrated no difference in graft function, incidence of complications, or graft and patient survival. In conclusion, the back-to-base approach was safe, did not compromise the overall benefit of NMP, and offers a practical alternative to portable normothermic ex situ machine transport.

Liver Transplantation 25 848–858 2019 AASLD.
Received December 12, 2018; accepted March 9, 2019.

With the hope of mitigating the worldwide shortage of donor organs, researchers in recent years have participated in an international resurgence in developing ex situ machine perfusion technology as an alternative to the established clinical standard of organ preservation with icebox static cold storage (SCS). Different ex situ machine designs have been used to evaluate different perfusate temperatures applied in various preservation strategies.[1-3]

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CIT, cold ischemia time; CVA, cerebrovascular accident; DBD, donation after brain death; DCD, donation after circulatory death; DRI, donor risk index; EAD, early allograft dysfunction; HTK, histidine-tryptophan-ketoglutarate; IC, ischemic cholangiopathy; ICH, intracranial hemorrhage; ICU, intensive care unit; INR, international normalized ratio; MELD-Na, Model for End-Stage Liver Disease–sodium; NASH, nonalcoholic steatohepatitis; NMP, normothermic machine perfusion; PNF, primary nonfunction; SCS, static cold storage; UNOS, United Network for Organ Sharing; WIT, warm ischemia time.

In one approach, normothermic machine perfusion (NMP; 35°C-38°C) aims to provide a near-physiologic environment for a potential liver graft, maintaining it in a fully functional metabolic state. NMP is the temperature modality that provides the most compelling theoretical advantage of ex situ organ viability assessment, allowing for more optimal graft selection before transplantation.

Large animal studies have demonstrated the superiority of NMP compared with SCS in liver preservation.[4-8] One experimental porcine study previously demonstrated that delaying initiation of NMP resulted in inferior graft function.[9] This provided a compelling rationale for the development of a portable NMP device that could be transported to the donor center, minimizing the cold ischemic injury. Indeed, some have advocated and clinically tested the feasibility of in situ donor liver perfusion to completely avoid any cold ischemia phase, which is an approach that may hold a particular advantage in severely steatotic grafts.[10]

Rapid clinical translation confirmed the formative evidence from experimental animal studies, and

Original article published after peer review in *Liver Transplantation* 2019 ; (25): 848-858

Original Paper

Title: A 'Back-to-base' Experience of Human Normothermic Ex Situ Liver Perfusion: Does the chill kill?

Authors: Mariusz Bral, MD[1,5,6], Khaled Dajani MD PhD[1,5,6,], Dayne Leon Izquierdo MD[1], David Bigam MD[1], Norman Kneteman MD[1,5], Carlo D.L. Ceresa[3], Peter J Friend MD [3,4], and A.M. James Shapiro MD PhD[1,5]

[1] Department of Surgery, University of Alberta, Edmonton, Canada

[2] Institute of Biomedical Engineering, University of Oxford, UK

[3] Nuffield Department of Surgical Sciences, University of Oxford, UK

[4] OrganOx UK

[5] Members of the Canadian Donation and Transplantation Research Program (CDTRP)

[6] Denotes joint first author

Corresponding Author:

A.M. James Shapiro, MD, PhD, Canada Research Chair in Transplantation Surgery and Regenerative Medicine, Professor, Director of Clinical Islet and Living Donor Liver Transplant Programs, Clinical Islet Transplant Program, University of Alberta. 2000 College Plaza, 8215-112th St, Edmonton T6G 2C8, Alberta, Canada. amjs@islet.ca

Telephone: +1-780-4077330, Fax: +1-780-4078259

5.1 Abstract

Rationale: Normothermic machine perfusion (NMP) has been shown to protect livers from injury between procurement and transplantation in a randomized controlled trial, where the machine was transported to and from the donor center. The aim of this study was to determine whether an alternative, more practical 'Back-to-base' approach after initial static cold storage (SCS) would compromise beneficial outcomes.

Methods: Between February 2015 and June 2018, a non-randomized pilot study was performed at a single site. Outcomes of 'Back-to-base' livers (n=26) were compared to grafts procured locally having undergone immediate NMP (n=17). The primary outcome measure (safety) was defined as 30-day patient and graft survival.

Results: A total of 46 liver grafts were perfused with NMP, of which three were discarded based on poor *ex situ* perfusion function. The 30-day patient and graft survival in the 'Back-to-base' and 'Local NMP' groups were both 100% (primary outcome, safety). Despite significantly prolonged mean cold ischemia time (6 vs 3.2 hours) (p=0.001), 'Back-to-base' livers demonstrated no difference in graft function, incidence of complications, or graft and patient survival.

Conclusions: The 'Back-to-base' approach was safe, did not compromise overall benefit of NMP, and offers a practical alternative to portable normothermic *ex situ* machine transport

5.2 Introduction

With hope of mitigating the worldwide shortage of donor organs, recent years have demonstrated an international resurgence in developing *ex situ* machine perfusion technology as an alternative to the established clinical standard of organ preservation with static 'ice-box' cold storage. Different *ex situ* machine designs have been utilised to evaluate different perfusate temperatures applied in various preservation strategies (1-3).

In one approach, normothermic perfusion (NMP) (35°C-38°C) aims to provide a near-physiologic environment for a potential liver graft, maintaining it in a fully functional metabolic state. NMP is the temperature modality which provides the most compelling theoretical advantage of *ex situ* organ viability assessment, allowing for more optimal graft selection before transplantation.

Large animal studies have demonstrated superiority of NMP compared to Static Cold Storage (SCS) in liver preservation (4-8). One experimental porcine study had previously demonstrated that delaying initiation of NMP resulted in inferior graft function (9). This provided compelling rationale for development of a portable NMP device that could be transported to the donor center, minimizing cold ischemic injury. Indeed, some have advocated and tested clinically the feasibility of *in situ* donor liver perfusion to completely avoid any cold ischemic phase, an approach that may hold particular advantage in severely steatotic grafts (10).

Rapid clinical translation confirmed the formative evidence from experimental animal studies, and a first-in-human pilot study conducted in the UK by the Oxford Group and OrganOx Ltd. confirmed feasibility and safety of this technology (11). These findings were re-confirmed in two other pilot clinical series (12, 13). The recent publication of a 220 subject multi-center randomized controlled trial comparing NMP with SCS conducted across the UK and Europe has now definitively established NMP as a clinically relevant and beneficial technology. This landmark study clearly demonstrated less graft injury with NMP, despite much longer preservation times, with overall higher graft utilization rates (14). These positive findings may be attributed to reduced ischemia-reperfusion injury during NMP, as a consequence of the combination of inflammation inhibition and upregulation of a regenerative profile of gene expression by the perfused graft (15). In most prior clinical NMP studies, machine perfusion was initiated as early as possible at the donor center, utilizing a 'Preservation Machine Perfusion' strategy in order to derive maximal benefit from *ex situ* NMP conditions (11-14, 16). This required transportation of the device with a technical team to and from the donor center, and maintenance of NMP throughout transportation back to the recipient center. All of the accrued experience to date mandated transportation by road using a dedicated and modified ambulance with built in power supply. While technically possible to transport by plane, the weight of the loaded device (approximately 80kg) and the confines of the cabin provide additional technical, physical and cost challenges We therefore sought to determine whether an alternative *ex situ* preservation strategy was feasible, using a 'Post Static Cold Storage Machine Perfusion' approach. Liver grafts were procured and transported from distant sites in SCS using routine transportation, with delayed initiation of NMP at our recipient center. Positive findings from such a strategy (termed 'Back-to-base') would potentially obviate the need for the

transport phase of the NMP process, simplifying organ preservation logistics and potentially lowering cost. In a previous publication, Watson *et al* described a clinical series of NMP perfused livers transplanted after a period of cold storage with mixed outcomes, confirming the need for further studies in this area (17).

5.3 Methods

5.3.1 Study design

Between February 2015 until June 2018, a non-randomized pilot study was performed at a single liver transplant site (University of Alberta Hospital) to evaluate outcomes of livers transported from distant retrieval sites in SCS with delayed initiation of NMP (termed 'Back to base'), compared to locally procured livers with immediate initiation of NMP post retrieval ('Local NMP'). The study was approved by the Health Research Ethics Board at the University of Alberta (MS3_Pro00058909 and MS8_Pro00043239) and by Health Canada and registered with clinicaltrials.gov (NCT03089840). The decision to include a graft into the NMP intervention group was made depending on the site of procurement, the logistics surrounding the operating room and the transplant team as well as the type of donor. All grafts procured from distant sites were considered for inclusion in the 'Back to base' group, and only those grafts procured at the local transplant centre (University of Alberta) were considered for inclusion in the 'Local NMP' group. We did not transport the perfusion machine to any distant centers. As the study progressed we elected to put the majority of DCD grafts on machine perfusion as most DCD withdrawals took place in the late evening. A schematic of our study and enrolment is provided in **Figure 1**.

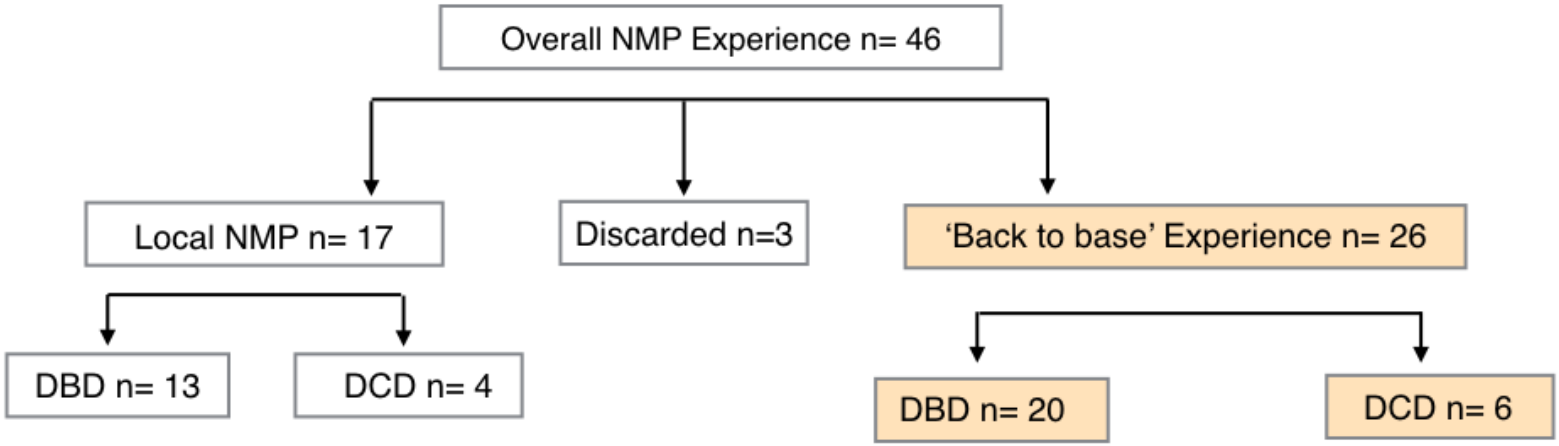


Figure 5-1. Study overview schematic.

(Donation after Brain Death= DBD, Donation after Circulatory Death= DCD, Normothermic Machine Perfusion= NMP)

The primary objective was to assess safety and efficacy of NMP for liver preservation applied in a 'Back-to-base' strategy. The primary outcome measure was safety as defined by 30-day patient and graft survival. Secondary outcome measures included: 1) Patient and graft survival at 90 days and 6 months, 2) Peak AST and peak serum alanine aminotransferase (ALT) in the first 7 days post-transplant, and months 1 and 6, 3) Incidence of early allograft dysfunction (EAD) in the first 7 days; and 4) Peak alkaline phosphatase (ALP), and total bilirubin in the first 7 days, and months 1 and 6, 5) International normalized ratio (INR) in the first 7 days, 6) Biliary and arterial complications at 6 months. EAD was defined as the presence of one or more of the following biochemical markers post-transplant: 1) Total bilirubin >170 μmol/L on day 7, 2) INR ≥1.6 on day 7 and 3) Peak AST >2000 IU/L in the first 7 days (18). Ischemic cholangiopathy was defined as diffuse biliary structuring in the absence of significant arterial stenosis. Arterial stenosis were suspected on the basis of doppler ultrasound imaging and confirmed with CT angiography. Significant stenosis were investigated further and managed with angiography, balloon angioplasty or stenting where indicated.

All deceased donors (≥40 Kg in weight) were considered irrespective of donation type (DBD and DCD), and irrespective of standard or extended criteria status. DCD graft warm ischemia time was calculated from onset of hemodynamic instability (mean arterial pressure < 50 and oxygen saturations of <70%) until the start of cold flush in the donor. Living donors, and split transplant livers were excluded. All indications for liver transplant were accepted. Selection of potential NMP grafts in this study was conditional upon recipient consent and availability of the perfusion team.

5.3.2 Normothermic Machine Perfusion

Livers allocated to the intervention groups were preserved using the NMP system (*metra*®, OrganOx. Oxford, UK), as previously described (11, 12). All procured liver grafts were flushed *in situ* using Histidine-Tryptophan-Ketoglutarate (HTK) solution (Methapharm Inc., Brantford, Canada). Locally procured livers were cannulated promptly and placed on the NMP circuit. Livers procured at distant sites were transported using SCS until arrival at the recipient center where NMP was initiated. Cold ischemic time (CIT) was calculated from donor cross clamp time until the liver was brought into the operative field in the recipient. For NMP livers, CIT was from donor cross clamp to initiation of NMP.

5.3.3 Metabolic Parameters

Liver grafts were monitored during NMP at hourly intervals for determination of pH, lactate, ALT, AST and total bilirubin. Liver graft quality was evaluated through observed trends of perfusate biochemistry, need for bicarbonate correction, perfusion flow stability, and hourly bile production as previously suggested (14, 17, 19, 20).
Once the recipient hepatectomy was completed, NMP was discontinued, and grafts were flushed with chilled HTK solution before implantation. Surgical implantation techniques were identical between in all the groups (bicaval anastomoses, venous reperfusion after portal reconstruction, no venovenous bypass, and subsequent arterial then biliary reconstruction).

5.3.4 Viability assessment

Prior to transplant, on circuit livers were assessed for damage and viability using a number of previously reported parameters, none of which have been clinically validated to-date. At the time that we initiated our study, there were few publications in this area. As the study progressed we accrued more experience, and our decisions to accept a liver for implantation became more informed. We utilized a number of parameters including: opening lactate level, lactate clearance, necessity of bicarbonate pH correction, and bile production. We did not measure bile biochemistry.

5.3.5 Post-Transplant Care

Post-transplant care was standardized according to center-specific protocols including steroid-free tacrolimus-based maintenance immunosuppression after basiliximab induction. Sirolimus was used early and selectively in recipients with renal dysfunction, and later (after one month) in those with pre-existing hepatocellular carcinoma in the explanted grafts. All recipients of DCD liver grafts underwent magnetic resonance cholangiopancreatography (MRCP) at 6 months to assess for ischemic cholangiopathy (IC).

5.3.6 Statistical analysis

Data are represented as median and ranges. The Mann-Whitney U-test, was used to analyze differences between continuous variables. Fisher's Exact test was used to compare proportions between groups for categorical outcomes. Spearman's correlation was used to assess relationship between continuous variables and outcome.

A p-value < 0.05 was considered significant and all the analysis was performed using Stata GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

5.4 Results

5.4.1 Primary Outcome Measures

The primary outcome safety measure of 30-day patient and graft survival in the 'Back-to-base' and 'Local NMP' groups were both 100%.

5.4.2 Donor and Graft Characteristics

A total of 46 potential liver grafts were procured and perfused with NMP. Four of these were poor quality livers which we would not have transplanted based on donor characteristics. They were accepted provisionally to assess their function during ex situ NMP, with a view to transplantation had their function been adequate. All of these livers were retrieved at distant sites, and so would have been included in the 'Back to base' group had they been transplanted. On the basis of *ex situ* assessment, we successfully transplanted one graft (previously rejected by all centers in UNOS) with excellent post-operative outcome and normal graft function at 18 months of follow up. The remaining three were discarded. All remaining grafts were transplanted successfully (**Figure 1**). The three discarded livers all had on circuit levels of AST > 2600 U/L and ALT of > 5400 U/L at start of perfusion, all of which increased over the duration of the perfusion. These livers also failed to clear lactate during the perfusion**.**

Transplanted NMP grafts (n=43) were classified into two groups. The first was comprised of livers procured locally with immediate initiation of NMP ('Local NMP', n=17). The second was comprised of livers procured from distant sites, transported in SCS to the recipient center, where NMP was initiated ('Back-to-base', n=26). No difference in donor characteristics existed between these two groups (**Table 1**).

Table 5-1. Donor and graft characteristics for 'Back to base' and 'Local NMP' grafts.

	Back to base (n=26)	Local NMP (n=17)	p Value
DCD donor	6 (23%)	4 (24%)	> 0.99
Donor DRI median (range)	1.71 (1.08 – 2.93)	1.28 (0.92 – 2.39)	0.05
Donor gender: Male Female	 19 (73%) 7 (23%)	 11 (65%) 6 (35%)	 0.74 0.74
Donor age median (range)	37 years (15 – 67)	40 years (14 – 71)	0.66
Donor BMI median (range)	24.6 (20.2 – 41.1)	27.5 (19.6 – 37.3)	0.42
Donor cause of death: Trauma Anoxia CVA Other	 4 (15%) 12 (46%) 8 (31%) 2 (8%)	 4 (26%) 6 (35%) 5 (29%) 2 (12%)	 0.69 0.54 >0.99 >0.99
Warm Ischemia Time median (range)	20 min (14 - 42)	21 min (18- 25)	>0.99
Arterial Variant Reconstructions	2 (8%)	4 (23%)	0.19
Cold ischemia time median (range)	6 hrs (3.9- 8.4)	3.2 hrs (1 - 5.4)	**<0.0001**
Machine perfusion time median (range)	7.8 hrs (4- 16.8)	10.3 hrs (3.3- 22.4)	0.19
Total preservation time from cross-clamp to organ reperfusion median (range)	14.3 hrs (10.5- 24.4)	13.3 hrs (6.1- 27.3)	0.06

Ten of the 43 NMP grafts (21%) were procured from DCD donors (Maastricht category III), 4 of which were included in the Local NMP group, the remaining 6 in the 'Back-to-base' group.

Normothermic machine perfusion

The OrganOx *metra*® was primed concurrently while the liver graft 'back table' preparation surgery was being completed; NMP was established successfully in all 46 cases. While on the NMP circuit, all livers exhibited stable portal vein and hepatic arterial flow rates. Median NMP duration was 8.12 hrs (range 3.27 – 22.48 hrs). This was not significantly different in the 'Local NMP' group, 10.3 hrs (range 3.3 – 22.4) compared to the 'Back-to-base' group, 7.8 hrs (range 4 – 16.8, p=0.19). (**Table 1**). Perfusion vascular parameters and perfusate biochemistry of both groups are summarized in **Table 2**.

Of the 46 livers perfused with NMP, three were discarded based on poor *ex situ* function (**Figure 2, Table 3**). All three discarded livers were from DCD donors. There were no significant differences in median warm ischemic time (WIT) or CIT between discarded liver grafts (WIT 23 min, CIT 5.7 (5.6- 5.9 hrs)) compared to those that were implanted (WIT 20, CIT 5.5 (2.4-7.6 hrs), p=0.67). The median DRI however, was significantly higher in the discarded liver grafts (2.96 (2.74 – 3.62) compared to those that were ultimately implanted (2.0 (1.49 – 2.93), p=0.03). All discarded grafts failed to clear lactate within the first 5 hours of *ex situ* perfusion and had higher on-circuit transaminase levels (AST greater than 2600 U/L and ALT greater than 5000 U/L) (**Table 3**, **Figure 2**). At perfusion termination, the median lactate and ALT levels were significantly higher in the discarded livers (p=0.04 and p=0.01).

Table 5-2. Perfusion vascular parameters and perfusate biochemistry.

	'Back to base' (n=26)	Local NMP (n=17)	P Value
Peak Perfusion AST (U/L) Median (IQR)	927 (674-2600)	585 (522 - 2107)	0.13
Peak Perfusion ALT (U/L) Median (IQR)	623 (454 – 2160)	454 (324 – 1430)	0.17
Peak Perfusion Lactate (mmol) Median (IQR)	5.3 (3.5 – 7.3)	5.3 (1.9 – 9.9)	0.86
End Perfusion Lactate (mmol) Median (IQR)	1.5 (1 – 2.3)	0.7 (0.4 – 1.3)	0.01
Bile production (ml/hr) Median (IQR)	13.1 (2.4 – 24.3)	8.0 (4.6 – 14.3)	0.27
Hepatic artery flow (l/min) Median (IQR)	0.6 (0.5 – 0.62)	0.47 (0.4 – 0.5)	0.001
Portal Vein Flow (l/min) Median (IQR)	0.97 (0.9 – 1.0)	1.08 (1.07 – 1.1)	0.001

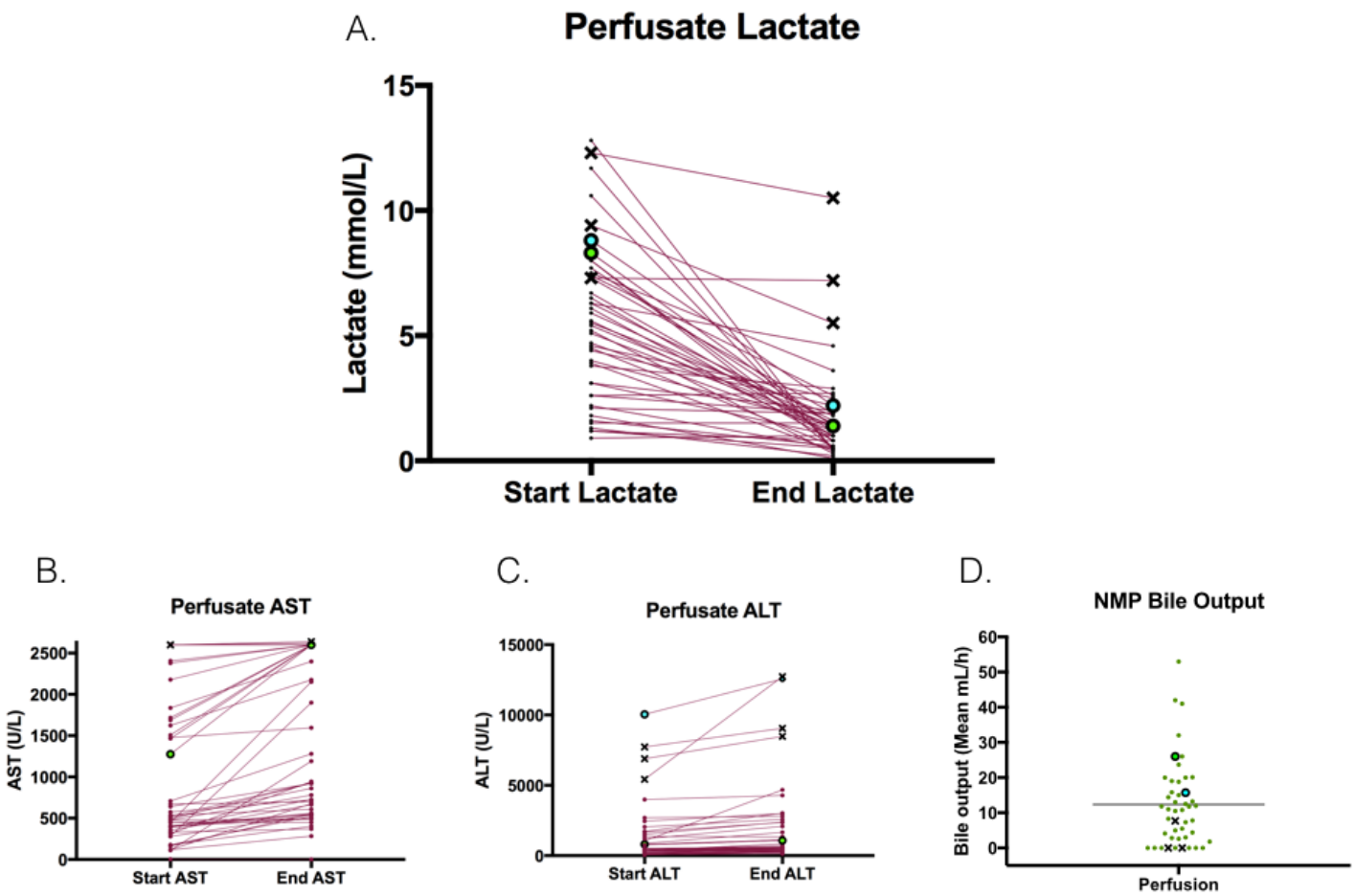


Figure 5-2. *Ex situ* perfusate during NMP.

A. Circulating perfusate lactate (mmol/L) levels at initiation and end of NMP; B. Circulating aspartate transaminase AST (U/L) perfusate levels at initiation and end of NMP; C. Circulating alanine aminotransferase (ALT) (U/L) perfusate levels at initiation and end of NMP; D. Bile output (mean mL/hour) for all livers during NMP. Discarded liver grafts are marked with an X. Blue dots indicate a liver graft which was transplanted but failed at 3 months, requiring re-transplant. Green dots indicate a graft which was rejected by all of UNOS, and ultimately successfully transplanted with normal graft function at 15 months follow-up

Table 5-3. Donor and perfusion characteristics of discarded and marginal liver grafts perfused with NMP.

