Normothermic Machine Perfusion of Kidneys: Optimization of Perfusate

by

Yilun Wu

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Department of Physiology
University of Alberta

Abstract

Chronic kidney disease and end-stage renal disease (ESRD) are becoming increasingly prevalent in the world. The World Health Organization estimates that in 2015, 1.2 million people died from kidney failure, an increase of 32% from a decade ago. An estimated 2 million people worldwide are currently receiving lifesaving renal replacement therapy; however, the true prevalence of end-stage renal disease is estimated to be significantly higher. Two forms of renal replacement therapy currently exist: dialysis or transplantation. Kidney transplantation has demonstrated significant improvements in quality of life, survival, and cost-savings over dialysis. However, there are major drawbacks facing kidney transplantation, such as the availability and quality of donor grafts. With an ever-increasing demand for kidney allografts, it is necessary to improve the preservation, evaluation, and intervention of the recovered donor graft prior to transplantation. Traditional methods of kidney preservation rely upon hypothermia to reduce ischemic injury, but its lack of metabolic activity does not allow the evaluation of renal function or injury metrics prior to implantation. A novel method of normothermic *ex situ* machine perfusion (NMP) may be able to provide the metabolic support and re-establish renal function (waste removal and electrolyte homeostasis). Kidney NMP can offer a metabolically active environment that allows improved evaluation and intervention opportunities before the graft is transplanted. However, due to its relative immaturity as a clinical intervention, kidney NMP lacks an established gold standard perfusate composition and protocol. *This research project investigates the effects of different perfusate components and their effects on proposed evaluation metrics during kidney NMP.* The first study establishes hypothermic and normothermic machine perfusion protocols using a porcine kidney model and investigates perfusion dynamics and urine and perfusate compositions. The second study investigates the

effects of two oncotic agents on kidney NMP metrics. And the last study investigates the effects of two oxygen carriers on kidney NMP metrics. Research within this thesis aims to elucidate the efficacy of different perfusate components and their effects on various perfusion, inflammatory, and injury metrics within kidney NMP. With the optimization of perfusate composition and evaluation methods, normothermic kidney perfusion could improve clinical outcomes of kidney transplant recipients and improve the utilization of donor grafts.

Preface

This thesis is an original work by Yilun Wu. No part of this thesis has been previously published. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board and the Institutional Animal Care and Use Committee. University of Alberta Animal Use Protocol #00000943.

The research performed in this thesis was a part of a larger collaborative research project on *ex situ* organ perfusion by Dr. Darren Freed, Dr. Jayan Nagendran. The project aims to improve organ preservation for the purposes of transplantation.

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List of Abbreviations

AKI	Acute Kidney Injury
ANOVA	Analysis of Variance
ATN	Acute Tubular Necrosis
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
CIT	Cold Ischemia Time
CKD	Chronic Kidney Disease
COPE	Consortium for Organ Preservation in Europe
COR	Controlled Oxygenated Rewarming
CRP	C-Reactive Protein
DAMP	Damage-Associated Molecular Patterns
DBD	Donation after Brain Death
DCD	Donation after Circulatory Death
DGF	Delayed Graft Function
DNA	Deoxyribose Nucleic Acid
EC	Euro-Collins
ECD	Expanded Criteria Donor
ELISA	Enzyme-Linked Immunosorbent Assay
ESRD	End-Stage Renal Disease
GFR	Glomerular Filtration Rate
HES	Hydroxyethyl Starch
HLA	Human Leukocyte Antigen
HMP	Hypothermic Machine Perfusion
HOC	Hyper-Osmolar Citrate

HTK	Histidine-Tryptophan-Ketoglutarate
IL	Interleukin
IRR	Intra-Renal Resistance
KDPI	Kidney Donor Profile Index
KIM-1	Kidney Injury Molecule-1
MAPI	Maryland Aggregate Pathology Index
MSC	Mesenchymal Stem Cell
NF-kB	Nuclear Factor kappa-light-chain-enhancer of activated B-Cells
NGAL	Neutrophil Gelatinase-Associated Lipocalin
NMP	Normothermic Machine Perfusion
PRU	Peripheral Resistance Units
RBC	Red blood cell concentrate
RCT	Randomized Controlled Trial
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SCS	Static Cold Preservation
SIRNA	Small Interfering Ribonucleic Acid
TLR-4	Toll-Like Receptor-4
TNF-α	Tumour Necrosis Factor- α
UW	University of Wisconsin
WB	Whole Blood
WIT	Warm Ischemia Time

Chapter 1: Introduction

To introduce the research projects, the background chapter will begin with a brief exploration of the rationale and history behind kidney preservation for the purposes of transplantation, followed by a review of the current standard methods of preservation, and a review of the current literature on normothermic *ex situ* kidney perfusion. The chapter will also discuss the different metrics that can be used to evaluate normothermic *ex situ* kidney perfusion and the background behind certain methodological choices in our experimental model.

Brief History of Kidney Transplantation

Chronic Kidney Disease (CKD) is a persistent loss of renal function, often with histological changes in renal structure, that can often progress to end-stage renal disease (ESRD)[1,2]. Over 35 thousand Canadians (Outside of Quebec) live with ESRD, and that number has increased by 36% in the last decade[2], making kidney disease a major public health concern. ESRD occurs with chronic degeneration of renal function in CKD or acute development of renal failure through nephrotoxicity, pre-renal, or post-renal injury; if left untreated ESRD has a rapid and high mortality rate. Therapeutic options to replace renal function include peritoneal dialysis, hemodialysis, or kidney transplantation, with transplantation being the preferred treatment for quality of life, long-term survival, and healthcare cost-savings.

Throughout the history of medicine, the idea of transplanting organs and limbs has existed. Even though there are many recorded attempts since the 19th century, it wasn't until early 20th century that the medical community came to the consensus that long-term allografts survival requires immune factors to be overcome[3]. Towards the latter half of the 20th century, these

immune factors would be understood as human leukocyte antigens (HLA) triggering the rejection process within the recipient[3]. The first successful human kidney transplantation with long term graft function was performed in 1954 by Joseph Murray in Boston, where Murray bypassed immune factors by transplanting from a living donor who was an identical twin of the recipient[3]. In 1963 Dr. Tom Starzl revealed his immunosuppressive cocktail made of the traditional azathioprine with added prednisone; this was the first of several successive immunosuppressive cocktail therapies that eventually evolved into the modern iteration[3]. Sir Roy Calne discovered the use of calcineurin inhibitor cyclosporine as a key immunosuppressant that is now a component of immunosuppressive therapy used in many other organ transplants[4]. Alongside the usage of immunosuppressive therapy, a deeper understanding of tissue typing was also developed, allowing the appropriate matching of donors to recipients for minimized acute/hyperacute rejection. Currently recipients are classified as low, intermediate, or high immunologic risk, and post-operative immunosuppression therapies are accordingly differentiated with different combinations of drugs (according to BC Transplant for Clinical Kidney Transplantation, revised 2018).

The invention of organ perfusion pumps by Charles Lindberg and Dr. Alexis Carrel allowed machine replacement of the physiological functions of heart and lung (provide blood circulation and gas exchange) for both surgery, and *ex situ* maintenance of organs recovered for the purposes of transplantation[5]. In 1969, Collins et al. developed a flush solution mimicking intracellular ionic composition (Collins solution) for flushing and subsequent cold storage of kidneys using a dog model which ushered in the development of different cold flush solutions and Static Cold Storage (SCS) that largely remains a clinical standard today. The Collins solution was later modified by the EuroTransplant Foundation and led to the Euro-Collins (EC)

solution widely used until the 1980s. Following the Euro-Collins solution, several additional solutions were devised for both static preservation and machine perfusion, which will be discussed in further detail for both hypothermic and normothermic machine perfusion systems in their respective sections.

Kidney transplantation has now evolved into a state-of-the-art therapy for end-stage renal disease, with a 90% five-year survival rate for living donor transplants and 82% for deceased donor transplants compared to the 35% five-year survival rate for patients relying on dialysis[2]. Kidney transplantation also has lower costs (5-year cost-saving of $313 000 for a kidney transplantation instead of continued hemodialysis[3]) and enhanced quality of life metrics (hemodialysis takes 3-5 hours three days a week in a dialysis clinic)[2].

However, kidney transplantation therapy faces many challenges, chiefly the lack of donor organs suitable for transplant: with over 4 million Canadians facing chronic kidney disease, the number of Canadians with ESRD continues to increase while the median wait time for a deceased donor transplant is 4 years, with variations between blood groups[6]. With increased demand, there is also an apparent increase in percentage of procured donor kidneys being discarded (~5% in the USA in 1988 to ~19% in 2015)[7], which is likely due to higher Kidney Donor Risk Index found in the increased utilization of “expanded criteria donors” (ECD) over the last three decades as an effort by transplant programs to meet increasing demands[8]. These higher discard rates can potentially be reduced by improved evaluation of grafts during preservation, thus making more kidneys available for transplant.

Issues Facing Kidney Transplantation:

Increased warm ischemia is the main contributing factor to acute kidney injury (AKI) during transplantation procedures; while cold ischemia times are significantly lengthier, it has been demonstrated that hypothermia reduces the rate of AKI allowing for significant cold ischemia before the graft becomes unacceptable for transplant. The ideal graft procurement occurs in a controlled environment where the donor is determined to be deceased by neurological criteria (NDD), and procurement occurs with minimal warm ischemia before an *in situ* cold flush of the graft induces cold ischemia. However, because most a significant number of allografts come from donation after circulatory death (DCD), hypoxia and acute kidney injury occurs with declining cardiovascular function long before the *in situ* flush can transition into cold ischemia; DCD organ procurement also often require a period of observation for official determination of death which differs from region to region[9]. Although reporting is possibly flawed, a USA study on kidney transplant warm ischemia times (WIT) reported a range from 1 minute to over 120 minutes for WIT, with the largest variation occurring during DCD graft procurements; the authors also concluded significantly poorer post-operative 5 to 10-year survival outcomes with longer WIT's[10].

After cold flush, cold ischemia time (CIT) continues until the organ is reperfused in the recipient. Studies have demonstrated a significant correlation between CIT and delayed graft function (DGF). Delayed graft function (DGF) is a clinical diagnosis made based on the need for dialysis support post-transplant, DGF has been independently associated with decreased long-term graft survival[11]. Along with its association with decreased long-term graft survival, post-operative DGF also increases the risks of further complications as the patient has to return to dialysis until the new graft starts to function. CIT's vary from 0-2 hours in live donor transplants

to longer than 24 hours in less controlled transplant scenarios (NDD or DCD where WIT also increases); increased ischemia times play a role in DGF in all situations[12,13]. The almost four-fold increase in graft discard rate over the last three decades is likely a result of a complex mixture of shifts in attitude, technology, and policy; however, it is also symptomatic of a need for novel preservation methods to improve pre-operative graft evaluation and lower both warm and cold ischemia times[7].

A recent major logistical concern in kidney transplantation is the transport of the donor kidney when the living donor does not wish to or cannot travel to the recipient's local hospital. In the recent Kidney Paired Donation programs in Canada, kidney shipment has become more frequent as cross-country matches often deter donor travel due to lack of convenient transportation and the added complications of changing surgical care teams for the living donor after transplant [14]. There are various considerations in the logistics of transporting a kidney across five time zones, however a self-contained, automated, and mobile system physically capable to be transported in small spaces such as a commercial airline would be essential to the success of any normothermic machine perfusion system. There are fewer logistical concerns when it comes to using static cold storage or hypothermic machine perfusion of the kidney graft for preservation and transport, as these methods often require less supporting equipment and limited perfusionist expertise during the pre-transplant transport and management.

With a basic discussion of the need for novel preservation methods to improve evaluation of grafts and shorten ischemia times, we will briefly review the development of the current state of hypothermic preservation methods, followed by a review of normothermic preservation.

Chapter 2: Literature Review

1) Review of Static Cold Storage (SCS) and Hypothermic Machine Perfusion (HMP):

Modern kidney preservation methods began with the technically difficult hypothermic machine perfusion (HMP), followed by the development of increasingly effective static cold storage (SCS) solutions, and finally with a return of more refined HMP systems. The idea of using hypothermia to preserve organs for the purposes of transplantation dates back to 1905, and although homograft transplants had no real successes until the 1950's and 1960's, it has been used in transplant experiments[3]. The first attempts at organ perfusion were made by Alexis Carell and Charles Lindbergh in the early 20th century using a sterile organ perfusion pump system to create an *in vitro* "culture of whole organs" by providing a physiologically analogous environment[5]. Their landmark perfusion machine, which evolved into the heart-lung machine, would be the basis for many future machine perfusion systems. The idea of preserving or "culturing" organs outside of the body also inspired research into a simpler version of static preservation using the same principles of hypothermia[15]. The first successful experimental model of kidney transplantation after cold preservation was claimed by Dr. Lapchinsky using a canine model and a hypothermic blood based perfusate[15]. It was quickly followed by successful *ex situ* kidney preservations in canine models using variations of acellular hypothermic solutions including the University of Wisconsin solution which is still used today[15,16].

Many of the preservation solutions used for SCS are also used for HMP, which is a design goal of groups such as Southard et al. who created the University of Wisconsin solution, variations of which are currently used for both SCS and HMP. The main goals of most SCS solutions aim to counteract different deleterious effects of cold ischemia and the reperfusion

thereafter, including: 1) prevention of cellular edema, 2) prevention of metabolic depletion, 3) prevention of accumulation of reactive oxygen species (ROS) during cold ischemia and after reperfusion. Due to the relative simplicity of SCS application, we will mostly focus on the development and rationale behind each SCS solution.

1.1 SCS Solutions

SCS solutions were developed over several decades in the second half of the 20th century as solid organ transplantation became a more realistic intervention, and improved preservation quality and duration was needed in the implementation of larger organ transplant programs. To improve preservation, the three previously mentioned aims had to be tackled.