Graft Type	Age	DRI	Cause of Death	BMI	WIT	Retrieval Location	Cold Ischemic Time (hours)	Perfusion Start/End AST* (U/L)	Perfusion Start/End ALT (U/L)	Perfusion Start/End Lactate	Perfusion Mean Bile Production (mL/hour)	Perfusion Duration (hours)	Graft Outcome
DCD	51	2.29	Anoxia	21.1	24	International (Rejected by UNOS)	8	1276/ 2600	805/ 1131	8.3/ 1.4	26	16	Transplanted- good graft function
DCD	29	1.78	ICH	22.3	30	Regional	7.6	2600/ 2600	10041/ 12562	8.8/ 2.2	15.7	12.03	Graft failed. Successful re-transplant at 3 months
DCD	57	2.74	Physician Assisted Suicide	24.7	23	National	5.7	2600/ 2600	5428/ 5839	7.3/ 7.2	26	5	Discarded- poor lactate clearance
DCD	64	2.96	ICH	33.6	21	National	5.6	2600/ 2600	6883/ 8484	12.3/ 10.5	0	5	Discarded- poor lactate clearance
DCD	60	3.62	Anoxia	27.2	23	National	5.9	2600/ 2600	7725/ 9050	9.4/ 5.8	7.6	9	Discarded- poor lactate clearance

The single graft that required re-transplantation from our NMP experience also had high *ex situ* perfusate transaminase levels, however it did clear lactate and produce bile, which ultimately led to a decision to proceed with implantation. This liver failed at three months post-transplant due to combined arterial and venous stenoses with secondary biliary strictures, and the recipient underwent a successful re-transplant (**Table 3**). None of the other transplanted livers had ALT levels above 5000 U/L on the NMP circuit. This also included one DCD graft which was rejected by all transplant centers across the United States through the United Network for Organ Sharing (UNOS), which was ultimately transplanted successfully at our site based on acceptable *ex situ* NMP function (**Table 3**).

We found significant correlation between the NMP circuit AST level and the subsequent development of EAD in recipients (r= 0.33 95% CI 0.02 - 0.58, p= 0.03). None of the other perfusate biochemistry (ALT, bilirubin and lactate), nor bile output correlated with post-transplant EAD (p= 0.33, 0.69, 0.8 and 0.32 respectively).

5.4.3 Preservation time

The 'Back-to-base' group had significantly longer CIT compared to the local NMP (6.0 vs 3.2 hrs, p= 0.001), and although the total preservation time was higher in the 'Back-to-base' group this did not reach statistical significance (14.3 vs 13.3 hrs, p=0.06) (**Table 1**).

5.4.4 Recipients

A total of 43 subjects underwent transplantation with livers perfused by NMP. There were no significant differences in recipient characteristics between 'Back-to-base' and 'Local NMP' groups (**Table 4**).

Biliary reconstructions in NMP grafts were duct-to-duct in 28 (65%) cases, of which 17 were in the 'Back-to-base' group and 11 were in the "Local NMP' group (p=>0.99). The decision to perform a hepatico-jejunostomy was dependent upon recipient characteristics, the main indications being size discrepancy, previous surgery including re-transplants and recipient with PSC.

Postoperatively, both the duration of intensive care unit (ICU) and total hospital stay were significantly shorter in recipients of 'Back-to-base' compared to 'Local NMP' grafts (2 vs 6 days p=0.004, and 16 vs 43 p=0.001, respectively) (**Table 4**).

5.4.5 *Complications*

Biliary complications (anastomotic and non-anastomotic) were experienced by 4 (15%) of 'Back-to-base' recipients compared to 4 (24%) of 'Local NMP' recipients (p=0.69) (**Table 4**). These were managed by endoscopic or percutaneous drainage with biliary stenting procedures in all cases. None of the recipients of NMP grafts developed ischemic cholangiopathy (IC) within the 6 months follow-up (p=0.55) **(Table 4).**

There were no hepatic arterial or portal vein thrombosis in either group. Anastomotic arterial stenoses were noted in 5 (19%) of recipients of ‘Back-to-base’, compared to 1 (6%) of ‘Local NMP’ grafts (p=0.37), as determined by doppler ultrasound,

Table 5-4. Recipient characteristics and graft outcome measures for 'Back to base' and 'Local NMP' grafts.

	'Back to base' (n=26)	Local NMP (n=17)	p Value
Recipient age median (IQR)	57 (40-63)	59 (50-63)	0.31
Recipient MELD-Na median (IQR)	22 (17-24)	25 (21-32)	0.07
Transplant Indication:			
Hepatocellular Carcinoma	7 (27%)	5 (29%)	0.56
Hepatitis C Cirrhosis	1 (4%)	1 (6%)	0.64
Autoimmune Hepatitis	1 (4%)	3 (18%)	0.28
Non-alcoholic Steatohepatitis	2 (8%)	3 (18%)	0.36
Alcohol Cirrhosis	5 (19%)	2 (12%)	0.68
Primary Sclerosing Cholangitis	3 (12%)	1 (6%)	0.64
Primary Biliary Cholangitis	3 (12%)	0 (0%)	0.26
Other	4 (15%)	2 (12%)	>0.99
PNF	0 (0%)	0 (0%)	-
EAD incidence	5 (19%)	6 (35%)	0.29
EAD incidence (DCD)	3/5 (60%)	3/6 (50%)	>0.99
Biliary Complications	4 (15%)	4 (23.5%)	0.69
Anastomotic	2 (7.5%)	4 (23.5%)	0.19
Non-anastomotic	2 (7.5%)	0	0.51
Ischemic Cholangiopathy Incidence	0 (%)	0 (0%)	-
Arterial Stenosis Incidence	5 (19%)	1 (6%)	0.37
ICU stay median (IQR)	2 days (2-4)	6 days (3-48)	**0.004**
Hospital stay median (IQR)	16 days (12-20)	43 days (22-61)	**0.001**
30-day graft survival	100%	100%	>0.99
30-day patient survival	100%	100%	>0.99
90-day graft survival	100%	100%	>0.99
90-day patient survival	100%	88%	0.1
6-month patient survival	100%	88%	0.1
6-month graft survival	94%	93%	>0.99

computerized arterial tomography or by angiography where indicated. These were managed by balloon angioplasty or stenting with no operative reconstruction required.

A single patient was re-transplanted within 6 months in the ‘Back to base” NMP group. The re-transplant was related to primary arterial and venous stenoses leading to recurrent biliary sepsis.

5.4.6 Three and Six-month Patient and Graft Survival

The 3 month and 6-month patient survival in the ‘Back-to-base’ group was 100% which was not significantly different from that of recipients of ‘Local NMP’ grafts at 88% (p=0.1) (**Table 4**). There were no deaths in the ‘Back-to-base’ group. There were two deaths in the ‘Local NMP’ group. The first patient died at 3 months of early aggressive cholestatic hepatitis secondary to uncontrolled recurrent hepatitis C infection. A second patient died with normal liver function at 3 months from recurrent sepsis after a combined liver/kidney transplant.

Graft Survival was not different between the two groups. We had one retransplant in the ‘Back to base” due to biliary structuring secondary to arterial and venous stenosis. In the ‘Local NMP’ group, one patient lost his graft from early aggressive cholestatic hepatitis secondary to uncontrolled recurrent hepatitis C infection, and eventually died from this.

5.4.7 Assesment of post-transplant injury and function

The peak AST in the first 7 days post-transplant was not significantly different in recipients of ‘Back-to-base’ and ‘Local NMP’ grafts (863 vs 709 U/L, p=0.63) (**Table 5**). No difference existed in the peak levels of the other liver function parameters assessed during the first week following transplantation (Bilirubin, ALT, ALP, and INR) when comparing the recipients of ‘Back-to-base’ and ‘Local NMP’ grafts (**Table 5**).

Table 5-5. Post transplant liver function parameters for recipients of 'Back to base' and 'Local NMP' grafts.

	'Back to base' (n=26)	Local NMP (n=17)	P Value
Peak AST (U/L) day 1 -7 median (IQR)	863 (460-1640)	709 (283-1921)	0.63
Peak ALT (U/L) day 1 -7 median (IQR)	522 (303-733)	353 (252-1536)	0.95
Peak ALP ((U/L) day 1 -7 median (IQR)	236 (130-305)	173 (99-377)	0.43
Peak Bili (μmol/L) day 1 -7 median (IQR)	74 (39-157)	124 (45-170)	0.43
Peak INR day 1 – 7 median (IQR)	1.4 (1.2-1.7)	1.4 (1.3-1.7)	0.95
1 month AST (U/L) median (IQR)	25 (18-34)	28 (18-37)	0.91
1 month ALT ((U/L) median (IQR)	23 (18-43)	26 (14-45)	>0.99
1 month ALP (U/L) median (IQR)	111 (92-163)	196 (110-275)	0.07
1 month Bili (μmol/L) median (IQR)	13 (9-25)	16 (14-23)	0.21
6 month AST (U/L) median (IQR)	31 (22-37)	24 (20-42)	0.61
6 month ALT (U/L) median (IQR)	34 (21-56)	27 (20-47)	0.35
6 month ALP (U/L) median (IQR)	96 (71-132)	149 (78-227)	0.24
6 month Bili (μmol/L) median (IQR)	9 (8-12)	12 (7-14)	0.63

Primary non-function was not observed in any group. The incidence of EAD in recipients of 'Back-to-base' grafts was 19%, which was not statistically different to that of the recipients of local NMP grafts at 35% (p= 0.29) (**Table 5**).

5.4.8 Overall NMP Experience

We further compared our overall NMP experience (n=43) with matched controls transplanted with livers preserved in static cold (n=86). NMP facilitated more daylight transplant procedures (NMP groups 84% vs SCS controls 65%, p=0.04). We further confirmed the findings of Nasralla *et al* in that the peak AST in the first 7 days was significantly lower in recipients of all NMP grafts compared to SCS grafts (784 vs 1082 U/L, p=0.04). None of the recipients of NMP grafts, compared to 3 (3.5%) of the SCS controls developed ischemic cholangiopathy (IC) within the 6 months follow-up (p=0.55).

5.5 Discussion

The first randomized controlled clinical trial comparing NMP to SCS demonstrated significant reductions in peak AST and EAD rates in NMP livers despite longer preservation times, with no differences in graft or patient survival (14). In their trial, the NMP device was transported to all donor centers, with initiation of NMP promptly following organ procurement. We sought herein to determine whether liver grafts could be transported from donor centers in SCS, until NMP could be commenced at the recipient center which would simplify the logistics and reduce the cost of the transplant process. Simultaneously with this trial but independently, a parallel study using a very similar protocol was carried out in the UK, addressing the same question.

While the prompt initiation of NMP is theoretically attractive, there are practical challenges and costs associated such a strategy. Considering our large geographic donor catchment area in Alberta, Canada, we rely on air transportation for many of our retrievals, unlike in the UK and Europe. Transporting the NMP device by air would require a fleet of larger planes, modified appropriately to accommodate power sources, fixation and grounding devices as well as radio-interference modification. Managing graft threatening perfusion problems midflight would almost certainly result in graft loss. Hence, transporting grafts from distant sites in SCS would seem to be a reasonable and practical compromise. Recently, Watson et al, described a series of transplanted livers (n=13) using an alternative NMP LiverAssist™ device (Organ Perfusion Systems, Groningen NL) with ≥ 6 hours of SCS prior to initiation of machine perfusion. This provided evidence for the potential role of a 'post-Static Cold Storage-NMP' strategy (17). In this series however, 3 of the 12 livers had serious adverse outcomes (one death following primary non-function (PNF), and two instances of ischemic cholangiopathy (IC), one requiring re-transplantation) (17). These results may be attributed to the higher risk grafts used in their series, however we have shown herein that post static cold storage NMP may be safely performed in lower risk DCD and DBD grafts.

Based on our findings, acknowledging the limited sample size and non-randomized nature of our study, we found no disadvantage to the additional period of SCS preservation time before initiating NMP. In our study, there was no statistical difference in early or late graft function, EAD, biliary or arterial complications, graft function, or patient survival noted between 'Back-to-base' and 'Local NMP' grafts.

Due to our routine use of jets for transport, the CIT accrued by grafts in the 'Back-to-base' group was 3 hours longer than those in the 'Local NMP' group, with no adverse consequences.

We are unable to state whether more extreme CIT would also be tolerated before initiation of NMP as currently no current evidence exists that NMP can reverse severe pre-existing graft damage.

There was a notable but non-significant higher incidence of anastomotic arterial stenoses in the 'Back-to-Base' group, for which there is no obvious rational explanation. The application of NMP intervention *per se* does not explain this, as the 'Local NMP' group had a lower incidence of arterial stenosis. A larger dataset would be needed to determine if this is a real effect, although we believe this is unlikely, especially given that the large randomized controlled trial from the European group observed a low incidence of arterial stenosis (14).

ICU and hospital stays were more prolonged in the 'Local NMP' group. While the MELD-Na scores were not significantly different between groups, there were clear patient-related factors in the 'Local NMP' cases that clearly contributed to their prolonged ICU and hospitalization stays, likely unrelated to NMP (pre-operative BMI >40 with mobility and rehabilitation issues; poor respiratory function (non-hepatopulmonary) requiring prolonged intubation; pre-existing unrecognized cognitive deficit from prior traumatic brain injury labeled incorrectly as encephalopathy; and, abdominal compartment syndrome with acute kidney injury requiring dialysis, prolonged ventilation and tracheostomy in ICU. Further, two of the recipients in 'Local NMP' group received a combined liver and kidney transplants.

A comparison of our overall NMP experience compared to SCS controls replicated the findings of the randomized controlled trial by Nasralla *et al*, with lower peak AST levels (11, 13). Compared to SCS, NMP offers significantly longer graft preservation time, with no obvious compromise in graft function or patient outcomes. This was also seen in previous clinical

studies (12, 14). There was no statistically significant difference overall in biliary or arterial complications, ICU or hospital stay, graft or patient survival between NMP and SCS livers. In our experience, NMP changes the logistics of the transplantation process and allows for more transplants to occur during daylight hours. This potentially allows better resource utilization in fully staffed hospital departments where assistance is readily available. A detailed cost analysis is necessary to assess whether these logistical changes can ultimately confer a cost-saving benefit.

We discarded three livers which failed to clear lactate during NMP and were obvious outliers in our experience. All discarded liver grafts were from distant DCD donors, with significantly higher median DRI. We cannot predict the outcome had these potential grafts actually been transplanted. In our analysis, the only perfusate parameter that correlated with any early post-transplant graft function was AST, which was correlated with EAD. To date, no viability markers have been validated clinically, and this remains a priority for the field.

An incidental benefit of normothermic viability assessment pre-transplant is that more grafts from 'extended criteria' donors may be accepted, with the possibility of implantation dependent on *ex situ* graft performance. In our series, we accepted 4 livers that we would not have considered for transplantation without graft ex situ viability assessment. Of these we transplanted one liver graft that was rejected by all US recipient centers participating in UNOS. This graft (51-year-old DCD donor, WIT 24 min, DRI 2.29, 10% macrosteatosis with mild fibrosis) was transplanted based on acceptable *ex situ* function and the recipient continues to have normal liver function at 18 months of follow-up. The other three exhibited poor ex situ machine perfusion function and were discarded.

We acknowledge that our study has several limitations. The non-randomized, limited sample size makes it difficult to draw definitive conclusions, and ultimately, we are not adequately powered to prove non-inferiority of a 'Back-to-base' approach vs. 'Local NMP', however, the study was primarily designed to assess safety. For our biochemical analysis the hospital laboratory cut-off for AST is 2600 U/L, which limits the *ex situ* perfusate and post-transplant assessment. We did not store samples for dilution and reanalysis to overcome this limitation of the cut-off value. We acknowledge that the three discarded livers, which were all procured from distant centres, would have been included in the 'Back to base' group had they been transplanted. Exclusion of these livers from the statistical analysis may have introduced bias into the final results.

This is the first clinical study to describe the outcome of liver grafts transplanted in a 'post static cold storage' preservation strategy in comparison to immediate normothermic *ex situ* machine preservation. Safe extension of preservation time alters transplant logistics, and our data demonstrates significantly more daytime operating as a result of NMP. Further, NMP may inform pre-transplant decisions for more optimal graft selection, which provided reassuring data to support transplantation of extended-criteria grafts. We have demonstrated that the 'Back-to-base' preservation strategy is safe, with comparable post-transplant levels of liver injury and functional parameters to NMP at the donor centre, as long as low risk DBD or DCD donors are selected. 'Back-to-base' preservation is a viable alternative to NMP device transport, which may increases uptake of this technology in more geographically disparate regions.

5.6 References

1. Guarrera JV, Henry SD, Samstein B, Odeh-Ramadan R, Kinkhabwala M, Goldstein MJ, et al. Hypothermic machine preservation in human liver transplantation: the first clinical series. Am J Transplant. 2010;10(2):372-81.
2. Dutkowski P, Schlegel A, de Oliveira M, Mullhaupt B, Neff F, Clavien PA. HOPE for human liver grafts obtained from donors after cardiac death. J Hepatol. 2014;60(4):765-72.
3. Knaak JM, Spetzler VN, Goldaracena N, Boehnert MU, Bazerbachi F, Louis KS, et al. Subnormothermic ex vivo liver perfusion reduces endothelial cell and bile duct injury after donation after cardiac death pig liver transplantation. Liver Transpl. 2014;20(11):1296-305.
4. Fondevila C, Hessheimer AJ, Maathuis MH, Munoz J, Taura P, Calatayud D, et al. Superior preservation of DCD livers with continuous normothermic perfusion. Ann Surg. 2011;254(6):1000-7.
5. Butler AJ, Rees MA, Wight DG, Casey ND, Alexander G, White DJ, et al. Successful extracorporeal porcine liver perfusion for 72 hr. Transplantation. 2002;73(8):1212-8.
6. Schon MR, Kollmar O, Wolf S, Schrem H, Matthes M, Akkoc N, et al. Liver transplantation after organ preservation with normothermic extracorporeal perfusion. Ann Surg. 2001;233(1):114-23.
7. Boehnert MU, Yeung JC, Bazerbachi F, Knaak JM, Selzner N, McGilvray ID, et al. Normothermic acellular ex vivo liver perfusion reduces liver and bile duct injury of pig livers retrieved after cardiac death. Am J Transplant. 2013;13(6):1441-9.

8. Imber CJ, St Peter SD, Lopez de Cenarruzabeitia I, Pigott D, James T, Taylor R, et al. Advantages of normothermic perfusion over cold storage in liver preservation. Transplantation. 2002;73(5):701-9.
9. Reddy S, Greenwood J, Maniakin N, Bhattacharjya S, Zilvetti M, Brockmann J, et al. Non-heart-beating donor porcine livers: the adverse effect of cooling. Liver Transpl. 2005;11(1):35-8.
10. He X, Guo Z, Zhao Q, Ju W, Wang D, Wu L, et al. The first case of ischemia-free organ transplantation in humans: A proof of concept. Am J Transplant. 2018;18(3):737-44.
11. Ravikumar R, Jassem W, Mergental H, Heaton N, Mirza D, Perera MT, et al. Liver transplantation after ex vivo normothermic machine preservation: a Phase 1 (first-in-man) clinical trial. Am J Transplant. 2016.
12. Bral M, Gala-Lopez B, Bigam D, Kneteman N, Malcolm A, Livingstone S, et al. Preliminary Single-Center Canadian Experience of Human Normothermic Ex Vivo Liver Perfusion: Results of a Clinical Trial. Am J Transplant. 2016.
13. Selzner M, Goldaracena N, Echeverri J, Kaths JM, Linares I, Selzner N, et al. Normothermic ex vivo liver perfusion using steen solution as perfusate for human liver transplantation: First North American results. Liver Transpl. 2016;22(11):1501-8.
14. Nasralla D, Coussios CC, Mergental H, Akhtar MZ, Butler AJ, Ceresa CDL, et al. A randomized trial of normothermic preservation in liver transplantation. Nature. 2018;557(7703):50-6.
15. Jassem W, Xystrakis E, Ghnewa YG, Yuksel M, Pop O, Martinez-Llordella M, et al. Normothermic machine perfusion (NMP) inhibits proinflammatory responses in the liver and promotes regeneration. Hepatology. 2018.

16. Karangwa SA, Dutkowski P, Fontes P, Friend PJ, Guarrera JV, Markmann JF, et al. Machine Perfusion of Donor Livers for Transplantation: A Proposal for Standardized Nomenclature and Reporting Guidelines. Am J Transplant. 2016;16(10):2932-42.

17. Watson CJE, Kosmoliaptsis V, Pley C, Randle L, Fear C, Crick K, et al. Observations on the ex situ perfusion of livers for transplantation. Am J Transplant. 2018;18(8):2005-20.

18. Olthoff KM, Kulik L, Samstein B, Kaminski M, Abecassis M, Emond J, et al. Validation of a current definition of early allograft dysfunction in liver transplant recipients and analysis of risk factors. Liver Transpl. 2010;16(8):943-9.

19. Mergental H, Perera MT, Laing RW, Muiesan P, Isaac JR, Smith A, et al. Transplantation of Declined Liver Allografts Following Normothermic Ex-Situ Evaluation. Am J Transplant. 2016;16(11):3235-45.

20. Guarrera JV, Henry SD, Samstein B, Reznik E, Musat C, Lukose TI, et al. Hypothermic machine preservation facilitates successful transplantation of "orphan" extended criteria donor livers. Am J Transplant. 2015;15(1):161-

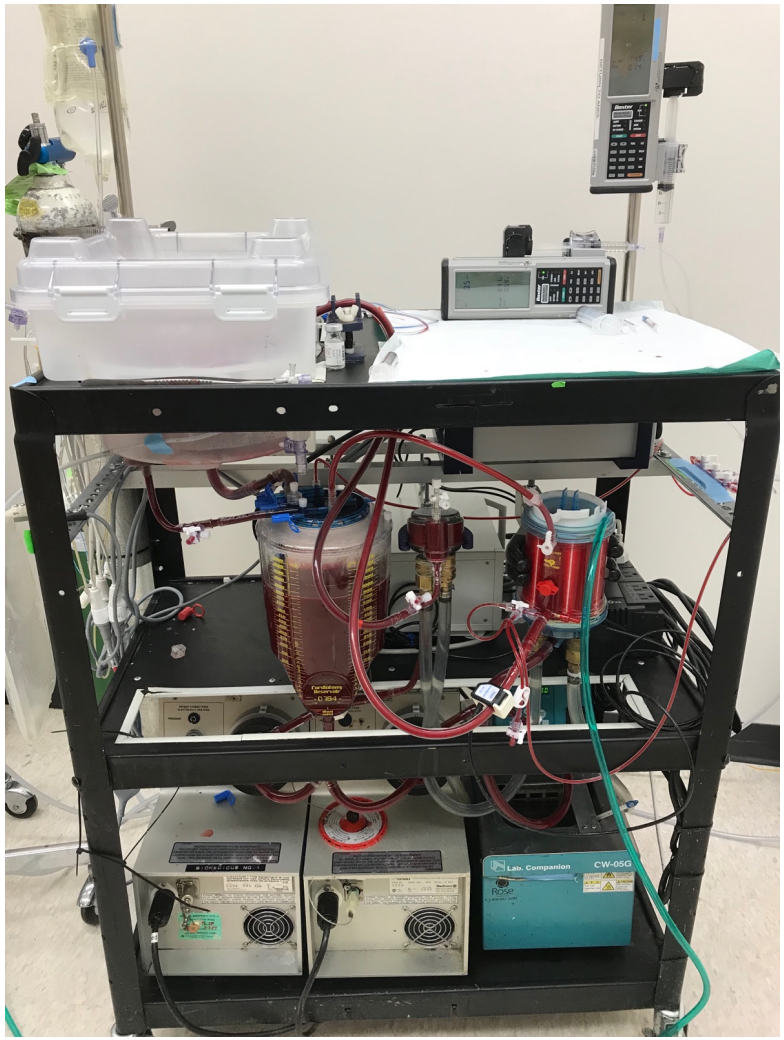
6 Chapter 6

General discussion, future directions, conclusions

6.1 Edmonton *ex situ* liver perfusion accomplishments

The Shapiro Laboratory in Edmonton, Alberta recognized the early potential of *ex situ* NMP liver perfusion to potentially augment and improve the quality and supply of clinical livers for transplantation. We relied heavily on major contributions from others, especially Professor Peter Friend's laboratory in Oxford UK, in adopting their technology in North America, and in parallel set up our own small and large animal experimental program to support and advance our clinical activity.

As part of this thesis, we have added substantially to the experimental and clinical literature on *ex situ* NMP. We helped define the minimal hemoglobin level required for liver NMP, literature that was not defined previously in the field[1]. In a large animal porcine liver model we showed for the first time the kinetics of transaminase clearance 'on circuit' to allow us and others to better understand and interpret perturbations in transaminase released from injured livers on NMP, and how that might contribute to the decision to use or discard a marginal graft. During perfusions lasting 48 hours, we demonstrated that healthy liver grafts reduce perfusate transaminases, further informing of their potential as surrogate markers of graft function [2]. A photograph of our experimental *ex situ* perfusion circuit is below **(Figure 6-1)**.