In the early investigation of cold ischemic injury, renal edema observed after ischemia-reperfusion injury was often attributed to hydrostatic forces of reperfusion[17]. However further understanding of cellular mechanisms of cold ischemia led to the theory that cold ischemia-induced cellular edema was caused the diffusion of ions into the cell down the electro-chemical gradient normally maintained by ATP-dependent pumps[18,19]. To address this, many SCS solutions either replicate intracellular ionic composition to remove the gradient that drives intracellular ionic accumulation or prevent the movement of water into cells through addition of impermeants or colloids[16]. Like the intracellular accumulation of sodium without the aid of ATP dependent pumps, calcium also accumulates intracellularly but with a significantly higher extracellular-intracellular gradient of 10000:1; intracellular calcium has been observed to increase significantly and in proportion to the duration of ischemic renal injury[20]. Although verapamil and other calcium entry blockers have shown success in improving renal function in renal failure models and transplanted grafts, its value may derive more from improved hemodynamics rather than reduced calcium accumulation[20,21]. Because of its relatively small

contribution to solute-induced swelling compared to sodium, it is hypothesized that calcium accumulation's mechanism of injury likely involves activation of highly calcium sensitive processes such as the enzymes calpain and protein kinase c[16,22], as well as activation of mitochondrial permeability transition which can lead to both cytosolic potassium influx-induced swelling and further loss of ATP synthesis potential[20,23]. Both cytosolic calcium overload and mitochondrial permeability transition are significant molecular events during cold renal ischemic injury, and may occur to different degrees causing tubular cell dysfunction or even necrosis[24]. When ATP depletion is not severe enough for necrosis to occur, DNA damage response has been observed prior to apoptosis in renal ischemia[25].

Metabolic depletion comes naturally during cold ischemia due to both reduction in cellular respiration and supply of substrates for ATP generation; ATP depletion can be as drastic as 20% of non-ischemic levels within 10 minutes of warm ischemia[20]. ATP depletion furthers the cellular swelling, but also lead to metabolic acidosis from anaerobic metabolism, cytoskeletal alterations, and alteration of mitochondrial structure. Metabolic acidosis is caused by the switch from reliance on aerobic metabolism to anaerobic metabolism for ATP synthesis during hypoxia. Resultant increase in glycolysis leads to increased lactate and hydrogen ion production, and lactate transport causes a net increase in extracellular hydrogen ions as cation (hydrogen) follows the anionic lactate[26]. Cytoskeletal alterations occur rapidly and reversibly because ATP depletion leads to an imbalance in dynamic regulation of different types of actin filaments and increases the activity of actin depolymerizing factor[27,28]. Some of these ATP depletion induced cytoskeletal alterations may be the cause of macroscopic pathology seen in ischemic acute kidney injury (endothelial dysfunction, vascular permeability, interstitial edema, and loss of tubular microvilli)[28,29].

Reactive oxygen species (ROS) have been identified as a contributing factor to ischemia-reperfusion injury in kidney grafts and therefore processes that generate ROS are often the targets of SCS and HMP solution additives. ROS is generated through physiological steps within the various redox reactions of mitochondrial respiration, however its generation is upregulated in pathophysiology[30]. Another source of ROS is nitric oxide synthase uncoupling (found in all isoforms of nitric oxide synthase) which can also lead to endothelial dysfunction and increased oxidative stress; the resultant reduction in nitric oxide bioavailability also exacerbates ischemic injury through vasoconstriction[31]. Increased ROS presence in the endothelium (due to endothelial nitric oxide synthase uncoupling) can cause further renal structural alterations through activation of matrix metalloproteinases[32]. ROS can damage proteins, lipids, and DNA contributing to apoptosis and necrosis in ischemia-reperfusion injury, though often secondary to the previous more direct causes[20]. Because of its dependency on oxygen and oxidative actions of the mitochondria, ROS generation and its associated damage to renal cells often occurs after reperfusion (*in situ* in transplants after SCS and *ex situ* in oxygenated HMP and NMP) and the return of normoxemia or hyperoxemia[33]. Although its impact on clinical renal tubular injury is unclear, ROS generation does occur during hypoxia paradoxically, therefore it may be possible that ROS plays a part during cold ischemia phase of SCS and HMP as well[34]. Overall ROS generation during cold ischemia and the following reoxygenation lead to functional and structural dysfunction of the vascular and renal tissues, its various deleterious effects are a target of antioxidant components within preservation solutions as well as novel pharmaceutical agents that will be discussed in later sections[35].

Both hypothermic preservation solutions and methodologies evolved and continue to evolve to address these three causes of injury in cold ischemia: cellular edema, metabolic

depletion, and oxidative stress. Electrolyte and biochemical compositions of different cold preservation solutions are summarized in Table 1.

Collins and Euro-Collins Solution

The initial development of a SCS solution revolved around mimicking *in vivo* conditions for cells and tissues, which then evolved as clinical and preclinical experience accumulated. In 1969, Collins et al. developed the first flush solution to be used in clinical SCS which mimicked physiologic intracellular ionic composition containing high potassium, low sodium and chloride, phosphate buffers, and 140 mmol glucose[18]. One aim of using an intracellular ionic composition was to prevent the creation of an electrochemical gradient across the cell membrane, therefore preventing the ionic fluxes (sodium, potassium, and calcium) that often lead to cellular edema and pathophysiologies related to cytosolic hypercalcemia[36]. Successful cadaveric human kidney transplantations after up to 24-45 hours of SCS was made possible using the "Collins solution" as a flush, and was quickly accepted clinically with modifications[37]. One notable condition of the study on Collins solution performed by Barry et al. was the lack of warm ischemia (ranging from 6-9 minutes) due to the use of "heart-pumping cadavers" or brain death donors, leaving the Collins solution largely untested for its ability to rescue organs that experienced long periods of warm ischemia like those found in donors after circulatory death[37].

The Collins solution was later modified by the Eurotransplant Foundation into the Euro-Collins (EC) solution, which was widely used until the 1980s: magnesium sulfate was removed because it did not contribute to preservation and glucose was increased to 195 mmol for a higher osmolarity of 406 mOsm/kg $_{H2O}$[38]. One disadvantageous component of EC solution was glucose, which could have led to cellular swelling after enzymatic breakdown and made it difficult to sterilize for clinical use; the replacement of glucose with metabolically inactive yet equally

osmotic variants such as sucrose or mannitol was a promising idea proposed by Dr. Collins himself[36,38,39].

Sacks Solution

Following the initial development of the Collins solution, another perfusate was proposed by Sacks et al. in 1973 named the Sacks solution[19]. The Sacks solution was similar to Collins solution in its intracellular ionic composition and osmolarity (410 mOsm/kg $_{H2O}$); however, glucose was replaced by mannitol, sodium bicarbonate was added as a buffer, and potassium phosphate was added to further mimic the hyperkalemic intracellular ionic composition[19]. Sacks hypothesized the mannitol would be an improvement upon the use of glucose as an osmotic agent because it is not actively metabolized, as previously discussed in proposals to improve upon the Collins solution[19,38]. The Sacks solution was used in a few transplant programs and was received variably during the 1970s-1980s, however record of its clinical successes were limited[40]. With studies showing magnesium phosphate deposits and significant animal model failures[41,42], its clinical viability soon became overshadowed by the successes of the University of Wisconsin solution introduced in the 1980s.

University of Wisconsin Solution

In the 1980s, Dr. Belzer and Dr. Southard developed the University of Wisconsin solution (UW solution), an alternative preservation solution offering versatile usage in both SCS and machine perfusion[16]. The UW solution had similarly mimicked the ionic composition of intracellular space like the EC solution, however it significantly changed the use of glucose to the metabolically inert yet osmotic components lactobionate and raffinose; using lactobionate, raffinose, and hydroxyethyl starch (HES), UW solution was designed to be approximately isotonic with an osmolarity of about 320 mOsm/kg $_{H2O}$[16]. The use of hydroxyethyl starch (HES)

in a solution designed for kidney preservation has been controversial, because of its increased viscosity and potential nephrotoxicity[16]. The increase in viscosity caused by HES was a concern cited by many studies, due to its reported effects on red blood cell hyperaggregation and reduced efficiency in flushing out red blood cells[43–47]. Because increased erythrocyte aggregation slows initial blood flush out and lowering of kidney temperature, it is theorized to cause increased warm ischemic injury[44,47]. The effect of viscosity of flush solution alone on the efficiency of flush and subsequent kidney preservation has been questioned, and evidence from recent studies appear to show a contradiction[48]. Boffa et al. found UW solution flushed out erythrocytes more effectively when compared to lower viscosity solutions, and found no correlation between viscosity and the penetration of kidney cortices for lowering of temperature[48]. In fact, Kay et al. have suggested that faster initial flush may cause increased endothelial injury and require more flush solution to induce desired cooling; the increased viscosity slowing down the flush may be beneficial[49]. The use of UW solution in clinical settings continue today, the potential for inefficient flushing can be addressed by increasing flush times and may be overshadowed by the superior renal preservation and evidence contradicting its true damage.

HES has also demonstrated nephrotoxicity which suggests it may be harmful to use in kidney preservation solutions, however the evidence in literature are almost exclusively from perioperative administration[50–52]. Although other studies have shown no difference in delayed graft function (DGF) after peri-operative administration of HES[53], a key difference may be the amount and concentration of HES administered, as Shaw and Kellum pointed out in a 2013 review, there can be nephrotoxicity as a result of qualitative (depending on the specific colloid or crystalloid used) or quantitative (amount and concentration of a specific oncotic/osmotic agent) factors[52]. HES and other colloids have been shown to be taken up by renal tubular cells via

pinocytosis, and subsequently cause osmotic nephrosis which is characterized by the reversible accumulation of colloids intracellularly and reduction of renal tubular function during colloid administration[54,55]. The osmotic nephrosis observed during colloid administration is hypothesized to occur by pinocytosis followed by increased vacuolization, where lower molecular weight HES is implicated to increase cellular uptake[55]. The osmotic nephrosis caused by HES and similar colloids (dextran, mannitol etc.) administration is observed to be reversible after stopping HES use[54,56]. Mechanistically, osmotic nephrosis may be caused by mRNA level adaptations to increase colloid presence in the tubules leading to increased uptake and decreased degradation and excretion[57]. The renal safety of HES is a hotly debated topic, though evidence of its nephrotoxicity is largely based on perioperative administration where the likelihood of AKI is already high without the administration of HES or other colloids for fluid resuscitation[53,58]. A systematic review by Wiedermann et al. found increased nephrotoxicity with administration of hyperoncotic HES compared to hyperoncontic albumin; however, it was also noted that due to non-overlapping situations, many of the studies cannot be compared directly (mainly sepsis or surgery for HES and cirrhotic patients for albumin)[59]. The lack of sufficiently conclusive pre-clinical evidence of nephrotoxicity, the reversibility of osmotic nephrosis, and continued clinical use of HES containing UW solution seem to suggest that HES is safe to use in a hypothermic static preservation system.

As many studies have pointed out, due to the lack of evidence that HES is beneficial and the possibility of its nephrotoxicity, it may be advantageous to seek out a replacement for the oncotic agent[51,60]. One alternative oncotic agent for use in UW solution is hyperbranched polyglycerol (HPG): a low viscosity, non-metabolized, but largely low molecular weight polymer[60–62]. HPG has shown effectiveness and increased biocompatibility (reduced aggregative

effects on erythrocytes and viscosity) in animal models of dialysis and kidney preservation[60,63]. Similar studies have used other polymers such as polyethylene glycol (PEG) to some success in pre-clinical trials[64,65]. A modified UW solution with lower potassium and HES replaced with PEG has been marketed as Institut George Lopez-1 solution, or IGL-1 (IGL©, Lyon, France). IGL-1 has been proven to be equivalent to UW solution in living kidney donor preservation with lower viscosity[60,66] and demonstrated equivalent post-operative function compared to UW solution in a multi-centre RCT[67,68]. IGL-1 is advertised to be a lower cost and lower viscosity version of UW solution with equivalent preservation abilities; it has also been successfully used in liver and pancreas SCS for transplantation[69,70]. The replacement of HES with HPG and PEG reduces the potential for nephrotoxicity from HES, but maintains sufficient oncotic pressure prevent cellular/interstitial edema and its associated injuries during ischemic, hypothermic storage. UW-modified (a UW solution with adenosine, allopurinol, and HES simply removed) has been found to be significantly cheaper and have comparable performance to UW solution[68,71]. In Baatard et al.'s study on UW-modified, only serum creatinine related parameters were reported and there was insufficient data to justify the outright removal of the oncotic agent. The development of new oncotic agents to replace HES may contribute to future iterations of a modified UW solution, though UW solution and IGL-1 solution are still widely used.

Other components of interest introduced in the UW solution include allopurinol (1mmol), glutathione (3 mmol), and adenosine (5 mmol), which were originally added for theoretical reasons postulated by Belzer and Southard[72]. It was proposed that allopurinol inhibits xanthine oxidase therefore preventing the production of reactive oxygen species (ROS), glutathione may scavenge ROS and is depleted during ischemia, and adenosine provided the substrate components and stimulated the production of adenosine triphosphate (ATP, the basic energetic

molecule used by cells) along with phosphates[72]. Allopurinol's role in reducing the deleterious impact of ROS through xanthine oxidase may be minimal in human kidneys, as although the specific enzyme xanthine oxidase significantly contributes to ROS generation in rodents, it is absent in humans (specifically absent in human kidneys and urine[20,73,74]. Later Southard further proposed that glutathione was also important for the preservation of the cell's ability to protect metabolic capacity and cellular membrane integrity as demonstrated by poor renal function in canine models when glutathione was removed from UW solution[75]. Similar work in liver models supports the effectiveness of glutathione in reducing ROS[76,77]. However, the active and reduced forms of glutathione are relatively unstable, leading to reduced effective ROS scavenging capacity after storage of UW solution and its derivatives thus requiring the addition of fresh reduced glutathione prior to use, or shorter storage period[78,79]. The introduction of ATP components into UW reflects the goal of reducing metabolic collapse often seen in cold ischemia, while the addition of antioxidant agents reflects the goal of reducing the damage of ROS produced during cold ischemia.