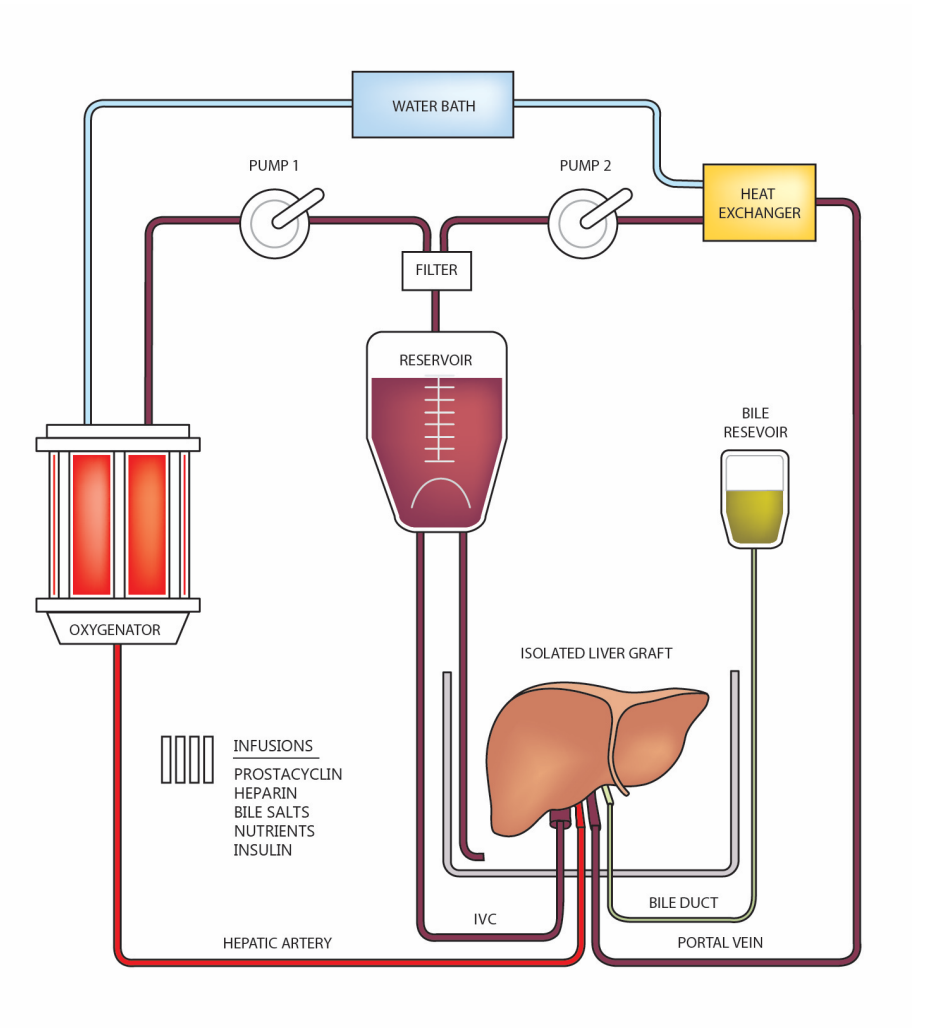


Figure 6-1 A. Photograph of the experimental *ex situ* perfusion circuit B. Schematic diagram of the experimental *ex situ* circuit *Transplantation.* 2018;102(8):1284-1292, with permission from Transplantation

Moving into the clinical realm, the initiation of our pilot clinical *ex situ* liver perfusion pilot trial was a major undertaking, requiring us to overcome considerable logistic and bureaucratic challenges. We were the first team in North America to use NMP technology to perfuse and then transplant a clinical liver on February 25, 2015 **(Figure 6-2)**. This publication, which was one of three worldwide at the time, confirmed the safety and feasibility of NMP in the Canadian setting, and we successfully demonstrated that NMP livers functioned equally as well to SCS controls post transplant, even with prolonged preservation times (23 hours) [3]. In this publication, we were both explicit and transparent about one specific isolated instance of graft loss, which led to changes in the OrganOx *metra*™ training manual and increased the awareness of the steep learning curve associated with the implementation of such technology. We were the first group to recognize that NMP technology had the advantage of being able to change the logistics of the transplant process, by increasing 'daylight' operating time, with strong overall potential to disrupt 'normal' transplant practice [3].

EDMONTON JOURNAL

Edmontonian is first to receive transplanted liver preserved in groundbreaking machine

BY OTIENA ELLWAND, EDMONTON JOURNAL

Feb 19 2015

Figure 6-2 Edmonton Journal publishes our *ex situ* normothermic accomplishment. (url: edmontonjournal.com/news/local-news/.)

In follow up to the publication of the large UK randomized trial, based on our local geographic and machine transport challenges, we sought to answer the question whether the accrual of 'cold ischemia time' (CIT) was detrimental to a liver graft prior to NMP, in a clinical setting. Previous experimental evidence in a porcine model had demonstrated that even one hour of CIT prior to NMP initiation had led to inferior outcomes [4]. For years, this had engendered emphasis on developing portable *ex situ* machine technology, and subsequently, in all reported series of clinical NMP prior, machine perfusion was initiated immediately following graft procurement in what is referred to as 'Preservation machine perfusion' [5]. Machine transport, with all attendant logistical complications and increased costs, is more challenging in large geographic catchment areas such as the province of Alberta, where organ procurement teams predominantly travel by air. In an alternative strategy, we transported liver grafts from donor centers using SCS, and then initiated NMP at the recipient center in a 'post-Static Cold Storage-NMP' preservation strategy. Our published results were the first to demonstrate that a modest increase in CIT (mean pre-NMP CIT 3 hours, maximum 6 hours) did not in any way compromise outcomes, compared to an SCS control group [6]. In Canada, where procurement teams and organs are potentially shipped over large distances, such simplified transport logistics can result in huge cost savings. Further, and perhaps more importatly, such a graft transport and machine preservation strategy can increase the accessibility of NMP to more transplant teams worldwide. If machine transport is indeed, not necessary, and if NMP technology does in fact significantly reduce liver graft discard rates, then these findings combined would strongly support more widespread application of NMP technology, particularly in large catchment areas.

At the time of writing, *ex situ* liver perfusion is transitioning from what was seen as a largely experimental preservation strategy to one that had been shown to be safe and feasable in the clinical setting, with increasingly more widespread worldwide use. Further, there exists a growing understanding and optimism that the NMP platform uniquely dovetails with current advances in the fields of biotechnology, biochemistry, and genetic bioengineering, allowing for a series of intervention strategies that could substantially alter prosective graft utilization. Such thinking sets the stage for a future where the practice of transplant surgery is highly personalized, and organ grafts can be extensively modified or repaired. Ensuing is a more detailed discussion of the likely directions that such graft intervention will proceed into, within the current evolution of parallel technologies.

6.2 Tenets of *ex situ* liver perfusion

In the context of the worldwide organ shortage, more recent *ex situ* organ machine perfusion technology was developed as a means to improve organ preservation, particularly for marginal organs. Different temperature modalities, and implementation strategies have been used to this end. Normothermic conditions are generally considered to provide the most physiologic, fully functioning metabolic state.
As experience with NMP as a preservation platform began to increase, a number of potential advantages started to become apparent, and hold promise of expanding the donor pool. These included: 1) offering the advantage of prolonged organ preservation 2) changing the operational logistics of current transplant practice 3) offering the potential of dynamic 'on circuit' graft assessment to help inform post-transplant graft function and 4) offering the possibility of graft resuscitation/ modification with targeted 'on circuit' application of therapeutic strategies.

6.2.1 Prolonged Preservation Time with Normothermic Preservation

It is now reasonably established that *ex situ* technology allows for extended preservation of liver grafts well beyond the possibilities of SCS. Experimentally, this has been demonstrated by a number of groups, with extended porcine and canine liver perfusions lasting up to 72 hours, and successful porcine liver transplants after 48 hours [7-9]. In our experimental perfusions, we were also successful at preserving grafts in NMP for 48 hours, without obvious injury. Such preservation times have recently been extended to 86 hours for a clinical liver, using a combination of NMP and SCS, but this

graft was not transplanted [10]. With further improvements in perfusate composition and additives, machine perfusate exchanges, iterative improvements in the machine circuits, it is conceivable that in the future perfusions may be performed for previously unseen durations, possibly days at time, especially if circuits, filters, blood components and other additive constituents are exchanged on frequent (perhaps daily) basis. Safe extended preservation imparts obvious logistic advantages for the transplant process, enabling the transport of organs over longer distances, across large geographic areas, and the possibility of matching of organs to 'ideal' recipients, irrespective of their location. It is indeed conceivable that a patient in Canada listed as Status 4F fulminant could receive a liver flown here from Australia using a commercial series of flights. Extended preservation times may also be both necessary and advantageous in future applications of additive strategies (eg. graft de-fattening), where the intervention may take days to achieve the desired effect.

Prolonged preservation inexorably introduces new challenges that currently may be of less consequence, including progressive degradation of oxygen carrying capacity of perfusate elements and buildup of toxic metabolic by-products. Over the duration of any perfusion, erythrocytes in currently employed blood-based perfusates are subjected to shear stresses from the circuit components and machine roller pumps, which results in unavoidable hemolysis. As an alternative, groups have tested hemoglobin-free oxygen carriers, with promising results. These solutions are less expensive, convey no infectious risk, and have the advantage of a prolonged shelf-life, and may be the readily employed in the future [11,12]. Although not obviously necessary at the moment, some groups have employed circuit dialysis in experimental perfusions, which may be incorporated into future circuit designs [13]. Such incremental advances

will further improve the usability and accessibility of MP technology, allowing for more widespread future use.

6.2.2 Altered Logistics of the Transplant Process

As an extension of prolonged preservation times, NMP technology allows for more optimal resource allocation within the hospital setting. With the available evidence to-date indicating that NMP preserved grafts perform at least as well as SCS stored grafts, surgical teams and OR staff can adjust operating times accordingly to ensure best outcome. At our institution, we now routinely place organs scheduled to arrive in the middle of the night on machine perfusion, ensuring that the implanting team is well rested. Effectively, this changes the transplant procedure from an emergent, time-constrained event, to almost the equivalent of an elective procedure. We were the first group worldwide to point towards this potential, and published a schematic example of these logistical changes as part of our first clinical experience **(Chapter 4)**.

6.2.3 Graft Viability Assessment

Dynamic *ex situ* graft functional assessment has been one of the ultimate goals of NMP technology. NMP is the only temperature modality that theoretically maintains a graft in at full metabolism, allowing for functional assessment. In the experimental setting, a number of groups have suggested markers for such viability testing, alone or in tandem [14-16]. Of these, a number of markers that have held promise in the experimental setting have not transitioned into clinical practice. As an example, bile output, which seemed a

rational choice as a surrogate marker of overall graft function, was repeatedly used in the experimental setting, but in the clinical scenario did not correlate well with post-transplant outcomes [17]. Nasralla *et al.* reported clinical transplantation of 18 livers that produced minimal or no bile, with no obvious correlation to post-transplant function or strictures. Only one of these transplanted livers went on to develop PNF, however this graft also did not clear lactate on-circuit [18].

More recently, it has come to light that bile quality may be of greater utility in graft assessment. Bile is modified by bile duct cholangiocytes, through secretion and resorption of bicarbonate, bile salts, water, glucose and amino acids. Monitoring bile content can then serve as an indicator of cholagiocyte metabolic function. Production of alkaline bile (pH >7.5) has been shown to correlate with decreased post-transplant cholangiopathy [19]. Recently, Matton *et al*. have reported that biliary bicarbonate and pH were significantly higher, and biliary glucose was significantly lower in livers with low histologic bile duct injury. They concluded that biliary bicarbonate, pH and glucose during *ex situ* liver NMP are reasonable and accurate biomarkers of bile duct injury and can be used as pre-transplant markers of bile duct viability [20].

In the clinical setting, most commonly, graft lactate clearance has been used in combination with pH and vessel flow stability for graft assessment [17,18]. Additionally, transaminases have also been suggested, with a previously reported significant correlation between the perfusate alanine transaminase (ALT) and peak ALT post-transplant levels in the first week [17]. It should be emphasized that the current viability parameters are not always convincing, as clinical livers have been discarded despite

favourable perfusion characteristics, even in the context of favourable donor and organ quality [18].

Similar to other reports, we also described our experience with on-circuit graft selection based on these parameters [3,6]. In the experimental setting, some groups have attempted more unusual graft assessment methods, such as perfusate indocianine green, or lidocaine clearance however, none of these have yet transitioned into clinical practice [21]. It is important to mention that no markers to date have been validated in the clinical setting, and this remains one of the challenges and future goals of the field. **Table 7.1** outlines the most up to date viability criteria currently in use for on-circuit graft assessment.

Looking towards the future, in addition to markers of viability, biomarkers that would predict acute cellular rejection, coupled with *ex situ* immunomodulation protocols would significantly advance the management of liver transplant recipients. In this regard, the most promising marker to date has been a tissue based gene expression profile of iron hemostasis. Although the mechanism is not described, preliminary testing suggests that iron hemostasis is important in regulating hepatic lymphocyte response [22]. A trial is currently ongoing to determine whether such a biopsy could in fact improve selection of candidates for immunosuppression withdrawal (NC-T02498977). The possibility of implementing such a test during *ex situ* machine perfusion would certainly add to the personalization of therapy for future transplant recipients. In the forseeable future, graft assessment will likely be based on '-omics' or microRNA analysis [23].

Table 6-1 Predicting post-transplant graft function from perfusion parameters and biochemistry

Author	Species	Predictive perfusion viability parameters
Matton et al. 2018 [20]	Human	Biliary bicarbonate > 18 mmol/L, biliary pH > 7.48, and biliary glucose < 16 mmol/L, and bile/perfusate glucose ratio <0.67
Mergental et al., 2018 [15]	Human	Within 2 hours of perfusion start >1 major criteria, >2 minor criteria must be met. Major criteria:
Watson et al. 2018 [24]	Human	Rate of lactate fall per unit liver weight, 'on-circuit' ALT levels, bile pH <7.4
Bral et al., 2017 [3]	Human	Stable physiologic pH and normalizing lactate.
Mergental et al., 2016 [25]	Human	Within 3 hours of perfusion: bile production or lactate less than 2.5 mmol/L, or two of the following: (1) perfusate pH greater than 7.3 (2) stable arterial flow > 150 mL and portal vein flow > 500 mL (3) homogenous graft perfusion with soft consistency and parenchyma
Guarrera et al., 2015 [26]	Human	AST and ALT levels measured during ex situ perfusion strongly correlate with post transplant peak AST and ALT

ALT (alanine aminotransferase), AST (aspartate transaminase), ATP (adenosine triphosphate), BE (base excess), HAR (hepatic artery resistance), LDH (lactate dehydrogenase), I-FABP (L-type fatty acid binding protein), PV (portal vein), PVR (portal vein resistance)

Although these findings have now preliminarily established NMP in the clinical forum, they have just begun to set the stage for the possibility of more widespread application, as well as the potential pre-transplant on-circuit graft interventions. Moving forward, efforts will likely be focused on graft bio-modulation and bio-engineering, with the obvious advantage that the graft is treated in isolation, and not the patient.

6.3 Pretransplant graft modulation during *ex situ* liver perfusion

6.3.1 Mitigating Ischemia Reperfusion Injury

During organ procurement, liver grafts are subjected to numerous stresses, including both warm and cold ischemia prior to and at the time of surgery, as well as progressive preservation injury in SCS. The cumulative effect of the these insults results in activation of cellular damage pathways, including production of free radicals, activation of damage associated molecular patterns, release of inflammatory cytokines, and caspase activation, eventually leading to cell death. Further, it has been demonstrated that orthotopic liver transplantation leads to a significant increase in directly measurable reactive oxygen species (ROS), downstream markers of lipid peroxidation (malondialdehyde (MDA) and isoprostanes), as well as consumption of antioxidative enzymes (superoxide dismutase, glutathione, and catalase) [27]. During reperfusion of transplanted livers, markers of oxidative stress increase significantly, attributed to, and consistent with ischemia reperfusion injury (IRI) injury.

Selected strategies hold potential to mitigate ischemia reperfusion injury but application of such interventions during *ex situ* NMP has not been widely investigated to date.

Cyclosporine A, for example, has previously shown efficacy in minimizing IRI in ischemic stroke and myocardial infarction, and is currently under investigation as a preconditioning drug administered to organ donors, with the expectation that this will reduce rates of delayed graft function in kidney transplantation [28-31]. **Table 7.2** outlines potential additive strategies to mitigate IRI, however, much work remains in regard to clarifying dosing approaches, and ultimate efficacy and possible toxicity during *ex situ* NMP.

The addition of potent antioxidants to the *ex situ* circuit could be a potentially highly effective way to minimize oxidative stress during NMP of injured human liver grafts. Using a porcine model, Goldaracena *et al*. applied various anti-inflammatory agents to NMP liver perfusions, including n-acetylcysteine, alprostadil, sevofluorane, and carbon monoxide. Levels of pro-inflammatory cytokines (TNF-alpha, beta galactosidase and IL-6 were demonstrated to be decreased and histology revealed decreased endothelial cell and hepatocyte injury [32]. In our laboratory, we previously had success in mitigating ischemic injury in islets, and this experience served as a rational basis for selecting potential compounds that would be effective in minimizing IRI in livers.

Based on our previous laboratory experience in protecting and enhancing islet engraftment, using a murine *in vivo* liver ischemia model, we tested a rational selection of these compounds, to as described in **the Appendix**. All groups demonstrated potency towards mitigating IRI, with the potent pan-caspase inhibitor F573, demonstrating the most efficacy, evidenced by statistically lower post-reperfusion transaminase levels and statistically less apoptosis, and improved cytokine profiles.

These findings have exciting potential in translating the use of these compounds during NMP.

Table 6-2 Additive strategies to mitigate ischemia reperfusion injury during *ex situ* liver NMP

Target	Category	Additive Examples
Oxidative Stress	Anti-apoptotic agents	IDN-6556, F573, Z-VAD-FMK
	Metaloproteinase Inhibitors	RXPO3, lactobionic acid
	Vasodilators	BQ123, Epoprosenol, Verapamil
	Anti-oxidants	glutathione-ethyl-ester, albumin, BMX-001
	Anti-inflammatory agents	acetylcysteine, carbon monoxide, sevofluorane, anti-aging glycopeptide, Etanercept
		Peroxiredoxin 6 (Prdx 6)
	Immunosuppressive agents	Cyclosporine A
	Anti-oxidant flavinoid	Baicalein- increases glutathione peroxidase activity, inhibits 12/15- LOX
	Vitamins	Vitamin C and E; potent antioxidants with diverse biological activity
	COX inhibitors	nimesulide- selective COX-2 inhibitor with anti-inflammatory, antipyretic, and analgesic properties
	Natural Polyphenols	Resveratrol- reduces inflammatory markers
	Co-Enzymes and Co-factors	Co-enzyme Q10- intracellular antioxidant. Alpha-lipoic acid- cofactor for mitochondrial alpha-ketoacid dehydrogenases, effects mitochondrial energy metabolism
		Dimethyl sulfoxide- increases glutathione peroxidase activity

6.3.2 De-fatting of Steatotic Liver Grafts

With the current worldwide obesity epidemic, the prevalence of nonalcoholic fatty liver disease (NAFLD) in western countries is estimated to be up to 24%. As a direct result, liver macrosteatosis is found in 15 to 25% percent of donor grafts [33]. Such grafts are more prone to preservation injury, and steatosis is one of the major reasons to decline a graft from the transplant process, often based on the opinion of the procurement surgeon. Although no guidelines exist, there is general consensus that macrosteatosis <30% may be safely transplanted, provided there are no other mitigating factors, and other publications have suggested that livers with moderate (30- 60%) and severe (>60%) steatosis may selectively be used (low MELD, short CIT, no re-transplant or DCD) [34].

Such overwhelmingly large numbers of steatotic grafts have spurred intense research into different approaches and drugs to reduce the fat content in livers. Several drugs have entered Phase 2 and 3 trials for treatment of NAFLD and non-alcoholic steato-hepatiitis (NASH)[34]. Although not attempted yet, these drugs in mono- or polytherapy may also have potency during *ex situ* NMP, which offers a unique platform for drug administration to explore the treatment of fatty livers before transplantation. NMP as a preservation strategy specifically, has long held promise in recovering steatotic grafts by minimizing cold ischemia time, and inducing fat metabolism through various pharmacologic interventions. Further, *ex situ* demonstration of acceptable function during NMP may be an especially helpful guide to the transplanting surgeon. A number of attempts have been made to de-fat steatotic grafts, although this has not translated into clinical practice yet. **Table 7.3** highlights selected promising drugs currently in

clinical trials, which may demonstrate efficacy during *ex situ* NMP. Doses and administration protocols have yet to be determined.

Nagrath *et al.* used NMP to perfuse stetaotic livers from obese Zucker rats, with added defatting agents including GW7647, forskolin, hypericin, scoparone, visfatin, and GW501516. They reported a 50% reduction in intracellular lipid content after only 3 hours of NMP. Metabolite analysis revealed up-regulated lipid oxidation and export and increased expression of transcription factors related to these processes [34].

In a porcine model, Jameson *et al.* investigated 48 hour continuous NMP of fatty livers, demonstrating a histologic decrease in hepatic fat content from 30% to 15% [35]. Somewhat surprisingly, in counterpoint to the above study, NMP of human livers with no additives, failed to clear any steatosis [36].

In a study using discarded human livers, Banan *et al.* used NMP for 8 hours, with the addition of defatting compounds L-carnitine and extendin-4. Compared to steatotic livers perfused without intervention, treated livers demonstrated significantly increased perfusate low-density lipoprotein and triglyceride levels. Histological analysis revealed a 10 percent decrease in macrosteatosis compared to baseline [36].

More recently, Boteon *et al.* solubilized fat in steatotic donor human livers by utilizing a defatting cocktail (10 μM forskolin, 1 μM GW7647, 10 μM of hypericin, 10 μM scoparone, 0.4 ng/mL visfatin, and 1 μM GW501516 supplemented with L-carnitine 0.8 mM diluted in DMSO) added to NMP perfusate. Livers in the treatment group had levels of tissue triglycerides dropped by 38%, and macrovesicular steatosis decreased by

40% over 6 hours. Treated livers also had enhanced metabolic functional parameters such as lower vascular resistance, lower release of alanine aminotransferase, higher bile production with higher bile pH, while concomitantly decreasing markers of cellular inflammation [15]. In all of these studies, the solubilized fat remained in the perfusate solution. Potentially the addition of a separate plasmafiltration centrifuge circuit could radically clear released and circulating fat droplets from the system, and could markedly improve the efficiency of fat clearance. No group has yet attempted such an approach.

Table 6-3 Potential additive strategies in *ex situ* NMP for defatting liver grafts

Target	Category	Additive Examples
Liver Defatting	Lipolytics	Forskolin, GW7646, scoparone, hypericin, visfatin, GW501516, L-carnitine
	Peroxisome proliferator-activated receptor agonists	Saroglitazar, IVA337, Pioglitazone (PPAR-gamma agonist), Elafibranor (PPAR- alpha/gamma agonist)
	Interstitial transporters	Volixibat (Sodium dependent bile acid transporter), LIK066 (sodium glucose co-transporter agonist);
	Glucagon receptor agonist	Liraglutide (GLP-1agonist)
	Chemokine Receptor blocker	Cenicriviroc (CCR2/CCR5 receptor blocker)
	Endocrine receptor agonists	Metreleptin (Leptin analog), Tesomorelin (GHRH analog)
	Bile Acid Receptor agonist	Obeticholic acid
	Apoptosis Signal regulating Kinase inhibitor	Selonsertib (ASK-1) inhibitor
	Vitamins	Vitamin E
	Lipid metabolism modifiers	Aramchol (Stearoyl-CoA desaturase inhibitor), Pradigastat (Diacylglycerol acyltransferase inhibitor),

6.3.3 Ischemia-free Liver Preservation

He *et al.* reported the first clinical case of 'ischemia free' organ transplantation. The authors report preserving a severely macrosteatotic graft (85-95%), under continuous NMP throughout procurement, preservation prior to transplant, and implantation without any cold phase in the process **(Figure A, B).** Liver inflow *in vivo* was established by cannulation of the portal vein (PV) via an iliac vein extension graft (24 Fr cannula) and gastroduodenal artery (8 Fr cannula), and outflow was ensured by cannulating the infrahepatic inferior vena cava (IVC) (34 Fr cannula). All cannulae were connected to a commercial Liver Assist device. Clamps were placed on the proximal PV and proximal hepatic artery, as well as the supra-renal IVC. The suprahepatic cava was clamped off, following which NMP was established by regulating inflow using machine perfusion. Although this approach interferes somewhat with multi-organ procurement, immediately following establishment of the NMP circuit, the kidneys were cold flushed via a cannula within the abdominal aorta and procured. The recipient recovered well, with a functioning liver graft, and without early biliary, vascular or immunologic complications. Post-transplant assessment demonstrated minimal injury on histology, minimal apoptosis (TUNEL staining), minimal inflammatory cytokine release and minimal inflammatory pathway activation, and importantly with rapid clearance of fat on the post-transplant core biopsies [37].

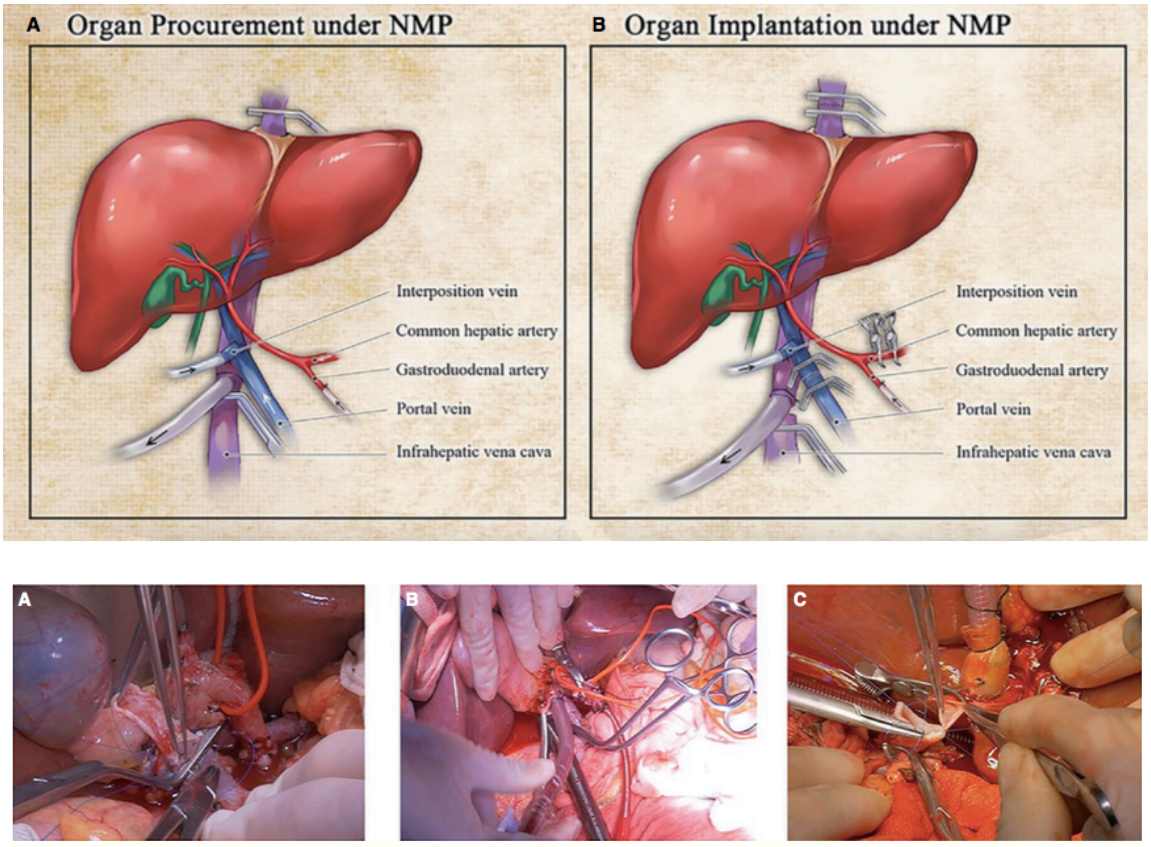


Figure 6-3 A) Ischemia-free liver transplant procedure.