The clinical introduction of the UW solution in 1987 led to a multi-center study comparing UW solution to EC solution; UW solution was found to reduce delay in graft function (DGF; 33% reduced to 23%) and suggested improvement of one-year graft survival (EC 82.5% to UW 88.2%)[80]. The 1992 study and continued accumulation of clinical evidence led to the shifting the standard SCS and HMP solution to UW solution which remains the gold standard in many transplant programs[16,81].

Histidine-Tryptophan-Ketoglutarate Solution

Another commonly used flush and preservation solution for SCS is the Histidine-Tryptophan-Ketoglutarate solution (HTK solution; also available commercially as Custodiol®

HTK) developed initially by Dr. Bretscheider in the 1970s with the goal of providing myocardial protection as a buffered solution to preserve cardiac tissue[82]. Unlike any of the previous preservation solutions which mimicked intracellular ionic concentrations, HTK solution had low sodium but also low potassium concentrations, and opted to have a strong buffer solution of histidine and histidine hydrochloride of 198mmol supplemented with 30mmol of mannitol to create a solution with 310 mOsm/kg H_2O[16,82]. Bretschneider et al. hypothesized that the buffered intra-renal pH, which does not drop below 6.7 due to the strong amino acid buffer histidine, prevents ischemic acidosis while still allowing lactate efflux from cells[26,83]. HTK solution is also theorized to reduce ischemic metabolic demands by using lower ionic concentrations (reducing demands on active transport components) and providing ketoglutarate as a substrate for anaerobic metabolism[16,83]. The initial design of HTK solution addressed edema through the use of strong buffers and mannitol, addressed metabolic depletion through reducing demand and providing additional substrate, and finally tryptophan acts as an antioxidant to reduce oxidative stress.

In a Eurotransplant multi-center study on 1180 heart-beating donor kidney transplants from 1990-1992, HTK solution was found to have similar rates of 3-year graft survival when compared to either UW solution or Euro-Collins solution[84]. While HTK solution had the same rates of delayed graft function (33%) when compared to UW solution, Euro-Collins solution had significantly higher rates of delayed graft function (43%) and Eurotransplant recommended against the further use of Euro-Collins solution for SCS of kidneys[84]. There are not many studies on the comparison of HTK to UW solutions in SCS of kidney grafts in extended criteria donations, however a single large (n=698) study on ECD livers demonstrated comparable success between the two solutions[85].

HTK solution has a lower viscosity when compared to the UW solution, which has often been cited as a reason why UW is not the optimal flush solution[38]. The combined approach of using an HTK solution for initial *in situ* or back table kidney flush followed by UW solution for long term SCS show favourable outcomes by ensuring a quick and effective flush with HTK solution and superior long-term preservation with UW solution[46]. Due to the simplicity of using one or the other as a dual purpose flush and preservation solution, this strategy is not as widely studied, especially with evidence contradicting the detrimental effects of UW solution's higher viscosity[48].

HTK solution has also found use in regional bypass of renal circulation during supra/intra-renal surgeries (aortic surgery or renal tumor removals), as well as its other uses in both cardiac and pulmonary protection in regional bypass and similar procedures that require *in situ* preservation[86–89].

Celsior Solution

Celsior solution is another hypothermic kidney preservation solution developed in the 1990s combining lactobionate and glutathione from UW solution, and histidine and mannitol from HTK solution; however, the Celsior solution uses a uniquely extra-cellular ionic composition that is hypo-osmolar (255 mOsm/kg $_{H2O}$)[16,90]. Celsior solution addressed the theoretical concerns that occur during cold ischemia, including: prevention of cellular edema using large impermeable molecules lactobionate and mannitol, stimulation of energy production using glutamate, and reduction of oxidative stress using glutathione[90]. Celsior solution had mixed results when used as cardioplegic solution and SCS solution for cardiac preservation as it was originally intended[91,92]. In clinical studies Celsior demonstrated comparable results with UW solution, but may have shortcomings in increased production of deleterious vasoactive agents

and increased need for post-operative inotropic support[93–95]. In clinical studies of Celsior solution as a kidney SCS solution, it demonstrated equivalent performance when compared to UW and HTK solutions in both graft survival and delayed graft function[68].

Hyperosmolar Citrate

Hyperosmolar Citrate (HOC or Marshall's solution) is a more extracellular solution derived from a Krebs-Henseleit buffer solution previously used in isolated kidney perfusion experiments[96]. HOC solution has now been modified to contain a large concentration of mannitol (185 mmol), citrate (80 mmol), 40 mmol magnesium, equal concentrations of sodium vs potassion (80 mmol), no calcium or chloride, and less bicarbonate (10 mmol) with an osmolarity of 400 mOsm/kg $_{H2O}$[16]. HOC addresses the theoretical concerns of edema through increasing its osmolarity and the use of the impermeant mannitol without any colloids; citrate is a calcium chelator, therefore reducing the risk of intracellular hypercalcemia[16]. When compared against UW solution, HOC provided comparable protection but seemed to have significant weight gain and histological injury during the flush[49]. The increased weight gain and injury during initial flush presents a concern for HOC solution, demonstrating the need for a colloid or a stronger impermeant component. HOC has been used as a renal preservation solution in the UK and Australia but has demonstrated limited viability when compared against UW and HTK solutions with a lack of data available in randomized controlled trials[68,97]. One practical advantage of HOC solution is its significant cost reduction and simplicity when compared to the other three equivalent solutions (UW, HTK, Celsior), with other solutions costing $190-250 USD per litre and HOC solution costing $15 USD per litre[68].

1.2 Hypothermic Machine Perfusion (HMP)

Hypothermic *Ex Situ* Kidney Perfusion, or Hypothermic Machine Perfusion (HMP), has a long history dating back to before the first successful human organ transplantation but the first clinical success was reported by Belzer et al. in 1968[98]. Belzer et al. perfused a human cadaveric kidney with 25 minutes of warm ischemia and 55 minutes of cold ischemia prior to being connected to their "extracorporeal perfusion apparatus" made up of a membrane oxygenator, pulsatile pump, chamber, filter, and heat exchanger similar to a heart-lung machine used in cardiopulmonary bypass[98]. Belzer et al. used a modified human plasma perfusate and perfused at 10 degrees Celsius and 110/40 mmHg for 15.5 hours before implanting the graft, and the patient experienced delayed graft function along with complications arising from severe rejection common as kidney transplantation was still an immature technique[98]. Since the 60's, equipment available for HMP has significantly improved but the basic principles and schematics remain largely unchanged, yet many perfusion solutions have developed alongside the various SCS solutions[99].