The diagram shows procurement (A) and implantation (B) of the donor liver under normothermic machine perfusion using Liver Assist with cannulation of the infrahepatic vena cava, portal vein, and gastroduodenal artery B) the key techniques used during ischemia-free liver transplantation procedure. Panel A shows the construction of an interposition vein on the portal vein using the right iliac vein. Panel B shows organ procurement under normothermic machine perfusion. Panel C shows organ implantation under normothermic machine perfusion (Reproduced with permission from *Am J Transplant.* 2018:737–744.)

Such studies demonstrate the potential for *ex situ* NMP intervention in salvaging fatty livers, but further efforts are needed to make such an approach clinically feasible.

6.3.4 Gene therapy during NMP

In recent years, adeno-associated viral (AAV) vectors have been recognized as safe and effective to introduce a transgene into a specific tissue or organ, with long-term gene and protein expression following a single injection of the vector[39]. AAV vectors are particularly good candidates for such therapy, in that they are recognized as non-pathogenic, are not immunogenic and have broad tropism. Further, AAVs can be genetically re-shuffled to enhance specific organ transduction potential. AAV gene delivery has already been used for treatment of inherited metabolic diseases.

Ex situ perfusion is an ideal platform for such transgenic therapy, and indeed, such treatments have already been successfully accomplished. In the first study of its kind, the Toronto group administered IL-10 gene therapy to injured human lungs during *ex situ* normothermic lung perfusion, and observed a decreased inflammatory response and improved function compared to untreated controls [40].

Translating this approach to *ex situ* liver perfusion, Goldaracena *et al.* applied microRNA technology to induce hepatitis C resistance in liver grafts during NMP. Using miravirsen, an anti-sense miRNA-122 oligonucleotide during NMP, they observed significant mRNA-122 uptake and mRNA-122 target gene depression, which did not occur in the SCS control group. This study served as the first proof of concept of

genetic graft modification during NMP, and opens up exciting possibilities for future applications [41].

Recently, it has been demonstrated that Angiotensin Converting Receptor 2 (ACE2) gene therapy holds promise in treating biliary fibrosis. There is ample evidence that angiotensin II (ANG II) is one of the main mediators of hepatic fibrosis, with levels elevated in cirrhosis, as well as activation of the local Renin-Angiotensin System (RAS) in response to injury. It has been speculated that that one way of achieving a therapeutic outcome in biliary fibrosis would be to increase the level of antifibrotic peptide Ang-(1-7), which mitigates many of the damaging effects of Ang II. Animal studies have shown that Ang-(1-7) peptide can reduce collagen secretion, leading to notable improvement in hepatic fibrosis. Enhancing ACE2 expression and activity would be expected to convey both the benefit of increasing the degradation of profibrotic peptide Ang II while concomitantly increasing antifibrotic Ang-(1-7). Using a murine model, Mak *et al* demonstrated that a single injection of AAV vector with a liver specific capsid, carrying murine ACE2 produced sustained elevation of liver ACE2 expression for up to 6 months [42]. Using such specific liver viral vector therapy administered during *ex situ* NMP, could in theory provide robust therapeutic protection against biliary fibrosis, and may be potentially effective in preventing recurrence of primary sclerosing cholangitis post-transplant [43].

6.3.5 Gene silencing with siRNA

A recent development in graft gene manipulation is the unique application of siRNA to *ex situ* liver perfusion. RNA interference (RNAi) is a process of post-transcriptional gene regulation, and RNAi based therapies have been used with success in experimental transplantation to modulate ischemia-reperfusion injury, and to silence genes of the innate immune system [44].

Utilization of RNAi for organ modification has ongoing challenges, some of which can be resolved by application of such technology to an *ex situ* circuit during graft perfusion. The administration of small interfering RNA (siRNA) to an *ex situ* perfusate allows for more efficient delivery, lower doses, cost savings, more targeted application and possible avoidance of the side effects of administering the drug systemically [44]. **Table 7.4** outlines possible additive strategies during *ex situ* NMP using RNAi. Dosing and duration of exposure have yet to be determined.

In a unique study, Thijssen *et al*. demonstrated uptake of siRNA during *ex situ* perfusion under both hypothermic and normothermic conditions. They demonstrated that siRNA against the Fas receptor added directly to perfusion solution could be successfully delivered to rat liver grafts during MP. Evidence of siRNA uptake was demonstrated by fluorescent confocal microscopy. This study provided proof-of concept that siRNA delivery during *ex situ* machine perfusion was feasible, and potentially improved target cell specificity in a unique delivery platform [44]. Such research shows promise for future *ex situ* applicability to decrease graft IRI, alter the risk of rejection (major histocompatibility complex antigens), achieve operational tolerance, or prevent viral infections..

Table 6-4 Potential additive strategies during *ex situ* NMP using RNAi

Therapy	Target	Additive Examples
Silencing Apoptotic Genes	Fas (CD95)	Hepatocytes express high numbers of Fas receptors. IRI was attenuated by suppressing Fas mediated apoptosis
	TNF-alpha	TNF-alpha plays a central role in liver IRI, can also activate hepatocyte apoptosis by prolonging c-Jun N-terminal kinase activation. Silencing TNF-alpha decreased liver injury and inflammation
	Caspase 3 (cas3) and Caspase 8 (cas8)	Activation of proteases cas3 and cas8 leads to cell death. Silencing cas3 and cas8 using siRNA attenuated IRI, decreased hepatocyte damage
	P53	Activation of p53 leads to up-regulation of cell death pathways.
Immune Modulation	Nuclear factor kappa B (NF-KB) via transcription factor RelB	Silencing RelB in the liver decreased IRI, and reduced formation of reactive oxygen species, decreased liver damage and cytokine production
	Interleukin-1 receptor associated kinase-4 (IRAK4)	IRAK4 signals innate immune responses from Toll-like receptors. Silencing IRAK4 leads to decreased biochemical and histologic evidence of liver injury
	C3	Increased murine survival, less damage evident on pathology
	C5a	

6.3.6 Altering the immunogenic potential of the graft

The liver is already an immune-privileged organ, which is well known. This is a complex, with multiple proposed mechanisms, including 1) microchimerism, 2) increased hematopoietic cells with immunoregulatory properties 3) generation of regulatory molecules, and 4) elimination of expression of Class II allo-target HLA antigens, or replacement with HLA-G epitopes that are immunologically silent [45].

To date, no group has yet published findings relating to *ex situ* machine perfusion immunomodulation therapy, however, previous groups have successfully weaned liver graft recipients off of immunosuppression (IS). *In vivo*, the ideal candidates for IS withdrawal and the achievement of 'operational tolerance' are those at lowest risk of acute cellular rejection. This requires consideration of candidate selection, as the etiology of liver disease prior to transplantation may affect the outcome. Patients with autoimmune compared to non-autoimmune liver diseases have increased rejection risk and as such are not good candidates for IS withdrawal [46].

Data supporting the clinical benefit of achieving operational tolerance are limited to date. Donkier *et al.* reported on a small case series in which living donor liver transplant (LT) recipients with intrahepatic malignancy were given pre-transplant treatment (cyclophosphamide, ATG) followed by donor stem cell infusion and then living donor liver transplant (LDLT). Both recipients were withdrawn from immunosuppression early after transplant. In a second series, recipients underwent LDLT, followed by ATG, steroids, sirolimus, and donor stem cell infusion. Two out of three patients were successfully weaned off of rapamycin [47].

Todo *et al.* utilized T regulatory cell (Treg) therapy in the development of tolerance in ten LDLT patients. Recipient autologous Tregs were expanded, and standard immunosuppression without induction was initiated at the time of transplant, with cyclophosphamide given on post-operative day 5 to deplete anti-donor lymphocytes. Treg therapy was induced two weeks after transplant. At 6 months post transplant, patients on tacrolimus monotherapy started a withdrawal protocol over a 1 year period. Seven out of ten were successfully withdrawn, and met pre-established criteria for tolerance [48].

Such studies are encouraging, and may have translational potential into the *ex situ* setting. If immune tolerance or modulation could be achieved by treating individual grafts on an *ex situ* circuit, the advantages would be numerous and profound.

The theoretical application of Treg therapy during *ex situ* perfusion represents a potentially promising strategy to mitigate the immunogenicity of the transplanted graft. Expansion of the recipient Treg population *ex vivo*, and then injecting these cells into the *ex situ* MP circuit during perfusion could readily be achieved, or alternatively, using pharmacologic expansion of recipient Treg cells 'on circuit', induced for example by exposure to IL-2 [49]. A number of studies have demonstrated that achieving graft operational tolerance depends on the ratio of Treg:Teff cells, with a higher balance of Treg cells favouring graft silence [50]. Theoretical 'washing out' of Teff cells during NMP could tip this balance into a more favourable ratio. In a further potential approach, directed Treg migration could be achieved during *ex situ* perfusion by using chemotactic factors, such as CCL22 [51]. Treg application in *ex situ* perfusion may be a

favourable strategy, as the local Treg up-regulation may be a more efficient and safer strategy to affect immune responses than systemic Treg up-regulation.

6.3.7 Stem cell therapies

The ability of mesenchymal stem cells (MSCs) to differentiate into different cell lines, as well as well as to locally release modulating bioactive compounds holds much potential for application in *ex situ* machine perfusion. MSCs secrete a large number of bio-chemicals, including various cytokines/chemokines and growth factors, all of which exert local tissue effects [52]. Through these chemical factors, MSCs influence regional regenerative responses that can promote native tissue recovery, effectively causing local cells to de-differentiate and replicate to restore damaged cells [53]. In this fashion, tissue repair occurs with cells derived from the tissue itself. Further, it has been demonstrated that MSCs are immunologically silent, and can therefore be infused without immunosuppression [54].

To date, few attempts have been published delivering MSCs into a graft during NMP, although expanding knowledge of the regenerative phase of tissue repair are pushing this field forward.

A recent paper by Brasile *et al.* demonstrated that DCD kidney grafts with warm ischemic damage, had improved function and decreased injury when perfused *ex situ* with MSCs for 24 hours. Treatment with MSCs resulted in reduced inflammatory cytokines, increased ATP levels and growth factors, and increased mitosis [54]. This study, which demonstrates the ability of *ex situ* NMP to serve as a platform for tissue repair

resulting in improved function, will likely have revolutionary impact on the practice of transplantation in the future, as well as the chronic organ shortage.

Future advanced therapy may involve decellularization of a liver grafts, followed by repopulation with patient specific induced pleuripotent stem cell (iPSC) cell populations, effectively bioengineering personalized organs. Already, in other organ systems, bioengineering of organs has been accomplished. Guenthart *et al.* applied *ex situ* lung perfusion (EVLP) to human lungs rejected from the transplant process, regionally decellularized the lungs by selectively removed the epithelium, followed by MSC and airway epithelial cell delivery and attachment [55].

6.3.8 Nanoparticle therapy delivery

Polymeric nanoparticles (NP) are gaining traction clinically as drug delivery vehicles for a number of illnesses. An interesting property of these systems is that the NP can be altered in ways specific to the proposed encapsulation of contents, with some nanoparticles engineered for sustained release. Despite these properties, administration of NPs in liver therapy has issues, as such molecules are rapidly eliminated systemically by phagocytes in the mononuclear phagocyte system. One method to overcome this issue is to create cell target specificity in an NP molecule through conjugation with a ligand specific for a receptor on the surface. Such modifications do not necessarily allow for a NP to seek out a specific cell, but may enhance binding of the NP to the target cell surface [56]. Such implementation of NP therapy in a liver *ex situ* NMP circuit, although directed, may be potentially challenging considering the abundance of phagocytes in that organ .

The utilization of nanoparticles as stable vehicles of therapy delivery has recently been applied in renal NMP. The authors showed that by conjugating an anti-CD31 antibody to therapeutic polymeric nanoparticles enhances the targeting of the NPs to human kidney graft endothelial cells during NMP [57]. Targeted NP therapy, if delivered to an NMP circuit prior to graft implantation could theoretically deliver drug or genetic therapy that would alter graft immunity, or limit damage from IRI.

6.3.9 Ex situ perfusion as multi therapy platform

Building on the array of potential interventions that could potentially be added to NMP perfusates, it is further plausible that such strategies could be used synergistically in combination. Stephenson *et al* demonstrated the potential of performing *ex situ* surgery on a graft during NMP, by performing a left lateral/right trisegmentectomy split [52]. Such advances only further enhance the utility of *ex situ* as a platform for multiple, varied therapeutic options, that have the potential of not just affecting global transplant practice, but also hepatobiliary surgery. It can be readily imagined for example, that a patient with hepatitis C fibrosis and a poorly situated hepatocellular carcinoma may be eventually ablated in such a fashion. After establishing bypass, the cancer would be resected *ex situ* under continuous NMP, and the liver remnant would be treated while 'on circuit' for a period of time, sparing the patient of potentially harmful drug side effects. Such an approach could markedly increase the therapeutic window of highly toxic systemic chemotherapeutics by limiting exposure only to the explanted liver. Indeed, Marcellin *et al.* demonstrated that curative antiviral therapy for hepatitis C can often reverse liver cirrhosis [53]. The treated liver graft would then be flushed of the

therapeutic medications, and re-implanted as an autologous transplant. Such an approach may also revolutionize treatment of other hepatic disorders, including correction of metabolic disease with gene therapy as an example, and may eliminate the need for an organ donor in such cases.

6.3.10 Ex situ restoration of brain circulation and cellular function

In a recent alarming and controversial publication, Vrselja *et al*. used normothermic *ex situ* machine perfusion to restore blood flow and neuronal function to pig brains 4 hours after retrieval post mortem from the abattoir. Using a NMP technology termed '*Brain Ex*', 6 hours of perfusion preserved cytoarchitecture, attenuated cell death, restored unorganized synaptic activity as well as active cerebral metabolism without global electrocorticographic activity. This study demonstrates that under specific circumstances, mammalian brains have a previously unrecognized capacity to maintain vitality, and calls into question established current guidelines concerning 'brain death'. This study may wreak havoc for neurological brain death criteria. In the end, we may have to disband the use of the term 'brain death' and replace it with 'irrecoverable neurological injury' if such research is not to disrupt the day-to-day care of organ recipients [54].

6.4 Cost analysis for widespread NMP application

NMP is expensive. Beginning with the cost of pump, the disposables ($20,000) and perfusate components, as well as special transport arrangements for the machine and the perfusion team. In the US, not infrequently, an extra surgeon travels with the procurement team to prepare and cannulate the liver, and establish perfusion.

In most clinical NMP publications to date, all hard clinical outcomes such as graft and patient survival, ICU and hospital length of stay, and rate of non-anastomotic biliary strictures has not been different between the NMP and SCS arms. This implies that any effects that NMP has on grafts, either as a preservation technique or as preservation method that informs the user about the transplantability of a particular organ seems to not be clinically significant.

What is significant, however, with potentially very important consequences, is that the publication of a randomized multi-center NMP trial demonstrated a 50 percent lower graft discard rate compared to NMP which equates to a 20% percent higher transplant rate. Assuming this is valid, in and of itself, could justify use and offset the cost of NMP technology.

In practice, a detailed and accurate cost analysis would be quite complex, as it would be offset by the (moral, ethical) reality of potentially saving individuals from dying and dropping off of the organ waitlist. Death is always more cost effective for payers but the price is too high for individuals and loved ones. Further, in consideration of the fiscal

cost, the routine replacement of cold storage by machine perfusion may be difficult to justify. What remains to be seen, is whether the relative benefit imparted to situations of increased risk of graft loss (advanced donor age, steatosis, prolonged donor warm ischemia), indeed offsets the cost, and what thresholds should be used to define the application of machine perfusion technology.

6.5 Conclusion

Over the past few years, interest in NMP has grown worldwide, owing to the organ shortage and increased use of marginal grafts. Transitioning from the experimental to the clinical realm, NMP is now coming forward as a means to assess grafts before transplant, to potentially recondition damaged or marginal organs, and to serve as a platform for more advanced graft modification. Throughout this evolution, we have made contributions to to the fundamental experimental understanding of this technology, and as a result have positiviely impacted advancement of clinical liver NMP. *Ex situ* machine perfusion technology is ripe with opportunity for progressive interventions including: 1) selective application of medications for liver defatting, amelioration of IRI, 2) genetic modification of grafts using adenoviral vectors and siRNA to possibly alter graft immunogenic potential, and susceptibility to infection and disease, 3) graft immunomodulation based on suppression or expansion of cell populations in machine perfusate such as Treg cells, 4) nanotherpeutic vectors engineered with target specificity to deliver medications or viral therapy and 5) using MSCs to promote regional regenerative responses for native tissue recovery in damaged organs. The rapidly increasing understanding of these therapies, coupled with iterative advances in NMP technology will likely set the future stage for intensely personalized organ transplant therapies. Although currently far from the case, it is conceivable that in the not too distant future, organs will rarely be discarded, but rather will pass repeatedly through reparative treatments, only to be reused indefinitely for those in need.

6.6 References

1. Bral M, Gala-Lopez B, Thiesen A, et al. Determination of Minimal Hemoglobin Level Necessary for Normothermic Porcine Ex Situ Liver Perfusion. *Transplantation.* 2018;102(8):1284-1292.
2. Bral M, Aboelnazar N, Hatami S, et al. Clearance of transaminases during normothermic ex situ liver perfusion. *PLoS One.* 2019;14(4):e0215619.
3. Bral M, Gala-Lopez B, Bigam D, et al. Preliminary Single-Center Canadian Experience of Human Normothermic Ex Vivo Liver Perfusion: Results of a Clinical Trial. *Am J Transplant.* 2017;17(4):1071-1080.
4. Reddy S, Greenwood J, Maniakin N, et al. Non-heart-beating donor porcine livers: the adverse effect of cooling. *Liver Transpl.* 2005;11(1):35-38.
5. Karangwa SA, Dutkowski P, Fontes P, et al. Machine Perfusion of Donor Livers for Transplantation: A Proposal for Standardized Nomenclature and Reporting Guidelines. *Am J Transplant.* 2016;16(10):2932-2942.
6. Bral M, Dajani K, Leon Izquierdo D, et al. A 'Back-to-base' Experience of Human Normothermic Ex Situ Liver Perfusion: Does the chill kill? *Liver Transpl.* 2019.
7. Butler AJ, Rees MA, Wight DG, et al. Successful extracorporeal porcine liver perfusion for 72 hr. *Transplantation.* 2002;73(8):1212-1218.
8. Vogel T, Brockmann JG, Pigott D, et al. Successful transplantation of porcine liver grafts following 48-hour normothermic preservation. *PLoS One.* 2017;12(11):e0188494.
9. Watson CJ, Randle LV, Kosmoliaptsis V, Gibbs P, Allison M, Butler AJ. 26-hour Storage of a Declined Liver Before Successful Transplantation Using Ex Vivo Normothermic Perfusion. *Ann Surg.* 2017;265(1):e1-e2.

10. Liu Q, Nassar A, Buccini L, et al. Ex situ 86-hour liver perfusion: Pushing the boundary of organ preservation. *Liver Transpl.* 2018;24(4):557-561.
11. de Vries Y, van Leeuwen OB, Matton APM, Fujiyoshi M, de Meijer VE, Porte RJ. Ex situ normothermic machine perfusion of donor livers using a haemoglobin-based oxygen carrier: a viable alternative to red blood cells. *Transpl Int.* 2018;31(11):1281-1282.
12. de Vries Y, Matton APM, Nijsten MWN, et al. Pretransplant sequential hypo- and normothermic machine perfusion of suboptimal livers donated after circulatory death using a hemoglobin-based oxygen carrier perfusion solution. *Am J Transplant.* 2019;19(4):1202-1211.
13. Echeverri J, Goldaracena N, Kaths JM, et al. Comparison of BQ123, Epoprostenol, and Verapamil as Vasodilators During Normothermic Ex Vivo Liver Machine Perfusion. *Transplantation.* 2018;102(4):601-608.
14. Sutton ME, op den Dries S, Karimian N, et al. Criteria for viability assessment of discarded human donor livers during ex vivo normothermic machine perfusion. *PLoS One.* 2014;9(11):e110642.
15. Boteon YL, Stephenson BTF, Neil DAH, Mirza DF, Afford SC, Mergental H. Lipid metabolism and functional assessment of discarded human livers with steatosis undergoing 24 hours of normothermic machine perfusion. *Liver Transpl.* 2018;24(5):708-709.
16. op den Dries S, Karimian N, Sutton ME, et al. Ex vivo normothermic machine perfusion and viability testing of discarded human donor livers. *Am J Transplant.* 2013;13(5):1327-1335.
17. Watson CJE, Kosmoliaptsis V, Pley C, et al. Observations on the ex situ perfusion of livers for transplantation. *Am J Transplant.* 2018;18(8):2005-2020.

18. Nasralla D, Coussios CC, Mergental H, et al. A randomized trial of normothermic preservation in liver transplantation. *Nature.* 2018;557(7703):50-56.

19. Watson CJE, Jochmans I. From "Gut Feeling" to Objectivity: Machine Preservation of the Liver as a Tool to Assess Organ Viability. *Curr Transplant Rep.* 2018;5(1):72-81.

20. Matton APM, de Vries Y, Burlage LC, et al. Biliary Bicarbonate, pH and Glucose Are Suitable Biomarkers of Biliary Viability During Ex Situ Normothermic Machine Perfusion of Human Donor Livers. *Transplantation.* 2018.

21. Liu Q, Nassar A, Farias K, et al. Comparing Normothermic Machine Perfusion Preservation With Different Perfusates on Porcine Livers From Donors After Circulatory Death. *Am J Transplant.* 2016;16(3):794-807.

22. Bohne F, Martinez-Llordella M, Lozano JJ, et al. Intra-graft expression of genes involved in iron homeostasis predicts the development of operational tolerance in human liver transplantation. *J Clin Invest.* 2012;122(1):368-382.

23. Bruinsma BG, Sridharan GV, Weeder PD, et al. Metabolic profiling during ex vivo machine perfusion of the human liver. *Sci Rep.* 2016;6:22415.

24. Watson CJE, Hunt F, Messer S, et al. In situ normothermic perfusion of livers in controlled circulatory death donation may prevent ischemic cholangiopathy and improve graft survival. *Am J Transplant.* 2018.

25. Mergental H, Perera MT, Laing RW, et al. Transplantation of Declined Liver Allografts Following Normothermic Ex-Situ Evaluation. *Am J Transplant.* 2016;16(11):3235-3245.

26. Guarrera JV, Henry SD, Samstein B, et al. Hypothermic machine preservation facilitates successful transplantation of "orphan" extended criteria donor livers. *Am J Transplant.* 2015;15(1):161-169.

27. Senoner T, Schindler S, Stattner S, Ofner D, Troppmair J, Primavesi F. Associations of Oxidative Stress and Postoperative Outcome in Liver Surgery with an Outlook to Future Potential Therapeutic Options. *Oxid Med Cell Longev.* 2019;2019:3950818.

28. Orban JC, Fontaine E, Cassuto E, et al. Effects of cyclosporine A pretreatment of deceased organ donors on kidney graft function (Cis-A-rein): study protocol for a randomized controlled trial. *Trials.* 2018;19(1):231.

29. Nighoghossian N, Ovize M, Mewton N, Ong E, Cho TH. Cyclosporine A, a Potential Therapy of Ischemic Reperfusion Injury. A Common History for Heart and Brain. *Cerebrovasc Dis.* 2016;42(5-6):309-318.

30. Gill RS, Lee TF, Manouchehri N, et al. Postresuscitation cyclosporine treatment attenuates myocardial and cardiac mitochondrial injury in newborn piglets with asphyxia-reoxygenation. *Crit Care Med.* 2013;41(4):1069-1074.

31. Singh D, Chander V, Chopra K. Cyclosporine protects against ischemia/reperfusion injury in rat kidneys. *Toxicology.* 2005;207(3):339-347.

32. Goldaracena N, Echeverri J, Spetzler VN, et al. Anti-inflammatory signaling during ex vivo liver perfusion improves the preservation of pig liver grafts before transplantation. *Liver Transpl.* 2016;22(11):1573-1583.

33. Boteon YL, Afford SC, Mergental H. Pushing the Limits: Machine Preservation of the Liver as a Tool to Recondition High-Risk Grafts. *Curr Transplant Rep.* 2018;5(2):113-120.

34. Linares I, Hamar M, Selzner N, Selzner M. Steatosis in Liver Transplantation: Current Limitations and Future Strategies. *Transplantation.* 2019;103(1):78-90.

35. Nagrath D, Xu H, Tanimura Y, et al. Metabolic preconditioning of donor organs: defatting fatty livers by normothermic perfusion ex vivo. *Metab Eng.* 2009;11(4-5):274-283.

36. Jamieson RW, Zilvetti M, Roy D, et al. Hepatic steatosis and normothermic perfusion-preliminary experiments in a porcine model. *Transplantation.* 2011;92(3):289-295.

37. Banan B, Watson R, Xu M, Lin Y, Chapman W. Development of a normothermic extracorporeal liver perfusion system toward improving viability and function of human extended criteria donor livers. *Liver Transpl.* 2016;22(7):979-993.

38. He X, Guo Z, Zhao Q, et al. The first case of ischemia-free organ transplantation in humans: A proof of concept. *Am J Transplant.* 2018;18(3):737-744.

39. Yeung JC, Wagnetz D, Cypel M, et al. Ex vivo adenoviral vector gene delivery results in decreased vector-associated inflammation pre- and post-lung transplantation in the pig. *Mol Ther.* 2012;20(6):1204-1211.

40. Cypel M, Liu M, Rubacha M, et al. Functional repair of human donor lungs by IL-10 gene therapy. *Sci Transl Med.* 2009;1(4):4ra9.

41. Goldaracena N, Spetzler VN, Echeverri J, et al. Inducing Hepatitis C Virus Resistance After Pig Liver Transplantation-A Proof of Concept of Liver Graft Modification Using Warm Ex Vivo Perfusion. *Am J Transplant.* 2017;17(4):970-978.

42. Mak KY, Chin R, Cunningham SC, et al. ACE2 Therapy Using Adeno-associated Viral Vector Inhibits Liver Fibrosis in Mice. *Mol Ther.* 2015;23(9):1434-1443.

43. Rajapaksha IG, Angus PW, Herath CB. Current therapies and novel approaches for biliary diseases. *World J Gastrointest Pathophysiol.* 2019;10(1):1-10.

44. Thijssen MF, Bruggenwirth IMA, Gillooly A, Khvorova A, Kowalik TF, Martins PN. Gene Silencing With siRNA (RNA Interference): A New Therapeutic Option During Ex Vivo Machine Liver Perfusion Preservation. *Liver Transpl.* 2019;25(1):140-151.

45. Levitsky J. Operational tolerance: past lessons and future prospects. *Liver Transpl.* 2011;17(3):222-232.

46. Levitsky J, Goldberg D, Smith AR, et al. Acute Rejection Increases Risk of Graft Failure and Death in Recent Liver Transplant Recipients. *Clin Gastroenterol Hepatol.* 2017;15(4):584-593 e582.

47. Donckier V, Troisi R, Le Moine A, et al. Early immunosuppression withdrawal after living donor liver transplantation and donor stem cell infusion. *Liver Transpl.* 2006;12(10):1523-1528.

48. Todo S, Yamashita K, Goto R, et al. A pilot study of operational tolerance with a regulatory T-cell-based cell therapy in living donor liver transplantation. *Hepatology.* 2016;64(2):632-643.

49. Camirand G, Riella LV. Treg-Centric View of Immunosuppressive Drugs in Transplantation: A Balancing Act. *Am J Transplant.* 2017;17(3):601-610.

50. Safinia N, Scotta C, Vaikunthanathan T, Lechler RI, Lombardi G. Regulatory T Cells: Serious Contenders in the Promise for Immunological Tolerance in Transplantation. *Front Immunol.* 2015;6:438.

51. Montane J, Bischoff L, Soukhatcheva G, et al. Prevention of murine autoimmune diabetes by CCL22-mediated Treg recruitment to the pancreatic islets. *J Clin Invest.* 2011;121(8):3024-3028.

52. Lange C, Togel F, Ittrich H, et al. Administered mesenchymal stem cells enhance recovery from ischemia/reperfusion-induced acute renal failure in rats. *Kidney Int.* 2005;68(4):1613-1617.

53. Reinders MEJ, van Kooten C, Rabelink TJ, de Fijter JW. Mesenchymal Stromal Cell Therapy for Solid Organ Transplantation. *Transplantation.* 2018;102(1):35-43.

54. Brasile L, Henry N, Orlando G, Stubenitsky B. Potentiating Renal Regeneration Using Mesenchymal Stem Cells. *Transplantation.* 2019;103(2):307-313.

55. Guenthart BA, O'Neill JD, Kim J, Fung K, Vunjak-Novakovic G, Bacchetta M. Cell replacement in human lung bioengineering. *J Heart Lung Transplant.* 2019;38(2):215-224.

56. DiRito JR, Hosgood SA, Tietjen GT, Nicholson ML. The future of marginal kidney repair in the context of normothermic machine perfusion. *Am J Transplant.* 2018;18(10):2400-2408.

57. Tietjen GT, Hosgood SA, DiRito J, et al. Nanoparticle targeting to the endothelium during normothermic machine perfusion of human kidneys. *Sci Transl Med.* 2017;9(418).

58. Stephenson BTF, Bonney GK, Laing RW, et al. Proof of concept: liver splitting during normothermic machine perfusion. *J Surg Case Rep.* 2018;2018(3):rjx218.

59. Marcellin P, Gane E, Buti M, et al. Regression of cirrhosis during treatment with tenofovir disoproxil fumarate for chronic hepatitis B: a 5-year open-label follow-up study. *Lancet.* 2013;381(9865):468-475.

60. Vrselja Z, Daniele SG, Silbereis J, et al. Restoration of brain circulation and cellular functions hours post-mortem. *Nature.* 2019;568(7752):336-343.

Complete bibliography

1. Abt PL, Desai NM, Crawford MD, Forman LM, Markmann JW, Olthoff KM, et al. Survival following liver transplantation from non-heart-beating donors. Ann Surg. 2004;239(1):87-92.

2. Adam R, Bismuth H, Diamond T, Ducot B, Morino M, Astarcioglu I, et al. Effect of extended cold ischaemia with UW solution on graft function after liver transplantation. Lancet. 1992;340(8832):1373-6.

3. Angelico R, Perera MT, Ravikumar R, Holroyd D, Coussios C, Mergental H, et al. Normothermic Machine Perfusion of Deceased Donor Liver Grafts Is Associated With Improved Postreperfusion Hemodynamics. Transplant Direct. 2016;2(9):e97.

4. Axelrod DA, Dzebisashvilli N, Lentine KL, Xiao H, Schnitzler M, Tuttle-Newhall JE, et al. National assessment of early biliary complications after liver transplantation: economic implications. Transplantation. 2014;98(11):1226-35.

5. Banan B, Chung H, Xiao Z, Tarabishy Y, Jia J, Manning P, et al. Normothermic extracorporeal liver perfusion for donation after cardiac death (DCD) livers. Surgery. 2015;158(6):1642-50.

6. Banan B, Watson R, Xu M, Lin Y, Chapman W. Development of a normothermic extracorporeal liver perfusion system toward improving viability and function of human extended criteria donor livers. Liver Transpl. 2016;22(7):979-93.

7. Baskin-Bey ES, Washburn K, Feng S, Oltersdorf T, Shapiro D, Huyghe M, et al. Clinical Trial of the Pan-Caspase Inhibitor, IDN-6556, in Human Liver Preservation Injury. Am J Transplant. 2007;7(1):218-25.

8. Berendsen TA, Bruinsma BG, Lee J, D'Andrea V, Liu Q, Izamis ML, et al. A simplified subnormothermic machine perfusion system restores ischemically damaged liver grafts in a rat model of orthotopic liver transplantation. Transplant Res. 2012;1(1):6.

9. Bessems M, Doorschodt BM, Dinant S, de Graaf W, van Gulik TM. Machine perfusion preservation of the pig liver using a new preservation solution, polysol. Transplant Proc. 2006;38(5):1238-42.

10. Boehnert MU, Yeung JC, Bazerbachi F, Knaak JM, Selzner N, McGilvray ID, et al. Normothermic acellular ex vivo liver perfusion reduces liver and bile duct injury of pig livers retrieved after cardiac death. Am J Transplant. 2013;13(6):1441-9.

11. Boehnert MU, Yeung JC, Knaak JM, Selzner N, Selzner M. Normothermic acellular ex vivo liver perfusion (NEVLP) reduces liver and bile duct in DCD liver grafts. Am J Transplant. 2013;13(12):3290.

12. Bohne F, Martinez-Llordella M, Lozano JJ, Miquel R, Benitez C, Londono MC, et al. Intra-graft expression of genes involved in iron homeostasis predicts the development of operational tolerance in human liver transplantation. J Clin Invest. 2012;122(1):368-82.

13. Bohorquez H, Seal JB, Cohen AJ, Kressel A, Bugeaud E, Bruce DS, et al. Safety and Outcomes in 100 Consecutive Donation After Circulatory Death Liver Transplants Using a Protocol That Includes Thrombolytic Therapy. Am J Transplant. 2017;17(8):2155-64.

14. Boteon YL, Afford SC, Mergental H. Pushing the Limits: Machine Preservation of the Liver as a Tool to Recondition High-Risk Grafts. Curr Transplant Rep. 2018;5(2):113-20.

15. Boteon YL, Stephenson BTF, Neil DAH, Mirza DF, Afford SC, Mergental H. Lipid metabolism and functional assessment of discarded human livers with steatosis undergoing 24 hours of normothermic machine perfusion. Liver Transpl. 2018;24(5):708-9.

16. Boyer JL. Bile formation and secretion. Compr Physiol. 2013;3(3):1035-78.

17. Bral M, Aboelnazar N, Hatami S, Thiesen A, Bigam DL, Freed DH, et al. Clearance of transaminases during normothermic ex situ liver perfusion. PLoS One. 2019;14(4):e0215619.

18. Bral M, Dajani K, Leon Izquierdo D, Bigam D, Kneteman N, Ceresa CDL, et al. A 'Back-to-base' Experience of Human Normothermic Ex Situ Liver Perfusion: Does the chill kill? Liver Transpl. 2019.

19. Bral M, Gala-Lopez B, Bigam D, Kneteman N, Malcolm A, Livingstone S, et al. Preliminary Single-Center Canadian Experience of Human Normothermic Ex Vivo Liver Perfusion: Results of a Clinical Trial. Am J Transplant. 2016.

20. Bral M, Gala-Lopez B, Bigam D, Kneteman N, Malcolm A, Livingstone S, et al. Preliminary Single-Center Canadian Experience of Human Normothermic Ex Vivo Liver Perfusion: Results of a Clinical Trial. Am J Transplant. 2017;17(4):1071-80.

21. Bral M, Gala-Lopez B, Thiesen A, Hatami S, Bigam DL, Freed DM, et al. Determination of Minimal Hemoglobin Level Necessary for Normothermic Porcine Ex Situ Liver Perfusion. Transplantation. 2018;102(8):1284-92.

22. Braley AE, Sanders M. Aureomycin in ocular infections; a preliminary report. J Am Med Assoc. 1948;138(6):426.

23. Braly P, Garite T, German JC. Fetal heart rate patterns in infants in whom necrotizing enterocolitis develops: a preliminary report. Arch Surg. 1980;115(9):1050-3.

24. Brasile L, Henry N, Orlando G, Stubenitsky B. Potentiating Renal Regeneration Using Mesenchymal Stem Cells. Transplantation. 2019;103(2):307-13.

25. Brettschneider L, Daloze PM, Huguet C, Groth CG, Kashiwagi N, Hutchison DE, et al. Successful Orthotopic Transplantation of Liver Homografts after Eight to Twenty-Five Hours Preservation. Surg Forum. 1967;18:376-8.

26. Brettschneider L, Daloze PM, Huguet C, Porter KA, Groth CG, Kashiwagi N, et al. The Use of Combined Preservation Techniques for Extended Storage of Orthotopic Liver Homografts. Surg Gynecol Obstet. 1968;126(2):263-74.

27. Brockmann J, Reddy S, Coussios C, Pigott D, Guirriero D, Hughes D, et al. Normothermic perfusion: a new paradigm for organ preservation. Ann Surg. 2009;250(1):1-6.

28. Brokelman W, Stel AL, Ploeg RJ. Risk factors for primary dysfunction after liver transplantation in the University of Wisconsin solution era. Transplant Proc. 1999;31(5):2087-90.

29. Bruinsma BG, Avruch JH, Weeder PD, Sridharan GV, Uygun BE, Karimian NG, et al. Functional human liver preservation and recovery by means of subnormothermic machine perfusion. J Vis Exp. 2015(98).

30. Bruinsma BG, Berendsen TA, Izamis ML, Yarmush ML, Uygun K. Determination and extension of the limits to static cold storage using subnormothermic machine perfusion. Int J Artif Organs. 2013;36(11):775-80.

31. Bruinsma BG, Sridharan GV, Weeder PD, Avruch JH, Saeidi N, Ozer S, et al. Metabolic profiling during ex vivo machine perfusion of the human liver. Sci Rep. 2016;6:22415.

32. Bruinsma BG, Yeh H, Ozer S, Martins PN, Farmer A, Wu W, et al. Subnormothermic machine perfusion for ex vivo preservation and recovery of the human liver for transplantation. Am J Transplant. 2014;14(6):1400-9.

33. Bruni A, Pepper AR, Gala-Lopez B, Pawlick R, Abualhassan N, Crapo JD, et al. A novel redox-active metalloporphyrin reduces reactive oxygen species and inflammatory markers but does not improve marginal mass engraftment in a murine donation after circulatory death islet transplantation model. Islets. 2016;8(4):e1190058.

34. Butler AJ, Rees MA, Wight DG, Casey ND, Alexander G, White DJ, et al. Successful extracorporeal porcine liver perfusion for 72 hr. Transplantation. 2002;73(8):1212-8.

35. Camirand G, Riella LV. Treg-Centric View of Immunosuppressive Drugs in Transplantation: A Balancing Act. Am J Transplant. 2017;17(3):601-10.

36. Chung IS, Kim HY, Shin YH, Ko JS, Gwak MS, Sim WS, et al. Incidence and predictors of post-reperfusion syndrome in living donor liver transplantation. Clin Transplant. 2012;26(4):539-43.

37. Collins GM, Bravo-Shugarman M, Terasaki PI. Kidney preservation for transportation. Initial perfusion and 30 hours' ice storage. Lancet. 1969;2(7632):1219-22.

38. Croome KP, Lee DD, Keaveny AP, Taner CB. Improving National Results in Liver Transplantation Using Grafts From Donation After Cardiac Death Donors. Transplantation. 2016;100(12):2640-7.

39. Croome KP, Mathur AK, Lee DD, Moss AA, Rosen CB, Heimbach JK, et al. Outcomes of Donation After Circulatory Death Liver Grafts From Donors 50 Years or Older: A Multicenter Analysis. Transplantation. 2018;102(7):1108-14.

40. Cursio R, Gugenheim J. Ischemia-Reperfusion Injury and Ischemic-Type Biliary Lesions following Liver Transplantation. J Transplant. 2012;2012:164329.

41. Cypel M, Liu M, Rubacha M, Yeung JC, Hirayama S, Anraku M, et al. Functional repair of human donor lungs by IL-10 gene therapy. Sci Transl Med. 2009;1(4):4ra9.

42. Danial NN, Korsmeyer SJ. Cell death: critical control points. Cell. 2004;116(2):205-19.

43. De Carlis R, Di Sandro S, Lauterio A, Ferla F, Dell'Acqua A, Zanierato M, et al. Successful donation after cardiac death liver transplants with prolonged warm ischemia time using normothermic regional perfusion. Liver Transpl. 2017;23(2):166-73.

44. de Rougemont O, Breitenstein S, Leskosek B, Weber A, Graf R, Clavien PA, et al. One hour hypothermic oxygenated perfusion (HOPE) protects nonviable liver allografts donated after cardiac death. Ann Surg. 2009;250(5):674-83.

45. de Vera ME, Lopez-Solis R, Dvorchik I, Campos S, Morris W, Demetris AJ, et al. Liver transplantation using donation after cardiac death donors: long-term follow-up from a single center. Am J Transplant. 2009;9(4):773-81.

46. de Vries Y, Matton APM, Nijsten MWN, Werner MJM, van den Berg AP, de Boer MT, et al. Pretransplant sequential hypo- and normothermic machine perfusion of suboptimal livers donated after circulatory death using a hemoglobin-based oxygen carrier perfusion solution. Am J Transplant. 2019;19(4):1202-11.

47. de Vries Y, van Leeuwen OB, Matton APM, Fujiyoshi M, de Meijer VE, Porte RJ. Ex situ normothermic machine perfusion of donor livers using a haemoglobin-based oxygen carrier: a viable alternative to red blood cells. Transpl Int. 2018;31(11):1281-2.

48. Dindo D, Demartines N, Clavien PA. Classification of surgical complications: a new proposal with evaluation in a cohort of 6336 patients and results of a survey. Ann Surg. 2004;240(2):205-13.

49. DiRito JR, Hosgood SA, Tietjen GT, Nicholson ML. The future of marginal kidney repair in the context of normothermic machine perfusion. Am J Transplant. 2018;18(10):2400-8.

50. do Amaral AS, Pawlick RL, Rodrigues E, Costal F, Pepper A, Galvao FH, et al. Glutathione ethyl ester supplementation during pancreatic islet isolation improves viability and transplant outcomes in a murine marginal islet mass model. PLoS One. 2013;8(2):e55288.

51. Donckier V, Troisi R, Le Moine A, Toungouz M, Ricciardi S, Colle I, et al. Early immunosuppression withdrawal after living donor liver transplantation and donor stem cell infusion. Liver Transpl. 2006;12(10):1523-8.

52. Dutkowski P, de Rougemont O, Clavien PA. Alexis Carrel: genius, innovator and ideologist. Am J Transplant. 2008;8(10):1998-2003.

53. Dutkowski P, Guarrera JV, de Jonge J, Martins PN, Porte RJ, Clavien PA. Evolving Trends in Machine Perfusion for Liver Transplantation. Gastroenterology. 2019;156(6):1542-7.

54. Dutkowski P, Polak WG, Muiesan P, Schlegel A, Verhoeven CJ, Scalera I, et al. First Comparison of Hypothermic Oxygenated PErfusion Versus Static Cold Storage of Human Donation After Cardiac Death Liver Transplants: An International-matched Case Analysis. Ann Surg. 2015;262(5):764-70; discussion 70-1.

55. Dutkowski P, Schlegel A, de Oliveira M, Mullhaupt B, Neff F, Clavien PA. HOPE for human liver grafts obtained from donors after cardiac death. J Hepatol. 2014;60(4):765-72.

56. Echeverri J, Goldaracena N, Kaths JM, Linares I, Roizales R, Kollmann D, et al. Comparison of BQ123, Epoprostenol, and Verapamil as Vasodilators During Normothermic Ex Vivo Liver Machine Perfusion. Transplantation. 2018;102(4):601-8.

57. Eisenbach C, Encke J, Merle U, Gotthardt D, Weiss KH, Schneider L, et al. An early increase in gamma glutamyltranspeptidase and low aspartate aminotransferase peak values are associated with superior outcomes after orthotopic liver transplantation. Transplant Proc. 2009;41(5):1727-30.

58. Fang X, Neyrinck AP, Matthay MA, Lee JW. Allogeneic human mesenchymal stem cells restore epithelial protein permeability in cultured human alveolar type II cells by secretion of angiopoietin-1. J Biol Chem. 2010;285(34):26211-22.

59. Fondevila C, Hessheimer AJ, Maathuis MH, Munoz J, Taura P, Calatayud D, et al. Hypothermic oxygenated machine perfusion in porcine donation after circulatory determination of death liver transplant. Transplantation. 2012;94(1):22-9.

60. Fondevila C, Hessheimer AJ, Maathuis MH, Munoz J, Taura P, Calatayud D, et al. Superior preservation of DCD livers with continuous normothermic perfusion. Ann Surg. 2011;254(6):1000-7.

61. Fontes P, Lopez R, van der Plaats A, Vodovotz Y, Minervini M, Scott V, et al. Liver preservation with machine perfusion and a newly developed cell-free oxygen carrier solution under subnormothermic conditions. Am J Transplant. 2015;15(2):381-94.

62. Fontes PA. The Evolution of Oxygen Carrier Solutions for Machine Perfusion. Transplantation. 2017;101(11):2657-8.

63. Friend PJ, Imber C, St Peter S, Lopez I, Butler AJ, Rees MA. Normothermic perfusion of the isolated liver. Transplant Proc. 2001;33(7-8):3436-8.

64. Furukawa H, Todo S, Imventarza O, Casavilla A, Wu YM, Scotti-Foglieni C, et al. Effect of cold ischemia time on the early outcome of human hepatic allografts preserved with UW solution. Transplantation. 1991;51(5):1000-4.

65. Gala-Lopez BL, Pepper AR, Pawlick RL, O'Gorman D, Kin T, Bruni A, et al. Antiaging Glycopeptide Protects Human Islets Against Tacrolimus-Related Injury and Facilitates Engraftment in Mice. Diabetes. 2016;65(2):451-62.

66. Garcia-Valdecasas JC, Tabet J, Valero R, Taura P, Rull R, Garcia F, et al. Liver conditioning after cardiac arrest: the use of normothermic recirculation in an experimental animal model. Transpl Int. 1998;11(6):424-32.

67. Gilbo N, Monbaliu D. Temperature and oxygenation during organ preservation: friends or foes? Curr Opin Organ Transplant. 2017;22(3):290-9.

68. Gill RS, Lee TF, Manouchehri N, Liu JQ, Lopaschuk G, Bigam DL, et al. Postresuscitation cyclosporine treatment attenuates myocardial and cardiac mitochondrial injury in newborn piglets with asphyxia-reoxygenation. Crit Care Med. 2013;41(4):1069-74.

69. Goldaracena N, Echeverri J, Spetzler VN, Kaths JM, Barbas AS, Louis KS, et al. Anti-inflammatory signaling during ex vivo liver perfusion improves the preservation of pig liver grafts before transplantation. Liver Transpl. 2016;22(11):1573-83.

70. Goldaracena N, Spetzler VN, Echeverri J, Kaths JM, Cherepanov V, Persson R, et al. Inducing Hepatitis C Virus Resistance After Pig Liver Transplantation-A Proof of Concept of Liver Graft Modification Using Warm Ex Vivo Perfusion. Am J Transplant. 2016.

71. Goldaracena N, Spetzler VN, Echeverri J, Kaths JM, Cherepanov V, Persson R, et al. Inducing Hepatitis C Virus Resistance After Pig Liver Transplantation-A Proof of Concept of Liver Graft Modification Using Warm Ex Vivo Perfusion. Am J Transplant. 2017;17(4):970-8.

72. Gotts JE, Matthay MA. Mesenchymal stem cells and acute lung injury. Crit Care Clin. 2011;27(3):719-33.

73. Grewal HP, Willingham DL, Nguyen J, Hewitt WR, Taner BC, Cornell D, et al. Liver transplantation using controlled donation after cardiac death donors: an analysis of a large single-center experience. Liver Transpl. 2009;15(9):1028-35.

74. Guarrera JV, Estevez J, Boykin J, Boyce R, Rashid J, Sun S, et al. Hypothermic machine perfusion of liver grafts for transplantation: technical development in human discard and miniature swine models. Transplant Proc. 2005;37(1):323-5.

75. Guarrera JV, Henry SD, Chen SW, Brown T, Nachber E, Arrington B, et al. Hypothermic machine preservation attenuates ischemia/reperfusion markers after liver transplantation: preliminary results. The Journal of surgical research. 2011;167(2):e365-73.

76. Guarrera JV, Henry SD, Samstein B, Odeh-Ramadan R, Kinkhabwala M, Goldstein MJ, et al. Hypothermic machine preservation in human liver transplantation: the first clinical series. Am J Transplant. 2010;10(2):372-81.

77. Guarrera JV, Henry SD, Samstein B, Reznik E, Musat C, Lukose TI, et al. Hypothermic machine preservation facilitates successful transplantation of "orphan" extended criteria donor livers. Am J Transplant. 2015;15(1):161-9.

78. Guenthart BA, O'Neill JD, Kim J, Fung K, Vunjak-Novakovic G, Bacchetta M. Cell replacement in human lung bioengineering. J Heart Lung Transplant. 2019;38(2):215-24.

79. He X, Guo Z, Zhao Q, Ju W, Wang D, Wu L, et al. The first case of ischemia-free organ transplantation in humans: A proof of concept. Am J Transplant. 2018;18(3):737-44.

80. Hirayama S, Sato M, Liu M, Loisel-Meyer S, Yeung JC, Wagnetz D, et al. Local long-term expression of lentivirally delivered IL-10 in the lung attenuates obliteration of intrapulmonary allograft airways. Hum Gene Ther. 2011;22(11):1453-60.

81. Hirayama S, Sato M, Loisel-Meyer S, Matsuda Y, Oishi H, Guan Z, et al. Lentivirus IL-10 gene therapy down-regulates IL-17 and attenuates mouse orthotopic lung allograft rejection. Am J Transplant. 2013;13(6):1586-93.

82. Hoyer DP, Mathe Z, Gallinat A, Canbay AC, Treckmann JW, Rauen U, et al. Controlled Oxygenated Rewarming of Cold Stored Livers Prior to Transplantation: First Clinical Application of a New Concept. Transplantation. 2016;100(1):147-52.

83. Hoyer DP, Paul A, Luer S, Reis H, Efferz P, Minor T. End-ischemic reconditioning of liver allografts: Controlling the rewarming. Liver Transpl. 2016;22(9):1223-30.

84. Imber CJ, St Peter SD, de Cenarruzabeitia IL, Lemonde H, Rees M, Butler A, et al. Optimisation of bile production during normothermic preservation of porcine livers. Am J Transplant. 2002;2(7):593-9.

85. Imber CJ, St Peter SD, Lopez de Cenarruzabeitia I, Pigott D, James T, Taylor R, et al. Advantages of normothermic perfusion over cold storage in liver preservation. Transplantation. 2002;73(5):701-9.

86. Iwamoto H, Matsuno N, Narumi Y, Uchiyama M, Kozaki K, Degawa H, et al. Beneficial effect of machine perfusion preservation on liver transplantation from non-heart-beating donors. Transplant Proc. 2000;32(7):1645-6.

87. Jain S, Lee CY, Baicu S, Duncan H, Xu H, Jones JW, Jr., et al. Hepatic function in hypothermically stored porcine livers: comparison of hypothermic machine perfusion vs cold storage. Transplant Proc. 2005;37(1):340-1.