Within the HMP section, we will begin by discussing the rationale of the recent return of HMP over SCS as the gold standard of kidney preservation. Then we will discuss the different parameters of HMP perfusion systems and the evidence behind different options. Finally, the metrics used to evaluate graft preservation and function during HMP and the different interventions aiming to improve those metrics will be examined.

Reasons for HMP over SCS

The inconveniently large size and complexity of HMP equipment available in the earlier stages of kidney preservation created a significant logistical barrier, and with the advent of several successful SCS solutions (EC then UW and HTK), HMP fell out of favour as SCS

became the standard[99]. Cost-benefit and logistical feasibility have always been at the forefront of comparisons between HMP and SCS, as primary graft function, rejection, and patient mortality have not show definitive advantages to either side[99,100]. Until the refinement and miniaturization of extracorporeal perfusion equipment in recent decades, it was impossible to use for transportation of kidneys between distant transplant centers, and expensive to both purchase and maintain for hospitals[101]. Another monetary and quality of life cost to consider, is the need for post-operative dialysis due to delayed graft function (DGF): two systematic reviews by O'Callaghan and Tingle have shown that HMP reduces the frequency of DGF in both DBD and DCD transplants[68,102]. Keeping in mind the lack of world-wide standardization and a host of uncontrolled variables, upfront costs of implementing HMP is still likely higher with estimates ranging from ~$500 to $3000 USD per patient, but some have argued the graft life-time cost savings would be equal if not higher in HMP due to reduced incidences of DGF[99,103,104]. With a combination of increased monetary cost-benefit ratio and improved mobility/miniaturization, HMP has become a more favourable method of preservation compared to SCS.

Another epidemiological/public health perspective that contributed significantly to the return of HMP in recent decades, is the increased donor age and the increase in associated chronic diseases ailing said donors among higher KDPI donors (a major factor in KDPI is donor age)[105]. SCS does not offer sufficient opportunity to evaluate or repair a graft, however machine perfusion has the potential to offer these opportunities pre-implantation.

HMP Methodology and Modes

Most often used commercial kidney HMP systems available include LifePort® (Organ Recovery Systems Inc, Illinois, USA), Kidney Assist-Transport® (Organ Assist; Groningen, Netherlands) and RM3 Waters kidney preservation system (Waters Medical System ®; New

York, USA)[106]. Following information on specifications of the above three systems were extracted from their respective product user manuals and websites.

The LifePort Kidney Transporter® is the most well studied system. It is pressure controlled and offers HMP at 1-8 degrees Celsius, with recordings of perfusion parameters including pressure, flow, temperature, and calculated intra-renal resistance[99,102,107]. Although arterial cannula pressure can be varied from a range of 10 to 65mmHg provided by the peristaltic pump with 30mmHg being the default and most used, most studies tend to use lower pressures for HMP due to historical concerns about HMP-related graft edema caused by hydrostatic pressure[99]. Dimensions are: 24" x 14.5" x 14.25" (61.96cm x 36.83cm x 36.195cm), 45lbs (20.4kg) fully loaded.

Kidney Assist-Transport® is a pulsatile pressure-controlled hypothermic perfusion system, it is self contained and offers 0-4 degrees Celsius and oxygenation[108]. The same company offers a similar but less portable HMP/NMP system for kidneys[109]. Currently Kidney Assist-Transport® is being tested in two European multi-centre RCT's[102,110]. One advantage of Kidney Assist-Transport® is the option of performing oxygenated HMP, research indicating its beneficial effects will be outlined in the later section on HMP parameters.

RM3® kidney preservation system is a flow, pressure, and temperature-controlled system providing pulsatile HMP[111]. RM3 provides flow-controlled perfusion using an occlusive pulsatile perfusion pump with adjustable occlusion arms to control volume per stroke. Dimensions are (21.25" x 15.75" x 10.83" (54.0cm x 40.0cm x 27.5cm), 67.6lbs (30.73kg) fully loaded excluding kidney and perfusate (1L); RM3 is the only of the three to advertise volume-controlled perfusion instead of pressure-controlled perfusion[111].

Several solutions have been studied for use in kidney HMP: UW, HTK, and HTK-N, but the most commonly used clinical solution is a modified UW solution marketed under Kidney Perfusion Solution (KPS-1®) by Organ Recovery systems who also supply the previously mentioned LifePort® HMP system[99,102]. KPS-1 is similar to the UW solution marketed under Static Preservation Solution-1 (SPS-1®; Organ Recovery System, Itasca, USA), but is modified in several ways: raffinose and lactobionate is replaced with mannitol and gluconate as impermeable osmotic agents, adenosine is replaced with free base adenine and ribose (equivalent chemical components of adenosine), and allopurinol is removed. KPS-1 is the recommended perfusion solution used by the LifePort® system and is part of clinical protocols adopted by most of the transplant centres where HMP studies were carried out[102]. UW solution was originally created by Southard et al. with its use in HMP in mind and has been since adapted to be used in HMP. KPS-1 is also known as PERF-GEN® or Pulsatile Perfusion solution for kidney supplied by Institut Georges Lopez (IGL; Lissieu, France) and is the recommended perfusate for RM3® kidney preservation system (Waters Medical System; Rochester, USA).

Another solution that has been investigated for use in HMP is a modified HTK solution named HTK-N. HTK-N is said to be a less toxic version of HTK solution, with the titular component modification being the replacement of Histidine with a less toxic derivative N-acetyl-L-histidine due to the potential for toxicity of histidine when combined with oxygen[112,113] (Rauen 2008; Gallinat 2013). HTK-N aimed to improve protection against several aspects of hypothermic ischemia: cation leak, metabolic depletion, and oxidative stress. HTK-N added glycine and alanine to prevent the leak of sodium-like cations by inhibiting transporters[114]; HTK-N also lowered sodium concentrations to reduce the inward electrochemical gradient. The Krebs cycle substrates aspartate and alpha-ketoglutarate were added to prevent metabolic depletion

during hypothermic hypoxia[113]. The addition of iron chelator deferoxamine and permeable iron chelator LK 614 to reduce the oxidative stress induced by iron's actions on hydrogen peroxide; nitroxide synthase substrate L-arginine is added to support nitric oxide production and vasodilation during reperfusion[113]. HTK-N supplemented with 50g/L of dextran 40 has been used for a pre-clinical transplant study using a pig model, with results showing a trend towards lowered post-operative creatinine and significantly reduced tissue expression of endothelin-1 and toll-like receptor-4 which have been attributed to reduced endothelial stress response[115]. Clinical study of HTK-N has yet to be performed, and no large-scale comparisons between HMP solutions are available.

Pulsatile flow has been suggested since Belzer's 1969 experiment to better replicate physiological factors and provide improved endothelial protection during cold ischemia, while continuous flow is more easily controlled and require less complicated equipment. All three of the commercially available systems utilize pulsatile flow to provide more physiological microvascular environment, and there is evidence for improved vasodilatory protection through stimulating capacitative flow within vessels and increasing nitric oxide synthesis[116–119]. An original reason for using pulsatile perfusion pumps was the ready availability of roller pumps which provide an innately pulsatile pattern of perfusion; they were also thought to be able to reach high systolic pressures without causing tissue edema through constantly high hydrostatic pressure[120]. Since the early days of bypass and perfusion equipment development, there are now non-traumatic continuous bypass pumps, but because of historical dominance of roller pumps there was simply not enough evidence to warrant removing pulsatility clinically.

The benefits of pulsatile perfusion pattern on renal function have also been demonstrated in a setting of cardiopulmonary bypass during cardiac surgery, where non-pulsatile flow increased

renin secretion and showed poorer tissue oxygenation[121]. One mechanistic difference between surgical cardiopulmonary bypass and an isolated renal HMP system is the presence of stimulation in intact renal sympathetic nerves, which may explain the clinical renal protection seen in pulsatile cardiopulmonary bypass[122]. There are proponents of non-pulsatile flow with very limited pre-clinical and clinical evidence[123]. A benefit of non-pulsatile flow is its mechanistic simplicity, whereas pulsatility in a pulsatile pump head may have haemodynamic interactions with other components before reaching the kidney[124]. With the limited *in vitro* and pre-clinical evidence, the use of pulsatile flow mode is favoured and is reflected in its adoption by existing commercial HMP systems. RCT's comparing pulsatile and continuous perfusion are lacking and no conclusive evidence of any effect on graft survival or DGF exist, further clinical and pre-clinical investigations into tangible benefits of pulsatile pump mode is needed.

The first successful HMP performed by Belzer in 1969 used a pulsatile pressure of 110/40mmHg, and since then many HMP studies and indeed clinical practice have been carried out at higher, near physiological systolic pressures[98,125]. Recent studies have demonstrated improved endothelial preservation and reduced DGF at lower perfusion pressures (30/20mmHg vs 60/40mmHg)[126], and this is reflected with most HMP systems suggested lower than physiological default systolic pressures (LifePort 30mmHg; RM3 45mmHg). Doorschodt et al. reduced the perfusion pressure even further from 30mmHg to 25mmHg in a small pre-clinical study and suggested even lower perfusion pressure may be desirable[127]. Wszola et al. performed a small scale RCT comparing LifePort against RM3 systems showed reduced severity of DGF and reduced one-year post-transplant renal fibrosis and tubular atrophy in LifePort systems, which differs from RM3 in both a pressure driven (vs flow driven in RM3) and lower systolic pressure[128]. Wszola et al. found no significant graft survival differences. Larger RCTs comparing

different perfusion pressures within the same pump modalities are needed to find the optimal perfusion pressure for kidney preservation and long-term graft function.

Perfusate oxygenation during hypothermic kidney preservation has been tested as early as the 1980s to counteract the ischemic injury during hypothermia and provide protection against metabolic collapse and lactate accumulation[129,130]. Many forms of oxygenation exist for hypothermic preservation (oxygen sufflation, hyperbaric environment setups, perfluorocarbon oxygen delivery, but the main focus is on the delivery of oxygenation in machine HMP[130]. It was determined that even at 5 degrees Celsius of hypothermia, there is still 5-10% metabolic activity occurring, therefore creating a need for metabolite waste removal and support for the production of new energetic materials[120]. Oxygenation during HMP may help to reduce renal injury from metabolic depletion, as previously outlined, however it may also increase oxidative stress.

In a DCD porcine transplant model, oxygenated HMP was compared against non-oxygenated HMP and the oxygenated group found lower post-operative serum creatinine and reduced long term fibrosis[131]. Venema et al. perfused DCD porcine kidneys with HMP at either 0%, 21%, or 100% oxygen supplementation, and found insignificant differences between the groups after rewarming to normothermia, but oxygenated HMP reduced renal damage as measured by aspartate aminotransferase[132]. A systematic review of oxygenated vs non-oxygenated HMP by O'Callaghan et al. in 2017 identified a lack of quality human RCTs investigating the effect of HMP perfusate oxygenation and pointed out a lack of ability to make conclusive remarks on perfusate oxygenation in HMP[133]. The COPE framework of transplant RCTs in Europe has within it a study testing the effects of oxygenated HMP utilizing the Kidney Assist-Transport and its unique ability to offer oxygenated HMP, this large scale consortium

study may be able to shed further light on the beneficial effects of hypothermic oxygenation[110,134].

The various HMP modalities (perfusate, pump, pressures, and oxygenation) continues to be studied, especially the differences among the commonly available commercial HMP systems, however definitive evidence in favour of each side is still wanting as we look forward to more multi-centre RCT's; not all evidence may be readily appreciable, as monitoring of long-term function and survival requires years of study.

HMP Metrics for Graft Viability/Function

Many parameters and biomarkers have been explored in HMP for their predictive association with primary graft function, rejection, and initial graft function, however few have been conclusively found to be effective in being useful for outright accepting or rejecting a donor graft pre-implantation[99,135]. The same biopsy techniques done in SCS can occur with HMP and normothermic MP, however with perfusion and wash out of tissue markers of injury into perfusate or urine; machine perfusion allows the evaluation of kidney injury biomarkers and potentially use them as an objective metric to accept or reject a donor graft pre-implantation. Research into said biomarkers is limited, however several studies showed inconclusive association between many injury markers found in HMP and graft survival/DGF[135]. Moers et al. found an association between last hour HMP perfusate biomarkers glutathione-s-transferase (GST), N-acetyl-beta-D-glucosaminidase (NAG), and heart-type fatty acid binding protein (HFABP) to be independent predictors of DGF, but not primary non-function (PNF) or graft rejection[135]. Because the patient will still have a functioning kidney even though DGF is costly in terms of financial and quality of life costs, Moers et al. recommended against the use of those predictors as grounds for graft rejection, but rather additional information for post-operative

care[135]. There may be a difference between the predictive abilities of biomarkers in HMP when compared to normothermic machine perfusion (NMP) due to differences in when ischemia-reperfusion injury occurs and abilities to influence metabolic states of the graft, which will be discussed further in the section on NMP.

A perfusion parameter examined for association with post-operative graft performance is intra-renal resistance, which is thought to reflect endothelial damage but also vascular health and responsiveness[20]. The same Eurotransplant group (Jochman et al., 2011 study on predictiveness of biomarkers), performed a blinded prospective study on predictiveness of intra-renal resistance (IRR) of the same post-transplant outcomes (DGC, graft loss or PNF, and graft rejection) and found independent association with both DGF and one-year graft loss[136]. However, Jochmans et al. used a post-hoc analysis of intra-renal resistance thresholds as a metric for predicting DGF and graft loss and demonstrated a large likelihood of false predictions[136]. Jochmans et al. again recommended against using IRR as a sole determining factor in rejecting a graft, but rather adding it to the list of other factors considered as a contributing factor and inform the physicians for improved post-operative monitoring and care[136].