88. Jamieson RW, Zilvetti M, Roy D, Hughes D, Morovat A, Coussios CC, et al. Hepatic steatosis and normothermic perfusion-preliminary experiments in a porcine model. Transplantation. 2011;92(3):289-95.

89. Jassem W, Xystrakis E, Ghnewa YG, Yuksel M, Pop O, Martinez-Llordella M, et al. Normothermic machine perfusion (NMP) inhibits proinflammatory responses in the liver and promotes regeneration. Hepatology. 2018.

90. Kalisvaart M, de Haan JE, Polak WG, Metselaar HJ, Wijnhoven BPL, JNM IJ, et al. Comparison of Postoperative Outcomes Between Donation After Circulatory Death and Donation After Brain Death Liver Transplantation Using the Comprehensive Complication Index. Ann Surg. 2017;266(5):772-8.

91. Kamimoto Y, Horiuchi S, Tanase S, Morino Y. Plasma clearance of intravenously injected aspartate aminotransferase isozymes: evidence for preferential uptake by sinusoidal liver cells. Hepatology. 1985;5(3):367-75.

92. Karangwa SA, Dutkowski P, Fontes P, Friend PJ, Guarrera JV, Markmann JF, et al. Machine Perfusion of Donor Livers for Transplantation: A Proposal for Standardized Nomenclature and Reporting Guidelines. Am J Transplant. 2016;16(10):2932-42.

93. Kim WR, Flamm SL, Di Bisceglie AM, Bodenheimer HC, Public Policy Committee of the American Association for the Study of Liver D. Serum activity of alanine aminotransferase (ALT) as an indicator of health and disease. Hepatology. 2008;47(4):1363-70.

94. Kim WR, Lake JR, Smith JM, Schladt DP, Skeans MA, Noreen SM, et al. OPTN/SRTR 2017 Annual Data Report: Liver. Am J Transplant. 2019;19 Suppl 2:184-283.

95. Kim WR, Lake JR, Smith JM, Skeans MA, Schladt DP, Edwards EB, et al. OPTN/SRTR 2015 Annual Data Report: Liver. Am J Transplant. 2017;17 Suppl 1:174-251.

96. Knaak JM, Spetzler VN, Goldaracena N, Boehnert MU, Bazerbachi F, Louis KS, et al. Subnormothermic ex vivo liver perfusion reduces endothelial cell and bile duct injury after donation after cardiac death pig liver transplantation. Liver Transpl. 2014;20(11):1296-305.

97. Knaak JM, Spetzler VN, Goldaracena N, Louis KS, Selzner N, Selzner M. Technique of subnormothermic ex vivo liver perfusion for the storage, assessment, and repair of marginal liver grafts. J Vis Exp. 2014(90):e51419.

98. Kornasiewicz O, Bojarczuk K, Bugajski M, Golab J, Krawczyk M. Application of a proteomic approach to identify proteins associated with primary graft non-function after liver transplantation. Int J Mol Med. 2012;30(4):755-64.

99. Laing RW, Bhogal RH, Wallace L, Boteon Y, Neil DAH, Smith A, et al. The Use of an Acellular Oxygen Carrier in a Human Liver Model of Normothermic Machine Perfusion. Transplantation. 2017;101(11):2746-56.

100. Lange C, Togel F, Ittrich H, Clayton F, Nolte-Ernsting C, Zander AR, et al. Administered mesenchymal stem cells enhance recovery from ischemia/reperfusion-induced acute renal failure in rats. Kidney Int. 2005;68(4):1613-7.

101. Lee JW, Fang X, Gupta N, Serikov V, Matthay MA. Allogeneic human mesenchymal stem cells for treatment of E. coli endotoxin-induced acute lung injury in the ex vivo perfused human lung. Proc Natl Acad Sci U S A. 2009;106(38):16357-62.

102. Levitsky J. Operational tolerance: past lessons and future prospects. Liver Transpl. 2011;17(3):222-32.

103. Levitsky J, Goldberg D, Smith AR, Mansfield SA, Gillespie BW, Merion RM, et al. Acute Rejection Increases Risk of Graft Failure and Death in Recent Liver Transplant Recipients. Clin Gastroenterol Hepatol. 2017;15(4):584-93 e2.

104. Linares I, Hamar M, Selzner N, Selzner M. Steatosis in Liver Transplantation: Current Limitations and Future Strategies. Transplantation. 2019;103(1):78-90.

105. Liu Q, Nassar A, Buccini L, Grady P, Soliman B, Hassan A, et al. Ex situ 86-hour liver perfusion: Pushing the boundary of organ preservation. Liver Transpl. 2018;24(4):557-61.

106. Liu Q, Nassar A, Farias K, Buccini L, Baldwin W, Mangino M, et al. Sanguineous normothermic machine perfusion improves hemodynamics and biliary epithelial regeneration in donation after cardiac death porcine livers. Liver Transpl. 2014;20(8):987-99.

107. Liu Q, Nassar A, Farias K, Buccini L, Mangino MJ, Baldwin W, et al. Comparing Normothermic Machine Perfusion Preservation With Different Perfusates on Porcine Livers From Donors After Circulatory Death. Am J Transplant. 2016;16(3):794-807.

108. Lonze BE, Holzer HT, Knabel MK, Locke JE, DiCamillo GA, Karhadkar SS, et al. In vitro and ex vivo delivery of short hairpin RNAs for control of hepatitis C viral transcript expression. Arch Surg. 2012;147(4):384-7.

109. Lutz J, Thurmel K, Heemann U. Anti-inflammatory treatment strategies for ischemia/reperfusion injury in transplantation. J Inflamm (Lond). 2010;7:27.

110. Machuca TN, Cypel M, Bonato R, Yeung JC, Chun YM, Juvet S, et al. Safety and Efficacy of Ex Vivo Donor Lung Adenoviral IL-10 Gene Therapy in a Large Animal Lung Transplant Survival Model. Hum Gene Ther. 2017;28(9):757-65.

111. Mak KY, Chin R, Cunningham SC, Habib MR, Torresi J, Sharland AF, et al. ACE2 Therapy Using Adeno-associated Viral Vector Inhibits Liver Fibrosis in Mice. Mol Ther. 2015;23(9):1434-43.

112. Marcellin P, Gane E, Buti M, Afdhal N, Sievert W, Jacobson IM, et al. Regression of cirrhosis during treatment with tenofovir disoproxil fumarate for chronic hepatitis B: a 5-year open-label follow-up study. Lancet. 2013;381(9865):468-75.

113. Matton APM, Burlage LC, van Rijn R, de Vries Y, Karangwa SA, Nijsten MW, et al. Normothermic Machine Perfusion of Donor Livers Without the Need for Human Blood Products. Liver Transpl. 2017.

114. Matton APM, de Vries Y, Burlage LC, van Rijn R, Fujiyoshi M, de Meijer VE, et al. Biliary Bicarbonate, pH and Glucose Are Suitable Biomarkers of Biliary Viability During Ex Situ Normothermic Machine Perfusion of Human Donor Livers. Transplantation. 2018.

115. McCall MD, Maciver AM, Kin T, Emamaullee J, Pawlick R, Edgar R, et al. Caspase inhibitor IDN6556 facilitates marginal mass islet engraftment in a porcine islet autotransplant model. Transplantation. 2012;94(1):30-5.

116. Mergental H, Perera MT, Laing RW, Muiesan P, Isaac JR, Smith A, et al. Transplantation of Declined Liver Allografts Following Normothermic Ex-Situ Evaluation. Am J Transplant. 2016;16(11):3235-45.

117. Mergental H, Stephenson BTF, Laing RW, Kirkham AJ, Neil DAH, Wallace LL, et al. Development of Clinical Criteria for Functional Assessment to Predict Primary Nonfunction of High-Risk Livers Using Normothermic Machine Perfusion. Liver Transpl. 2018;24(10):1453-69.

118. Merion RM, Goodrich NP, Feng S. How can we define expanded criteria for liver donors? J Hepatol. 2006;45(4):484-8.

119. Merion RM, Pelletier SJ, Goodrich N, Englesbe MJ, Delmonico FL. Donation after cardiac death as a strategy to increase deceased donor liver availability. Ann Surg. 2006;244(4):555-62.

120. Minor T, Efferz P, Fox M, Wohlschlaeger J, Luer B. Controlled oxygenated rewarming of cold stored liver grafts by thermally graduated machine perfusion prior to reperfusion. Am J Transplant. 2013;13(6):1450-60.

121. Modanlou HD, Freeman RK, Braly P. A simple method of fetal and neonatal heart rate beat-to-beat variability quantitation: preliminary report. Am J Obstet Gynecol. 1977;127(8):861-8.

122. Monbaliu D, Liu Q, Libbrecht L, De Vos R, Vekemans K, Debbaut C, et al. Preserving the morphology and evaluating the quality of liver grafts by hypothermic machine perfusion: a proof-of-concept study using discarded human livers. Liver Transpl. 2012;18(12):1495-507.

123. Montane J, Bischoff L, Soukhatcheva G, Dai DL, Hardenberg G, Levings MK, et al. Prevention of murine autoimmune diabetes by CCL22-mediated Treg recruitment to the pancreatic islets. J Clin Invest. 2011;121(8):3024-8.

124. Morito N, Obara H, Matsuno N, Enosawa S, Watanabe K, Furukori M, et al. Regulated Oxygenation of Rewarming Machine Perfusion for Porcine Donation After Cardiac Death Liver Transplantation. Transplant Proc. 2016;48(4):1244-6.

125. Muller X, Marcon F, Sapisochin G, Marquez M, Dondero F, Rayar M, et al. Defining Benchmarks in Liver Transplantation: A Multicenter Outcome Analysis Determining Best Achievable Results. Ann Surg. 2018;267(3):419-25.

126. Nagrath D, Xu H, Tanimura Y, Zuo R, Berthiaume F, Avila M, et al. Metabolic preconditioning of donor organs: defatting fatty livers by normothermic perfusion ex vivo. Metab Eng. 2009;11(4-5):274-83.

127. Nasralla D, Coussios CC, Mergental H, Akhtar MZ, Butler AJ, Ceresa CDL, et al. A randomized trial of normothermic preservation in liver transplantation. Nature. 2018;557(7703):50-6.

128. Nassar A, Liu Q, Farias K, Buccini L, Baldwin W, Bennett A, et al. Impact of Temperature on Porcine Liver Machine Perfusion From Donors After Cardiac Death. Artif Organs. 2016;40(10):999-1008.

129. Nassar A, Liu Q, Farias K, D'Amico G, Tom C, Grady P, et al. Ex vivo normothermic machine perfusion is safe, simple, and reliable: results from a large animal model. Surg Innov. 2015;22(1):61-9.

130. Nassar A, Quintini C. Vasodilation During Normothermic Machine Perfusion; Preventing the No-Reflow Phenomena. Transplantation. 2018;102(4):548-9.

131. Nativ NI, Maguire TJ, Yarmush G, Brasaemle DL, Henry SD, Guarrera JV, et al. Liver defatting: an alternative approach to enable steatotic liver transplantation. Am J Transplant. 2012;12(12):3176-83.

132. Natori S, Higuchi H, Contreras P, Gores GJ. The caspase inhibitor IDN-6556 prevents caspase activation and apoptosis in sinusoidal endothelial cells during liver preservation injury. Liver Transpl. 2003;9(3):278-84.

133. Nighoghossian N, Ovize M, Mewton N, Ong E, Cho TH. Cyclosporine A, a Potential Therapy of Ischemic Reperfusion Injury. A Common History for Heart and Brain. Cerebrovasc Dis. 2016;42(5-6):309-18.

134. Olthoff KM, Kulik L, Samstein B, Kaminski M, Abecassis M, Emond J, et al. Validation of a current definition of early allograft dysfunction in liver transplant recipients and analysis of risk factors. Liver Transpl. 2010;16(8):943-9.

135. Oniscu GC, Randle LV, Muiesan P, Butler AJ, Currie IS, Perera MT, et al. In situ normothermic regional perfusion for controlled donation after circulatory death--the United Kingdom experience. Am J Transplant. 2014;14(12):2846-54.

136. op den Dries S, Karimian N, Sutton ME, Westerkamp AC, Nijsten MW, Gouw AS, et al. Ex vivo normothermic machine perfusion and viability testing of discarded human donor livers. Am J Transplant. 2013;13(5):1327-35.

137. Op den Dries S, Sutton ME, Karimian N, de Boer MT, Wiersema-Buist J, Gouw AS, et al. Hypothermic oxygenated machine perfusion prevents arteriolonecrosis of the peribiliary plexus in pig livers donated after circulatory death. PLoS One. 2014;9(2):e88521.

138. Op den Dries S, Sutton ME, Lisman T, Porte RJ. Protection of bile ducts in liver transplantation: looking beyond ischemia. Transplantation. 2011;92(4):373-9.

139. Orban JC, Fontaine E, Cassuto E, Baumstarck K, Leone M, Constantin JM, et al. Effects of cyclosporine A pretreatment of deceased organ donors on kidney graft function (Cis-A-rein): study protocol for a randomized controlled trial. Trials. 2018;19(1):231.

140. Otero A, Gomez-Gutierrez M, Suarez F, Arnal F, Fernandez-Garcia A, Aguirrezabalaga J, et al. Liver transplantation from Maastricht category 2 non-heart-beating donors. Transplantation. 2003;76(7):1068-73.

141. Pepper AR, Bruni A, Pawlick R, Wink J, Rafiei Y, Gala-Lopez B, et al. Engraftment Site and Effectiveness of the Pan-Caspase Inhibitor F573 to Improve Engraftment in Mouse and Human Islet Transplantation in Mice. Transplantation. 2017.

142. Perera T, Mergental H, Stephenson B, Roll GR, Cilliers H, Liang R, et al. First human liver transplantation using a marginal allograft resuscitated by normothermic machine perfusion. Liver Transpl. 2016;22(1):120-4.

143. Pienaar BH, Lindell SL, Van Gulik T, Southard JH, Belzer FO. Seventy-two-hour preservation of the canine liver by machine perfusion. Transplantation. 1990;49(2):258-60.

144. Radi ZA, Koza-Taylor PH, Bell RR, Obert LA, Runnels HA, Beebe JS, et al. Increased serum enzyme levels associated with kupffer cell reduction with no signs of hepatic or skeletal muscle injury. Am J Pathol. 2011;179(1):240-7.

145. Rajapaksha IG, Angus PW, Herath CB. Current therapies and novel approaches for biliary diseases. World J Gastrointest Pathophysiol. 2019;10(1):1-10.

146. Ravikumar R, Jassem W, Mergental H, Heaton N, Mirza D, Perera MT, et al. Liver Transplantation After Ex Vivo Normothermic Machine Preservation: A Phase 1 (First-in-Man) Clinical Trial. Am J Transplant. 2016;16(6):1779-87.

147. Ravikumar R, Leuvenink H, Friend PJ. Normothermic liver preservation: a new paradigm? Transpl Int. 2015;28(6):690-9.

148. Reddy S, Greenwood J, Maniakin N, Bhattacharjya S, Zilvetti M, Brockmann J, et al. Non-heart-beating donor porcine livers: the adverse effect of cooling. Liver Transpl. 2005;11(1):35-8.

149. Reddy SP, Bhattacharjya S, Maniakin N, Greenwood J, Guerreiro D, Hughes D, et al. Preservation of porcine non-heart-beating donor livers by sequential cold storage and warm perfusion. Transplantation. 2004;77(9):1328-32.

150. Reinders MEJ, van Kooten C, Rabelink TJ, de Fijter JW. Mesenchymal Stromal Cell Therapy for Solid Organ Transplantation. Transplantation. 2018;102(1):35-43.

151. Robertson FP, Bessell PR, Diaz-Nieto R, Thomas N, Rolando N, Fuller B, et al. High serum Aspartate transaminase levels on day 3 postliver transplantation correlates with graft and patient survival and would be a valid surrogate for outcome in liver transplantation clinical trials. Transpl Int. 2016;29(3):323-30.

152. Rojas-Pena A, Bartlett RH. Ex Situ Organ Preservation: The Temperature Paradigm. Transplantation. 2018;102(4):554-6.

153. Rosen HR, Martin P, Goss J, Donovan J, Melinek J, Rudich S, et al. Significance of early aminotransferase elevation after liver transplantation. Transplantation. 1998;65(1):68-72.

154. Safinia N, Scotta C, Vaikunthanathan T, Lechler RI, Lombardi G. Regulatory T Cells: Serious Contenders in the Promise for Immunological Tolerance in Transplantation. Front Immunol. 2015;6:438.

155. Schlegel A, Dutkowski P. Role of hypothermic machine perfusion in liver transplantation. Transpl Int. 2015;28(6):677-89.

156. Schlegel A, Rougemont O, Graf R, Clavien PA, Dutkowski P. Protective mechanisms of end-ischemic cold machine perfusion in DCD liver grafts. J Hepatol. 2013;58(2):278-86.

157. Schlegel A, Scalera I, Perera M, Kalisvaart M, Mergental H, Mirza DF, et al. Impact of donor age in donation after circulatory death liver transplantation: Is the cutoff "60" still of relevance? Liver Transpl. 2018;24(3):352-62.

158. Schon MR, Kollmar O, Wolf S, Schrem H, Matthes M, Akkoc N, et al. Liver transplantation after organ preservation with normothermic extracorporeal perfusion. Ann Surg. 2001;233(1):114-23.

159. Seal JB, Bohorquez H, Reichman T, Kressel A, Ghanekar A, Cohen A, et al. Thrombolytic protocol minimizes ischemic-type biliary complications in liver transplantation from donation after circulatory death donors. Liver Transpl. 2015;21(3):321-8.

160. Selzner M, Goldaracena N, Echeverri J, Kaths JM, Linares I, Selzner N, et al. Normothermic ex vivo liver perfusion using steen solution as perfusate for human liver transplantation: First North American results. Liver Transpl. 2016;22(11):1501-8.

161. Selzner M, Goldaracena N, Echeverri J, Kaths JM, Linares I, Selzner N, et al. Normothermic Ex Vivo Liver Perfusion Using Steen Solution as Perfusate for Human Liver Transplantation-First North American Results. Liver Transpl. 2016.

162. Senoner T, Schindler S, Stattner S, Ofner D, Troppmair J, Primavesi F. Associations of Oxidative Stress and Postoperative Outcome in Liver Surgery with an Outlook to Future Potential Therapeutic Options. Oxid Med Cell Longev. 2019;2019:3950818.

163. Shi S, Xue F. Current Antioxidant Treatments in Organ Transplantation. Oxid Med Cell Longev. 2016;2016:8678510.

164. Singh D, Chander V, Chopra K. Cyclosporine protects against ischemia/reperfusion injury in rat kidneys. Toxicology. 2005;207(3):339-47.

165. Spetzler VN, Goldaracena N, Echiverri J, Kaths JM, Louis KS, Adeyi OA, et al. Subnormothermic ex vivo liver perfusion is a safe alternative to cold static storage for preserving standard criteria grafts. Liver Transpl. 2016;22(1):111-9.

166. Starzl TE, Groth CG, Brettschneider L, Moon JB, Fulginiti VA, Cotton EK, et al. Extended survival in 3 cases of orthotopic homotransplantation of the human liver. Surgery. 1968;63(4):549-63.

167. Stephenson BTF, Bonney GK, Laing RW, Bhogal RH, Marcon F, Neil DAH, et al. Proof of concept: liver splitting during normothermic machine perfusion. J Surg Case Rep. 2018;2018(3):rjx218.

168. Sutton ME, op den Dries S, Karimian N, Weeder PD, de Boer MT, Wiersema-Buist J, et al. Criteria for viability assessment of discarded human donor livers during ex vivo normothermic machine perfusion. PLoS One. 2014;9(11):e110642.

169. Thijssen MF, Bruggenwirth IMA, Gillooly A, Khvorova A, Kowalik TF, Martins PN. Gene Silencing With siRNA (RNA Interference): A New Therapeutic Option During Ex Vivo Machine Liver Perfusion Preservation. Liver Transpl. 2019;25(1):140-51.

170. Tietjen GT, Hosgood SA, DiRito J, Cui J, Deep D, Song E, et al. Nanoparticle targeting to the endothelium during normothermic machine perfusion of human kidneys. Sci Transl Med. 2017;9(418).

171. Todo S, Yamashita K, Goto R, Zaitsu M, Nagatsu A, Oura T, et al. A pilot study of operational tolerance with a regulatory T-cell-based cell therapy in living donor liver transplantation. Hepatology. 2016;64(2):632-43.

172. Toso C, Merani S, Bigam DL, Shapiro AM, Kneteman NM. Sirolimus-based immunosuppression is associated with increased survival after liver transplantation for hepatocellular carcinoma. Hepatology. 2010;51(4):1237-43.

173. Tse HM, Milton MJ, Piganelli JD. Mechanistic analysis of the immunomodulatory effects of a catalytic antioxidant on antigen-presenting cells: implication for their use in targeting oxidation-reduction reactions in innate immunity. Free Radic Biol Med. 2004;36(2):233-47.

174. Tun-Abraham ME, Wanis K, Garcia-Ochoa C, Sela N, Sharma H, Al Hasan I, et al. Can we reduce ischemic cholangiopathy rates in donation after cardiac death liver transplantation after 10 years of practice? Canadian single-centre experience. Can J Surg. 2019;62(1):44-51.

175. Uchiyama M, Matsuno N, Hama K, Iwamoto H, Narumi Y, Kikuchi K, et al. Comparison between nonpulsatile and pulsatile machine perfusion preservation in liver transplantation from non-heart-beating donors. Transplant Proc. 2001;33(1-2):936-8.

176. Verhoeven CJ, Farid WR, de Jonge J, Metselaar HJ, Kazemier G, van der Laan LJ. Biomarkers to assess graft quality during conventional and machine preservation in liver transplantation. J Hepatol. 2014;61(3):672-84.

177. Vodkin I, Kuo A. Extended Criteria Donors in Liver Transplantation. Clin Liver Dis. 2017;21(2):289-301.

178. Vogel T, Brockmann JG, Coussios C, Friend PJ. The role of normothermic extracorporeal perfusion in minimizing ischemia reperfusion injury. Transplant Rev (Orlando). 2012;26(2):156-62.

179. Vogel T, Brockmann JG, Pigott D, Neil DAH, Muthusamy ASR, Coussios CC, et al. Successful transplantation of porcine liver grafts following 48-hour normothermic preservation. PLoS One. 2017;12(11):e0188494.

180. Vogel T, Brockmann JG, Quaglia A, Morovat A, Jassem W, Heaton ND, et al. 24-Hour Normothermic Machine Perfusion of Discarded Human Liver Grafts. Liver Transpl. 2016.

181. Vogel T, Brockmann JG, Quaglia A, Morovat A, Jassem W, Heaton ND, et al. The 24-hour normothermic machine perfusion of discarded human liver grafts. Liver Transpl. 2017;23(2):207-20.

182. Vrselja Z, Daniele SG, Silbereis J, Talpo F, Morozov YM, Sousa AMM, et al. Restoration of brain circulation and cellular functions hours post-mortem. Nature. 2019;568(7752):336-43.

183. Wanis KN, Ardiles V, Alvarez FA, Tun-Abraham ME, Linehan D, de Santibanes E, et al. Intermediate-term survival and quality of life outcomes in patients with advanced colorectal liver metastases undergoing associating liver partition and portal vein ligation for staged hepatectomy. Surgery. 2018;163(4):691-7.

184. Watson CJ, Kosmoliaptsis V, Randle LV, Russell NK, Griffiths WJ, Davies S, et al. Preimplant Normothermic Liver Perfusion of a Suboptimal Liver Donated After Circulatory Death. Am J Transplant. 2016;16(1):353-7.

185. Watson CJ, Randle LV, Kosmoliaptsis V, Gibbs P, Allison M, Butler AJ. 26-hour Storage of a Declined Liver Before Successful Transplantation Using Ex Vivo Normothermic Perfusion. Ann Surg. 2016.

186. Watson CJ, Randle LV, Kosmoliaptsis V, Gibbs P, Allison M, Butler AJ. 26-hour Storage of a Declined Liver Before Successful Transplantation Using Ex Vivo Normothermic Perfusion. Ann Surg. 2017;265(1):e1-e2.

187. Watson CJE, Hunt F, Messer S, Currie I, Large S, Sutherland A, et al. In situ normothermic perfusion of livers in controlled circulatory death donation may prevent ischemic cholangiopathy and improve graft survival. Am J Transplant. 2018.

188. Watson CJE, Jochmans I. From "Gut Feeling" to Objectivity: Machine Preservation of the Liver as a Tool to Assess Organ Viability. Curr Transplant Rep. 2018;5(1):72-81.

189. Watson CJE, Kosmoliaptsis V, Pley C, Randle L, Fear C, Crick K, et al. Observations on the ex situ perfusion of livers for transplantation. Am J Transplant. 2018;18(8):2005-20.

190. Watson CJE, Kosmoliaptsis V, Randle LV, Gimson AE, Brais R, Klinck JR, et al. Normothermic Perfusion in the Assessment and Preservation of Declined Livers Before Transplantation: Hyperoxia and Vasoplegia-Important Lessons From the First 12 Cases. Transplantation. 2017;101(5):1084-98.

191. White CW, Hasanally D, Mundt P, Li Y, Xiang B, Klein J, et al. A whole blood-based perfusate provides superior preservation of myocardial function during ex vivo heart perfusion. J Heart Lung Transplant. 2015;34(1):113-21.

192. Xu H, Berendsen T, Kim K, Soto-Gutierrez A, Bertheium F, Yarmush ML, et al. Excorporeal normothermic machine perfusion resuscitates pig DCD livers with extended warm ischemia. The Journal of surgical research. 2012;173(2):e83-8.

193. Yeung JC, Wagnetz D, Cypel M, Rubacha M, Koike T, Chun YM, et al. Ex vivo adenoviral vector gene delivery results in decreased vector-associated inflammation pre- and post-lung transplantation in the pig. Mol Ther. 2012;20(6):1204-11.

194. Zylstra B, Netscher G, Jacquemot J, Schaffer M, Shen G, Bowhay AD, et al. Extended, continuous measures of functional status in community dwelling persons with Alzheimer's and related dementia: Infrastructure, performance, tradeoffs, preliminary data, and promise. J Neurosci Methods. 2018;300:59-67.

A. Appendix

Pan-caspase inhibitor F573 mitigates liver ischemia reperfusion injury in a murine model

Article revisions submitted Aug 2, 2019, currently under review at PLoS One.

A.1 Original paper

Pan-caspase inhibitor F573 mitigates liver ischemia reperfusion injury in a murine model

Mariusz Bral[1], Rena Pawlick[1], Braulio Marfil-Garza [1,&], Nidheesh Dadheech[1,&], Aducio Thiesen[2], and A.M. James Shapiro[1*]

[1] Department of Surgery, University of Alberta, Edmonton, Canada

[2] Department of Pathology, University of Alberta, Edmonton, Canada

*Corresponding Author:

A.M. James Shapiro, MD, PhD, Canada Research Chair in Transplantation Surgery and Regenerative Medicine, Professor, Director of Clinical Islet and Living Donor Liver Transplant Programs, Clinical Islet Transplant Program, University of Alberta. 2000 College Plaza, 8215-112th St, Edmonton T6G 2C8, Alberta, Canada. amjs@islet.ca

Telephone: +1-780-4077330, Fax: +1-780-4078259

[&]These authors also contributed equally to this work.

A.2 Abstract

Background: Liver ischemia reperfusion injury (IRI) remains a challenge in liver transplantation. A number of compounds have previously demonstrated efficacy in mitigating IRI. Herein, we applied three specific additive strategies to a mouse IRI screening model to determine their relative potencies in reducing such injury, with a view to future testing in a large animal and clinical *ex situ* normothermic perfusion setting: 1) F573, a pan-caspase inhibitor, 2) anti-inflammatory anakinra and etanrecept and 3) BMX-001, a mimetic of superoxide dismutase.

Methods: A non-lethal liver ischemia model in mice was used. Additives in the treatment groups were given at fixed time points before induction of injury, compared to a control group that received no therapeutic treatment. Mice were recovered for 6 hours following the ischemic insult, at which point blood and tissue samples were obtained. Plasma was processed for transaminase levels. Whole liver tissue samples were processed for histology, markers of apoptosis, oxidative stress, and cytokine levels.

Results: In an *in vivo* murine IRI model, the F573 treatment group demonstrated statistically lower alanine aminotransferase (ALT) levels (p=0.01), less evidence of apoptosis (p=0.03), and lower cytokine levels compared to controls. The etanercept with anakinra treatment group demonstrated significantly lower cytokine levels. The BMX-001 group demonstrated significantly decreased apoptosis (p=0.01) evident on TUNEL staining.

Conclusions: The administration of pan-caspase inhibitor F573 in a murine *in vivo* model likely mitigates liver IRI based on decreased markers of cellular injury, decreased

evidence of apoptosis, and improved cytokine profiles. Anakinra with etanercept, and BMX-001 did not demonstrate convincing efficacy at reducing IRI in this model, and likely need further optimization. The positive findings set rational groundwork for future translational studies of applying F573 during normothermic *ex situ* liver perfusion, with the aim of improving the quality of marginal grafts.

A.3 Introduction

Ischemia reperfusion injury (IRI) is a well-recognized problem in liver transplantation. During the perioperative period, the liver graft is subjected to sequential insults, inexorably leading to degrees of reversible or irreversible graft damage. Such injury is then amplified upon reperfusion, when the ischemic organ comes into contact with warm, oxygenated blood, and abruptly resumes full metabolism.

Under such circumstances, progressing from ischemia to full physiologic function results in a cascade of injury, which defines IRI. This includes oxidative stress from generation of reactive oxygen species (ROS), inductional release of proinflammatory cytokines, release of damage associated molecular proteins (DAMPS), leading to caspase activation and potentially regulated or non-regulated cell death[1].

Several promising bioactive compounds have shown potential to mitigate liver IRI, and different groups worldwide have published these efforts [2-7]. In our laboratory, we previously explored the protective role of several potent molecules in minimizing ischemic injury to isolated and transplanted islets, and these experiences served as a rational basis for selecting specific compounds that held translational potential in

minimizing IRI in livers. To our knowledge, none of the compounds investigated herein had previously been applied in such a setting, or had been tested as less potent, older formulations.

One strategy for minimizing IRI is the use of anti-oxidants to protect livers from oxidative stress. Based on our previous experiments with pan-caspase inhibitors, we selected F573, a highly potent inhibitor that had previously demonstrated efficacy in islet preservation [8-11]. Indeed, we had taken this approach to a small pilot randomized trial in clinical islet transplantation previously.

In a second approach, we aimed to determine the efficacy of anakinra (an IL-1 receptor agonist) and etanercept (a tumor necrosis factor alpha blocker) in the murine IRI model. The administration of these two compounds in tandem had previously demonstrated remarkable improvement for islet engraftment and metabolic function, with decreased apoptosis[12]. These findings led to the implementation of these anti-inflammatory agents in clinical practice, and indeed, at our institution, all clinical islet transplant recipients routinely receive this treatment.

In an alternate strategy, islets treated with a mimetics of superoxide dismutase had also previously demonstrated improved survival and function in culture. Within this class of compounds, metalloporphyrin analogs have demonstrated particular efficacy and we had previously shown that islets cultured in the presence of BMX-001, a powerful metalloporphyrin anti-oxidant had also demonstrated improved function and engraftment [13]. With these promising findings, we sought to investigate whether this compound could alleviate liver IRI.

Herein, using a murine *in vivo* model, we tested a rational selection of protective compounds to mitigate liver IRI. Our plan is to use the *in situ* focal liver ischemia model in mice as a screening tool to look for compounds and strategies that we could promptly translate to our large animal and clinical liver transplant trials that utilize *ex situ* normothermic preservation before transplantation, as a means to recondition injured and otherwise marginal liver grafts.

A.4 Methods

A.4.1 Study design overview

The Institutional Animal Care Committee at the University of Alberta approved the experimental protocol (AUP00002033) in accordance with guidelines established by the Canadian Council on Animal Care Organization. C57BL/6 male mice were obtained from Charles River Laboratories (Quebec, Canada).

Twenty mice were allocated to the each group in a block randomization design to minimize bias. In all groups, under general anesthesia using isofluorane, mice underwent a laparotomy followed by a non-lethal 70 percent liver hilar clamp, as previously described[14]. Liver ischemia was confirmed by observed blanching of the left and middle liver lobes. Heparin sodium (5 U), and 500 ul of normal saline were administered intra-peritoneum. Duration of ischemia was 60 minutes, followed by unclamping of the liver and confirmation of immediate reperfusion of the left and middle lobe. Midline incisions were closed followed by the application of topical lidocaine (AstaZeneca Inc, Mississauga, Ontario) to the incision, and a 0.1 mg/Kg dose of subcutaneous bipuvicane (Sogeval UK Limited, Sherrif Hutton, York, UK). Several previous groups had published studies indicating that the peak liver injury following partial liver clamping manifests at 6 hours, with plasma levels of elevated transaminases decreasing by approximately 24 hours, establishing this model [2, 3, 5-7].

In all treatment groups, at a two hour time point prior to liver clamping, the intervention drug was administered, dosed optimally based on previous publications. The treatments were administered as follows: 1) pan-caspase inhibitor, F573, 10 mg/Kg

dose administered subcutaneously 2) Etanercept 5 mg/Kg administered intra-peritoneal, with Anakinra 100 mg/Kg administered intra-peritoneal 3) BMX-001, Maximum systemic dose with no adverse effect in mice 12 mg/Kg, given subcutaneously [10, 12, 15].

After 6 hours of recovery, general anesthesia was again induced as previously and mice were exsanguinated via cardiac puncture. Blood and liver tissue samples were obtained. Sham operations were also performed as additional controls (n=6). Blood samples were immediately centrifuged for 2 min at 15,000 x RPM and plasma was frozen at -80 °C until biochemical analysis was performed. Liver tissue samples were harvested and preserved both in 10% formalin and by flash freezing at -80 °C until further analysis..

A.4.2 Plasma biochemistry

Plasma biochemistry was performed using a VetTest Chemistry Analyzer (IDEXX, ME, USA) for levels of aspartate aminotransferase (AST), and alanine aminotransferase (ALT).

A.4.3 Histology

Liver tissue samples were obtained at the termination point, and were immediately fixed in 10% formalin. Tissue was embedded in paraffin, stained with hematoxylin and eosin, and examined in a blinded fashion by an independent expert pathologist, who assigned a semi-quantitative score to evaluate for hepatocyte injury and bile sequestration. Biopsy tissue was examined for necrosis (0- absent, 1- pericentral, 2- Zone 2 and 3, 3- panlobular); hemorrhage (score 0- absent, 1- focal, 2- zonal, 3- panlobular), cholestasis (score 0- absent, 1- present); and sinusoidal dilatation (score 0- none, 1- mild, 2- moderate, 3- severe), as published previously[15].

A.4.4 Markers of apoptosis

Tissue samples embedded in paraffin were stained for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using a DeadEnd Fluorometric TUNEL System (Promega, Washington, USA) to assess degree of apoptosis. Since apoptosis appeared to be heterogeneously dispersed within the tissue sections, displaying patchy fields of TUNEL positive cells, whole sections of clamped liver tissue were scanned to obtain an unbiased assessment. Slides were then analyzed in blinded fashion using Olympus VS ASW Imaging software. TUNEL positive nuclei were compared to all 4',6-diamidino-2-phenylindole (DAPI) stained nuclei for each given sample, and reported as a ratio.

Caspase-3 was measured by both Colorimetric Assay (R&D Systems, Minneapolis, MN, USA). Tissue was lysed in cell lysis buffer and protein concentration in the supernatant was determined by Bradford Dye Binding assay. 10mM of DTT and 100 μM of the chromophore DEVD-p nitroaniline (*p*NA) was added to each sample (200 μg protein) and incubated at 37 °C for 1.5 hours followed by detection at absorbance 405 nm.

A.4.5 Cytokine and biomarker analysis

Plasma and tissue samples were maintained frozen at -80 °C until analyzed for cytokine and biomarker content (Interleukin 1 (IL-1), Interleukin 4 (IL-4), Interleukin 5 (IL-5), Interleukin 6 (IL-6), Interleukin 10 (IL-10), Tumour necrosis factor alpha (TNF-α), Interferon gamma (IFN-γ), and KC/GRO) using Pro-inflammatory Panel (mouse) Kit (Meso Scale Discovery, Gaithersburg, MD, USA). Frozen tissue samples were lysed (50

nM Hepes, 1 mM EDTA, 150nM NaCl and 1% Triton-X) with homogenization prior to assay.

A.4.6 Oxidative stress measurement

Oxidative stress was quantified by measurement of lipid peroxidation malondialdehyde (MDA) assay (Abcam, Toronto, ON, Canada). Frozen tissue samples (-80 °C) were weighed and lysed in MDA lysis buffer + butylated hydroxytoulene (BTH) with homogenization then frozen at -20 °C until further analysis. Thiobarbituric Acid (TBA) was added to thawed samples, incubated for 60 minutes at 95 °C, cooled to room temperature in an ice bath for 10 minutes followed by colorimetric detection (532 nm).

A.4.7 Statistical analysis

Data are represented as means ± standard error of the means (SEM). Differences between continuous variables were compared using a Kruskall Wallis ANOVA or the Mann Whitney U-test. Overall comparison between groups was performed with a 95% confidence interval. A p-value of <0.05 was considered statistically significant and all the analysis was performed using Graphpad Prism (GraphPad Software Inc., La Jolla, CA, USA).

A.5 Results

Mice were anesthetized, and the laparotomy and liver clamping procedure was performed without complications. All mice tolerated anesthesia well, and recovered without undue visible duress. At the end of the 6-hour recovery period, mice were euthanized as described, and blood and tissue samples were harvested and processed until further analysis. Due to limitations in tissue and plasma sample size, only a subset of mice were tested in each post-procedural analysis.

A.5.1 F573 reduces transaminase levels post IRI

Plasma was analyzed for AST, and ALT (**Fig A1).** All intervention groups demonstrated lower mean ALT levels, but the F573 treatment group was the only one to reach statistical significance (p=0.01) **(Fig A1(A)**. AST was similarly lower in almost all treatment groups, with no statistical significance (p=0.42) **(Fig A1(B).**

A.5.2 F573 reduces hepatic apoptosis

Liver tissue slides were stained for apoptosis by TUNEL assay as described, and then analyzed by Olympus VS ASW Imaging software. TUNEL staining analysis demonstrated notably reduced levels of apoptosis in all intervention groups compared to the control, with F573 and BMX-001 both reaching statistical significance (p=0.03 and p=0.01, respectively) (**Fig A2, Fig A3**).

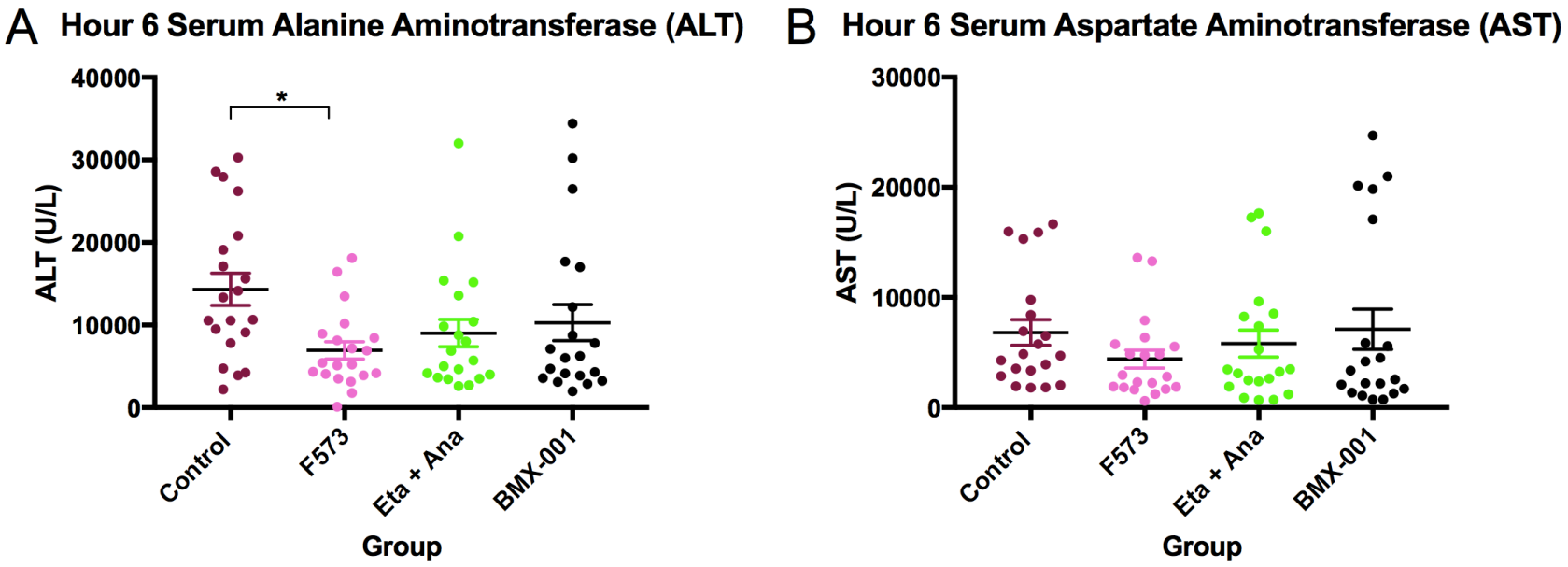


Figure A-1 Plasma biochemistry.

(A) ALT plasma levels compared to control, with F573 significantly lower (p=0.01). (B) AST plasma levels compared to control (p=0.42). Data points show means and SEM, 95% confidence interval (n=20 per group). Aspartate aminotransferase (AST), Alanine aminotransferase (ALT)

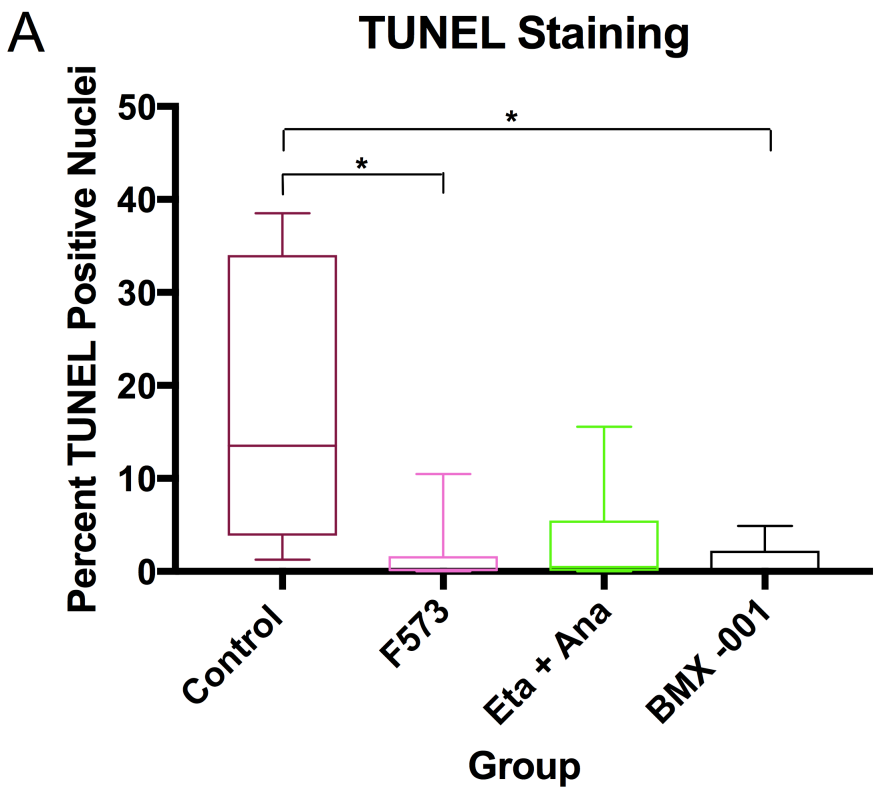


Figure A-2 Markers of apoptosis.

A) TUNEL staining of tissue samples, reporting relative percentage of TUNEL nuclei compared to DAPI. Significantly lower TUNEL staining was found in the F573 and BMX-001 groups (p=0.03 and p=0.01, respectively). Data points show means and SEM, 95% confidence interval (n=7 per group

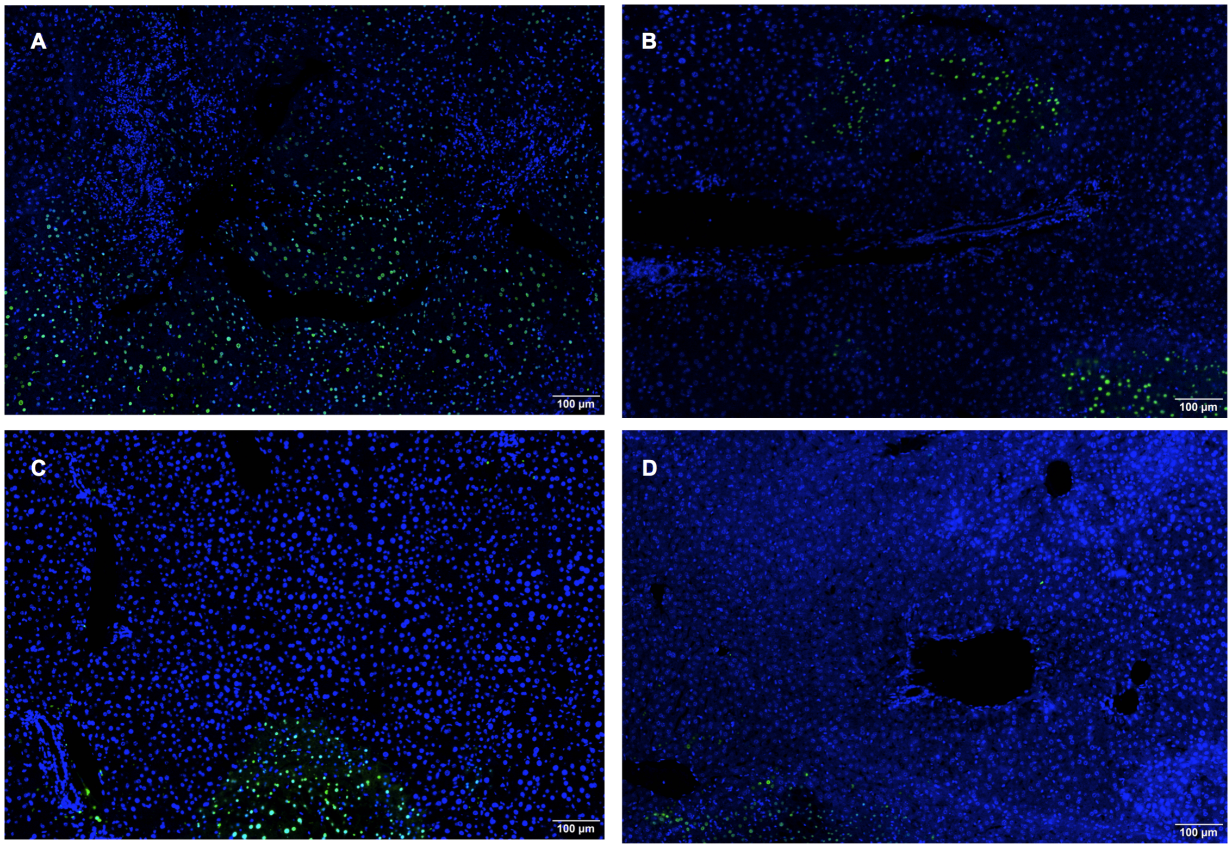


Figure A-3 Markers of apoptosis. Representative sections of liver parenchyma, stained with TUNEL

(A) Representative section of tissue from a liver in the control group. (B) Representative section of tissue from a liver in the F573 group. (C) Representative tissue from the anakinra and etanercept group. (D) Liver tissue from the BMX-001 group..

Measurement of oxidative stress using a malondialdehyde (MDA) assay and measurement of caspase 3 activity using calorimetric assay revealed no difference between groups (p=0.45 and p=0.84, respectively) **(Fig A4 (A and B)**

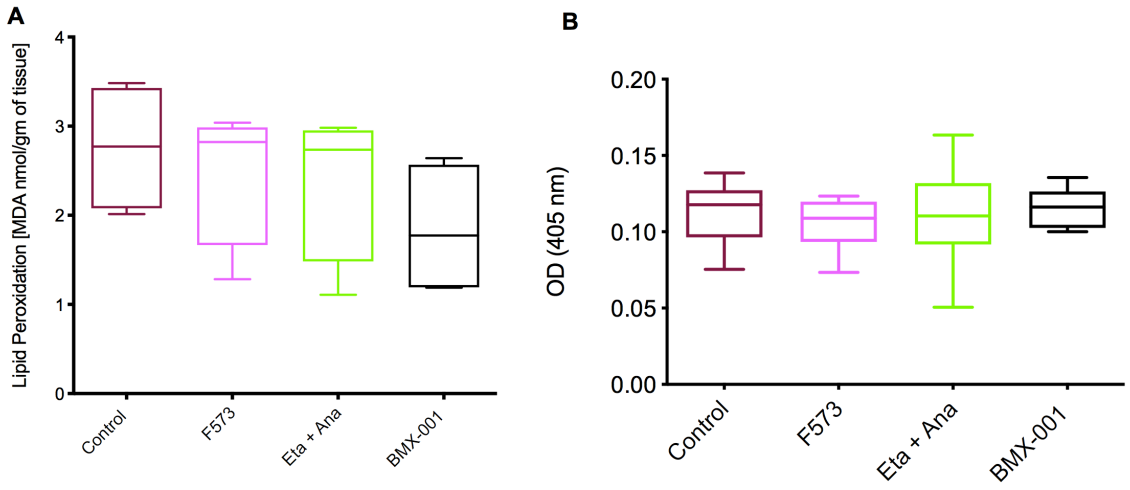


Figure A-4 Reactive oxygen species quantification and Caspase 3 activity.

A. Oxidative stress quantified by lipid peroxidation malondialdehyde (MDA) assay (p= 0.45) (B) Caspase 3 activity measured by calorimetric analysis of liver tissue homogenate (p=0.84

A.5.3 Histological analysis

Comparison of liver histology demonstrated reduced hepatocyte injury in all treatment groups, including less hemorrhage, necrosis and sinusoidal dilatation compared to controls, without reaching statistical significance (p=0.11) **(Fig A5 and Fig A6).**

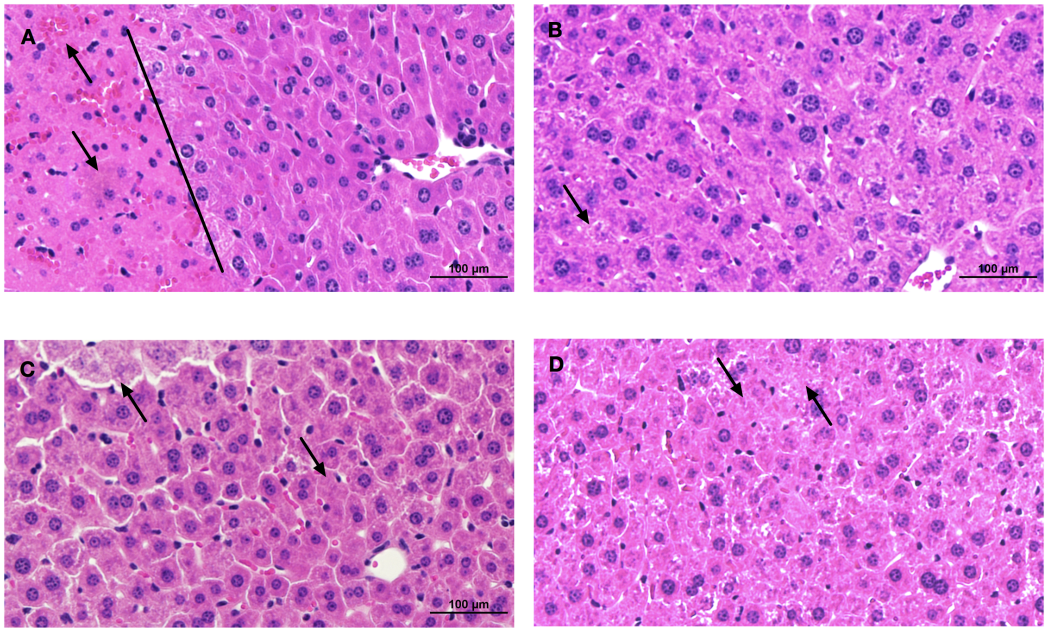


Figure A-5 Representative histologic sections of liver parenchyma, stained with hematoxylin and eosin (A) Representative section of tissue from a liver in the control group. The vertical black line indicates transition between normal tissue (right side of line) and necrotic liver (left of the line). (B) Representative section of tissue from a liver in the F573 group. (C) Representative tissue from the anakinra and etanercept group. (D) Liver tissue from the BMX-001 group. All photographs are shown at 20X magnification. Arrows indicate areas of liver damage relevant to the scoring system, including necrosis and hemorrhage

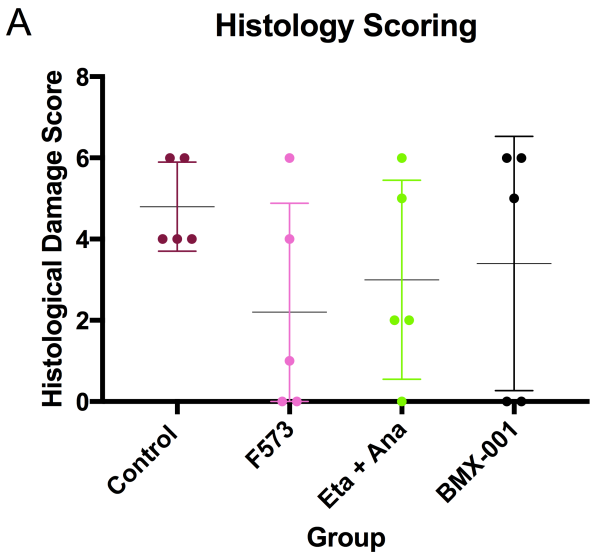
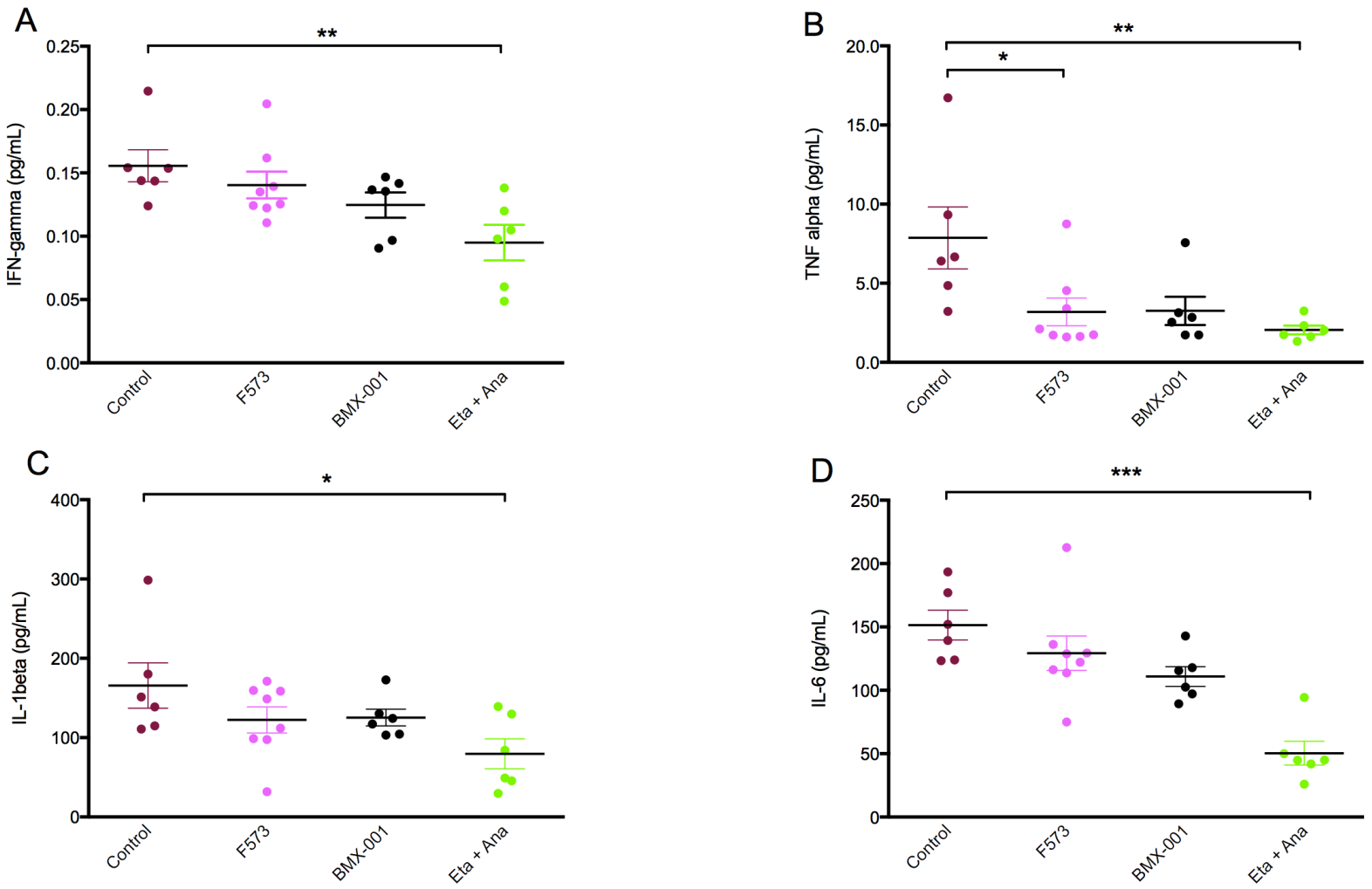


Figure A-6 Histological scoring of liver injury. (A) All tissue slides were examined and scored by an independent expert pathologist for hemorrhage, necrosis, sinusoidal dilatation, and bile sequestration. Treated groups all demonstrated less injury, without reaching statistical significance (p=0.11). Data columns show means and SEM, 95% confidence interval (n=6 per group).

A.5.4 F573 reduces liver tissue cytokine profile post-IRI

Liver tissue was analyzed for cytokine and biomarker activation and compared between groups. The anakinra with etanercept group had the lowest cytokine tissue profile when compared with controls (IFN-γ (p=0.006), TNF-α (p=0.009), IL-1B (p=0.047), KC/GRO (p= 0.0003), IL-4 (p=0.04). The F573 group demonstrated low pro-inflammatory activity for both IL-5 (p=0.02) and TNF- α (p=0.04), compared to controls **(Fig A7)**.


A
**
IFN-gamma (pg/mL)
0.25
0.20
0.15
0.10
0.05
0.00
Control
F573
BMX-001
Eta + Ana
B
**
*
TNF alpha (pg/mL)
20.0
15.0
10.0
5.0
0.0
Control
F573
BMX-001
Eta + Ana
C
*
IL-1beta (pg/mL)
400
300
200
100
0
Control
F573
BMX-001
Eta + Ana
D
***
IL-6 (pg/mL)
250
200
150
100
50
0
Control
F573
BMX-001
Eta + Ana

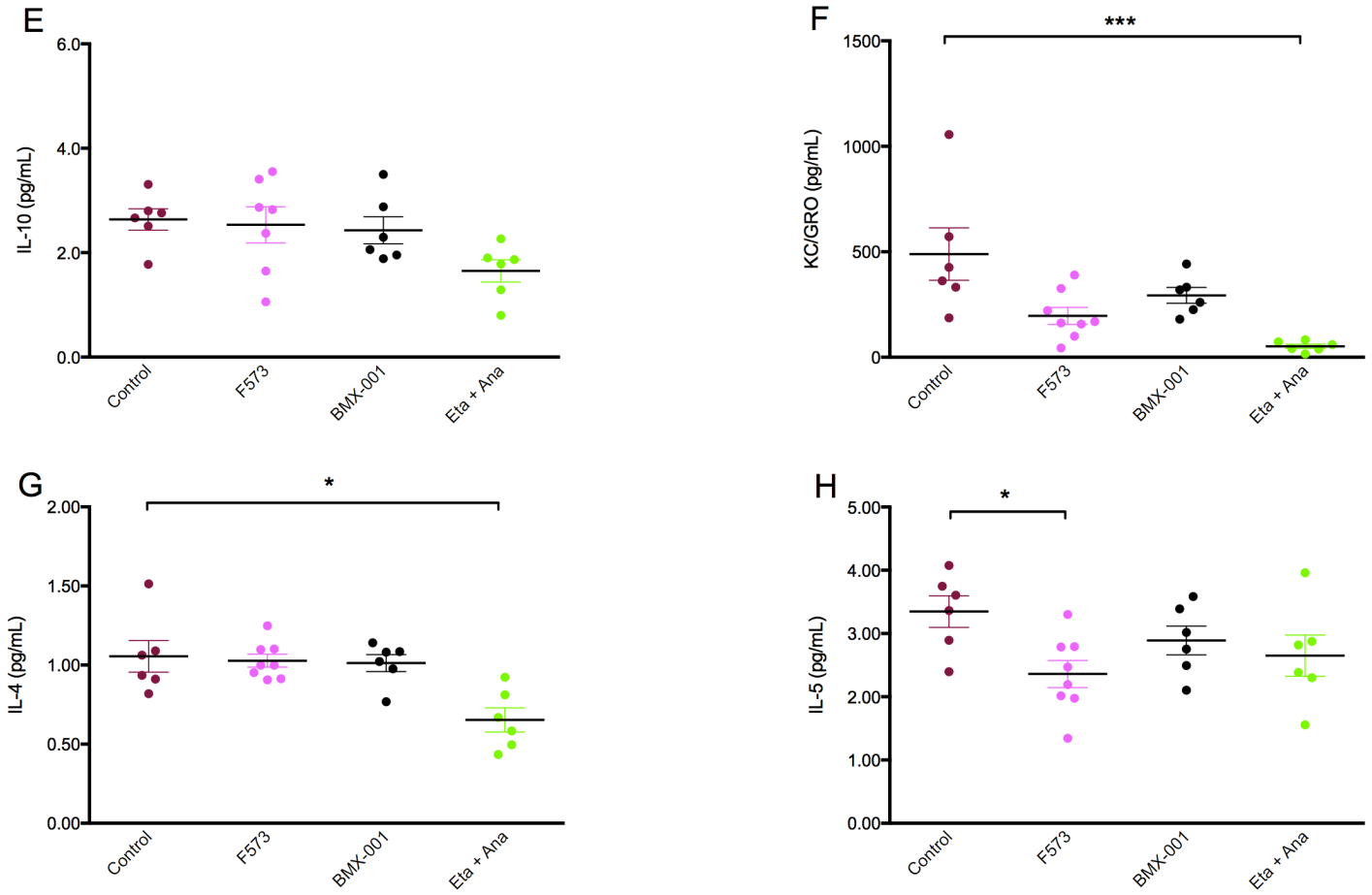


Figure A-7 Plasma cytokine and biomarker analysis.

All treatment groups are compared to the control. (A) IFN-γ levels (Eta+Ana p=0.006), (B) TNF-α levels (F573 p=0.04); Eta+Ana p=0.009), (C) IL-1 levels (Eta+Ana p=0.47), (D) IL-6 levels (Eta+Ana p=0.0003), (E) IL-10 levels (p=0.09), (F) KC/GRO levels (Eta+Ana p=0.0003), (G) IL-4 levels (Eta+Ana p= 0.04), (H) IL-5 levels (F573 p=0.02). Data columns show means and SEM, 95% confidence interval (n=6-8 per group). Etanercept and Anakinra (Eta+Ana).

A.6 Discussion

Liver ischemia reperfusion injury is a well-known component of graft damage in liver transplantation, which potentially leads to both immediate and long-term adverse outcomes. Further, the worldwide trend of utilizing marginal and extended criteria grafts, which have a poor tolerance of cold ischemia, can lead to increased IRI events and sequelae post-transplant.

In an effort to mitigate such organ damage, different groups worldwide have administered intervening strategies, with varying success. Based on previous efficacy in islet cultivation and transplantation, we sought to determine whether a group of specifically targeted and highly potent compounds could be used to ameliorate IRI in a murine *in vivo* liver ischemia model. Should any of these additives prove to be effective in reducing such injury, this would set the stage for future application in liver *ex situ* normothermic machine perfusion (NMP).

Over the past few years, *ex situ* NMP technology has emerged as a promising platform for liver graft modification prior to transplant [17]. Such interventions have the obvious benefit of treating a graft in isolation, while minimizing or obviating any treatment side effects for the patient. Despite this potential, and the long list of possible anti-inflammatory or anti-apoptotic compounds to be added to *ex situ* perfusate, application of such interventions during NMP has not been widely published to date.

In this study, we applied three specific, selected treatments in an attempt to mitigate IRI in a murine *in vivo* model.

Pan-caspase inhibitors, a group of compounds which act on the molecular pathways leading to apoptosis, had previously been shown to reduce liver IRI [18]. F573 is a newer, more potent compound from this family, and to our knowledge had previously not been evaluated in a liver IRI model. In our laboratory, F573 had previously demonstrated reduced human and mouse islet apoptosis after *in vitro* culture, and also improved islet engraftment and significantly improved post-transplant islet function. The F573 treated group demonstrated significantly reduced apoptosis, with decreased TUNEL-positive nuclei, and decreased caspase-3 activity when compared to islets in standard culture media [10]. Further, effective pan-caspase inhibition with at least 3 alternative pan-capsase inhibitors resulted in far more effective marginal mass islet transplant engraftment in mice, including human islets, where diabetes was reversed with a 70% reduction in infused islet mass in the presence of pan-caspase inhibition [8,9].

In our murine *in vivo* liver ischemia model, F573 also significantly reduced IRI, as evidenced by reduced ALT, as well as reduced apoptosis on TUNEL staining. Cytokine analysis revealed less pro-inflammatory TNF-α and IL-5 activity. Surprisingly, there were no significant differences noted on caspase-3 ELISA or MDA staining. We speculate that this was due to the fact that caspase-3 activation events are very short lived, and the cleaved products likely were not captured at the time of tissue collection. Activation of caspases translates rapidly to DNA damage which is ultimately captured in TUNEL staining. The dose administered in this study, 10 mg/Kg, had previously shown efficacy in an islet model, and was considered to be the maximal therapeutic dose[10]. Our findings suggest that such therapeutic intervention may indeed have utility in

abrogating IRI, and such efficacy may be translatable into an *ex situ* machine perfusion platform.

The second intervention group was comprised of anakinra, an IL-1 receptor agonist, and etanercept, an anti-TNF-alpha agonist. Anakinra, specifically had previously demonstrated efficacy in reducing apoptosis after myocardial infarction in an experimental murine model[19]. Previous groups have administered anti-inflammatory agents in attempts to minimize liver IRI, with some therapeutics reaching clinical trials, and some anti-inflammatory therapeutic interventions also entering into the *ex situ* MP realm. Using a porcine model, Goldaracena *et al*. applied various anti-inflammatory agents to subnormothermic *ex situ* machine liver perfusions, including n-acetylcysteine, alprostadil, sevofluorane, and carbon monoxide. In the study, the treatment group had decreased AST and cytokines during perfusion, and lower bilirubin levels post transplant. The authors concluded that addition of anti-inflammatory agents did indeed augment warm machine perfusion [20].

In our previous experience in clinical and experimental islet transplantation, combined treatment using etanercept and anakinra in combination in a marginal islet mass transplant model led to remarkable improvement in islet engraftment, compared to single drug treated controls [12]. Treated islets demonstrated improved metabolic function, increased insulin secretion, and decreased apoptosis [12]. Such encouraging results allowed us to speculate that these compounds may in fact be effective in other injury models, such as IRI. In the current study, anakinra and etanercept did not demonstrate efficacy, with no statistical significance in either transaminases, multiple apoptosis assays or histology. Interestingly, despite the specificity of action of both

anakinra and etanercept, cytokines were almost all decreased (IFN-γ, TNF-α, IL-1, IL-6 , KC/GRO, IL-4 and IL-5) in this group. This can potentially be explained by the fact that TNF-α is one of the more potent cytokines, the activation and binding of which cascades into a myriad of metabolic inflammatory events. Inhibition of TNF-α, specifically would likely suppress these major downstream biochemical effects.

BMX-001 (MnTnBuOE-2 PyP5+ (Mn(III) meso-tetrakis-(N-b-butoxyethylpyridinium-2-yl) porhirin)) is a low molecular weight metalloporphyrin MnSOD mimetic that had previously demonstrated cyto-protective efficacy in a murine islet model. Islets cultured in BMX-001 supplemented media had improved insulin secretion, and exhibited significantly less apoptosis on TUNEL staining. Treated islets transplanted under the kidney capsule at a marginal dose manifested improved engraftment and function, as did human islets [13].

In this study however, the administration of BMX-001 did not abrogate IRI to a notable degree. Although the BMX-001 treated group demonstrated significantly less apoptosis as per TUNEL staining, no significant difference was seen in transaminase levels, histology or cytokine analysis. We feel that further optimization of BMX-001 would be required before utilizing the agent in an *ex situ* machine perfusion model to achieve results.

There are several limitations to our study. All of the investigated interventions were administered prior to the ischemic insult, and we cannot predict at this time how this will translate to a liver *ex situ* machine perfusion setting, which would involve drug administration after injury. At the moment it is not clinically permissible to administer

interventions to the donor prior to organ retrieval, which could theoretically mitigate injury upon graft reperfusion. We also cannot predict the effects of repeat drug administration, which may have additional positive effects. Considering the *in vivo* model, we are unable to predict if the additives tested have any effect on cold ischemic injury.

A.7 Conclusions

Herein, we have shown that the pan-caspase inhibitor, F573, administered at a dose of 10 mg/Kg SC two hours before ischemic injury, mitigates IRI in a murine *in vivo* model, based on improved markers of cellular injury, decreased evidence of apoptosis, as well as improved tissue cytokine profile. Based on this positive finding, this compound can be used during *ex situ* normothermic liver perfusion, with the intention of improving marginal graft quality**.**

A.8 References

1. Peralta C, Jimenez-Castro MB, Gracia-Sancho J. Hepatic ischemia and reperfusion injury: effects on the liver sinusoidal milieu. J Hepatol. 2013;59(5):1094-106. Epub 2013/07/03. doi: 10.1016/j.jhep.2013.06.017. PubMed PMID: 23811302.
2. Linares I, Farrokhi K, Echeverri J, Kaths JM, Kollmann D, Hamar M, et al. PPAR-gamma activation is associated with reduced liver ischemia-reperfusion injury and altered tissue-resident macrophages polarization in a mouse model. PLoS One. 2018;13(4):e0195212. Epub 2018/04/05. doi: 10.1371/journal.pone.0195212. PubMed PMID: 29617419; PubMed Central PMCID: PMCPMC5884549.
3. Cheng F, Li Y, Feng L, Li S. Effects of tetrandrine on ischemia/reperfusion injury in mouse liver. Transplant Proc. 2008;40(7):2163-6. Epub 2008/09/16. doi: 10.1016/j.transproceed.2008.07.082. PubMed PMID: 18790181.
4. Rong YP, Huang HT, Liu JS, Wei L. Protective Effects of Geniposide on Hepatic Ischemia/Reperfusion Injury. Transplant Proc. 2017;49(6):1455-60. Epub 2017/07/25. doi: 10.1016/j.transproceed.2017.02.063. PubMed PMID: 28736023.
5. Yang Y, Yang J, Jiang Q. The protective effect of huperzine A against hepatic ischemia reperfusion injury in mice. Transplant Proc. 2014;46(5):1573-7. Epub 2014/06/18. doi: 10.1016/j.transproceed.2014.01.018. PubMed PMID: 24935330.
6. Hochhauser E, Lahat E, Sultan M, Pappo O, Waldman M, Sarne Y, et al. Ultra Low Dose Delta 9-Tetrahydrocannabinol Protects Mouse Liver from Ischemia Reperfusion Injury. Cell Physiol Biochem. 2015;36(5):1971-81. Epub 2015/07/24. doi: 10.1159/000430165. PubMed PMID: 26202357.

7. Palumbo T, Nakamura K, Lassman C, Kidani Y, Bensinger SJ, Busuttil R, et al. Bruton Tyrosine Kinase Inhibition Attenuates Liver Damage in a Mouse Warm Ischemia and Reperfusion Model. Transplantation. 2017;101(2):322-31. Epub 2016/11/08. doi: 10.1097/TP.0000000000001552. PubMed PMID: 27820779; PubMed Central PMCID: PMCPMC5263143.
8. Emamaullee JA, Davis J, Pawlick R, Toso C, Merani S, Cai SX, et al. The caspase selective inhibitor EP1013 augments human islet graft function and longevity in marginal mass islet transplantation in mice. Diabetes. 2008;57(6):1556-66. Epub 2008/03/22. doi: 10.2337/db07-1452. PubMed PMID: 18356409.
9. McCall MD, Maciver AM, Kin T, Emamaullee J, Pawlick R, Edgar R, et al. Caspase inhibitor IDN6556 facilitates marginal mass islet engraftment in a porcine islet autotransplant model. Transplantation. 2012;94(1):30-5. Epub 2012/06/19. doi: 10.1097/TP.0b013e318257745d. PubMed PMID: 22706322.
10. Pepper AR, Bruni A, Pawlick R, Wink J, Rafiei Y, Gala-Lopez B, et al. Engraftment Site and Effectiveness of the Pan-Caspase Inhibitor F573 to Improve Engraftment in Mouse and Human Islet Transplantation in Mice. Transplantation. 2017;101(10):2321-9. Epub 2017/01/11. doi: 10.1097/TP.0000000000001638. PubMed PMID: 28072753.
11. Emamaullee JA, Stanton L, Schur C, Shapiro AM. Caspase inhibitor therapy enhances marginal mass islet graft survival and preserves long-term function in islet transplantation. Diabetes. 2007;56(5):1289-98. Epub 2007/02/17. doi: 10.2337/db06-1653. PubMed PMID: 17303806.
12. McCall M, Pawlick R, Kin T, Shapiro AM. Anakinra potentiates the protective effects of etanercept in transplantation of marginal mass human islets in

immunodeficient mice. Am J Transplant. 2012;12(2):322-9. Epub 2011/11/08. doi: 10.1111/j.1600-6143.2011.03796.x. PubMed PMID: 22053751.

13. Bruni A, Pepper AR, Pawlick RL, Gala-Lopez B, Gamble A, Kin T, et al. BMX-001, a novel redox-active metalloporphyrin, improves islet function and engraftment in a murine transplant model. Am J Transplant. 2018;18(8):1879-89. Epub 2018/02/22. doi: 10.1111/ajt.14705. PubMed PMID: 29464912.
14. Abe Y, Hines IN, Zibari G, Pavlick K, Gray L, Kitagawa Y, et al. Mouse model of liver ischemia and reperfusion injury: method for studying reactive oxygen and nitrogen metabolites in vivo. Free Radic Biol Med. 2009;46(1):1-7. Epub 2008/10/29. doi: 10.1016/j.freeradbiomed.2008.09.029. PubMed PMID: 18955130; PubMed Central PMCID: PMCPMC2740994.
15. Gad SC, Sullivan DW, Jr., Spasojevic I, Mujer CV, Spainhour CB, Crapo JD. Nonclinical Safety and Toxicokinetics of MnTnBuOE-2-PyP5+ (BMX-001). Int J Toxicol. 2016;35(4):438-53. Epub 2016/04/22. doi: 10.1177/1091581816642766. PubMed PMID: 27098749.
16. Brockmann J, Reddy S, Coussios C, Pigott D, Guirriero D, Hughes D, et al. Normothermic perfusion: a new paradigm for organ preservation. Ann Surg. 2009;250(1):1-6. doi: 10.1097/SLA.0b013e3181a63c10. PubMed PMID: 19561463.
17. Goldaracena N, Spetzler VN, Echeverri J, Kaths JM, Cherepanov V, Persson R, et al. Inducing Hepatitis C Virus Resistance After Pig Liver Transplantation-A Proof of Concept of Liver Graft Modification Using Warm Ex Vivo Perfusion. Am J Transplant. 2017;17(4):970-8. Epub 2016/11/03. doi: 10.1111/ajt.14100. PubMed PMID: 27805315.
18. Baskin-Bey ES, Washburn K, Feng S, Oltersdorf T, Shapiro D, Huyghe M, et al. Clinical Trial of the Pan-Caspase Inhibitor, IDN-6556, in Human Liver Preservation

Injury. Am J Transplant. 2007;7(1):218-25. doi: 10.1111/j.1600-6143.2006.01595.x. PubMed PMID: 17227570.

19. Abbate A, Salloum FN, Vecile E, Das A, Hoke NN, Straino S, et al. Anakinra, a recombinant human interleukin-1 receptor antagonist, inhibits apoptosis in experimental acute myocardial infarction. Circulation. 2008;117(20):2670-83. doi: 10.1161/CIRCULATIONAHA.107.740233. PubMed PMID: 18474815.

20. Goldaracena N, Echeverri J, Spetzler VN, Kaths JM, Barbas AS, Louis KS, et al. Anti-inflammatory signaling during ex vivo liver perfusion improves the preservation of pig liver grafts before transplantation. Liver Transpl. 2016;22(11):1573-83. Epub 2016/10/27. doi: 10.1002/lt.24603. PubMed PMID: 27556578.