The most likely reason for the difficulty in finding strong predictive metrics is the overwhelming effects of factors surrounding donor health, organ recovery surgery, and post-operative patient care. These factors include tissue and humoral immunity matching, donor age, donor overall health, warm and cold ischemia times, previous transplants, and recipient pre-renal factors (hypovolemia, thrombosis, and oligourina/anuria)[11]. Another technical concern with using renal resistance as a pre-implantation metric is the variations in HMP modes utilized with different machines and studies: Jochmans et al. used LifePort kidney transporter, a pressure-controlled pulsatile perfusion system that generates sinusoidally varying renal arterial pressure

waves, while others in the past used RM3 which is a flow controlled perfusion system that generates varying arterial pressures with a constant flow, which means the renal resistance calculated would not be directly comparable between machines[136]. Even with the many confounding variables, renal resistance at the end of HMP may still offer important insight into the graft's likely performance both immediately after implantation and in the long-term.

Urine production during HMP, in both biochemistry and quantity, is most likely not relevant for kidney function or preservation as there is little active secretion or reabsorption during hypothermia. Most studies do not discuss urine production as a metric, and many do not even cannulate the ureter but instead allow the "urine" to drain back into the venous reservoir. However, this metric may be significantly more revealing during NMP, as normothermia allows for active renal excretion and reabsorption.

Interventions during HMP (Alport syndrome, post-operative microvascular thrombosis)

During HMP, metabolic activity is facilitated, compared to SCS, and with a delivery system for genetic vectors and pharmacological agents, the opportunity exists to both prevent ischemia-reperfusion related injuries and address any pre-existing concerns about the donor graft. Gene therapy trials during kidney HMP include using small interference RNA (siRNA) to prevent ischemia-reperfusion injury and reduce ROS production, but also transgene correction of Alports syndrome. The use of pharmaceutical agent thrombalexin to prevent post-operative microvascular thrombosis will be discussed along with therapies targeting ROS generation.

Alport syndrome is a genetic disease that causes progressive renal failure and collagen related conditions in other parts of the body; Alport syndrome currently has no specific treatment although angiotensin-converting-enzyme inhibitors slow renal failure progression and individual

symptoms are treated[137]. Genetic mutations target collagen IV formation and prevent effective filtration of proteins and large molecules by the glomeruli, and while different mutations may cause different individual pathophysiology, they all lead to progressive renal failure[137,138]. Alport syndrome is an ideal candidate for renal gene therapy as its life-threatening effects are almost exclusively renal and its genetic mutations (on *COL4A3, COL4A4, COL4A5*) have been marked as clear targets for somatic genetic alterations; Tryggvason et al. have demonstrated initial success using an adenovirus vector in a porcine model of *ex situ* perfusion[139,140]. Lin et al. demonstrated that inducing transgene expression of *COL4A3* gene in 21 day old mice could repair collagen IV networks within the glomeruli[138]; although their method of transgene delivery (injection into pronucleus of embryos) is unrealistic for human therapy, it does offer proof of concept that given efficient delivery method, somatic gene therapy for Alport syndrome can repair the glomeruli collagen networks. There is very limited current pre-clinical or clinical research on gene therapy in HMP, potentially due to its low receptivity towards gene transfer which pales in comparison with the highly metabolically active NMP environment. However, HMP does still allow the possibilities of gene therapy in a safe and isolated environment.

Pharmaceutical interventions might depend less on metabolic activity, and several vascular pharmaceutical interventions have been tested in pre-clinical studies. One such study was the use of thrombalexin as a pre-treatment against microvascular thrombosis[141]. The use of systemic anti-thrombotic agents such as heparin is a valid approach, however some situations warrant hesitation due to either non-renal complications in the recipient or increased bleeding risks during and post surgery. Hamaoui et al.'s study demonstrated the effectiveness of relatively passive treatment of microvascular conditions during HMP, as the tissue is well perfused even if not metabolically active; thrombalexin has been found to be present for several days in the post-

implantation graft[142]. One advantage of not having a metabolically active tissue is the reduced rate of breakdown, and if the urine produced is simply recirculated, there is little clearance of any pharmaceutical or genetic agent introduced into the HMP system increasing efficiency of any therapy introduced.

As seen in the addition of components such as allopurinol and reduced glutathione to SCS and HMP solutions, ROS generation has always been a target for therapeutic interventions in renal transplant. Nicotinamide (nicotinic acid, NAM or vitamin B3) is a vitamin available as a supplement commercially, but it is also a phosphate binder used in hemodialysis patients experiencing hyperphosphatemia[143,144]. Recently Song et al. investigated NAM's ability to protect cells against oxidative stress and prevent mitochondrial permeability transition[145], presenting a promising candidate to tackle one of the three obstacles in cold ischemic injury during SCS and HMP.

A long list of antioxidants both endogenous (catalase, glutathione peroxidase, and superoxide dismutase) and exogenous (ascorbic acid, resveratrol, and phenols) have been used to various degrees of success to combat renal ischemia-reperfusion generation of ROS[146]. However these antioxidants may be difficult to administer therapeutically and/or unable to penetrate into the mitochondria to prevent cellular ROS injury, which has led to the creation of antioxidant molecules conjugated with triphenylphosphonium (a cation used in cancer and neurodegenerative diseases to deliver mitochondrial target agents) that accumulate inside of the mitochondria[136,137]. These conjugated molecules can deliver many potent ROS scavengers, and one such conjugate mitoQ (cation plus ubiquinol) has been investigated for reducing cardiac ischemia-reperfusion injury[149] and renal ischemia-reperfusion injury in rodents[35,150].

2) Review of Normothermic Machine Perfusion (NMP)

Normothermic *ex situ* Kidney Perfusion or Kidney Normothermic Machine Perfusion (NMP) is not a novel concept, yet it has only received significant attention in the recent decade as the increased demand that drove the adoption of HMP over SCS continues to rise. ECD kidney grafts are increasingly used in transplant centres across the world, and even more accurate pre-implantation evaluation of grafts is desired. Several small clinical studies have been performed using NMP that show improvements over SCS[151], and there is currently a large multi-centre clinical study being performed in the UK of NMP compared to SCS[152]. The potential benefits of NMP extend beyond the immediate clinical benefits of improved preservation and evaluation, because it also offers a unique model for the testing of experimental therapeutic interventions in a safe, isolated yet metabolically active environment that mimics normal physiology.

Because NMP is still in its infancy as a clinical intervention, there are no established protocols and as a result different groups have implemented different methods in their own pre-clinical/clinical NMP systems. Different modes of NMP and any pre-clinical animal model evidence and clinical evidence available to justify these choices will be evaluated. With the potential to replace SCS and HMP as the gold standard in kidney preservation for transplantation, NMP needs to not only preserve the graft, but also to offer predictive metrics that can provide either insight for post-operative care or sufficient evidence to reject a marginal graft. The proposed metrics and systems of graft evaluation during NMP will be examined.

2.1 History and Goal of NMP

Before the first successful human solid organ transplant, Carrel and Lindbergh had already created a prototype organ perfusion machine and explored both hypothermic and normothermic organ preservation[153]. Due to various political, technological, and supply and demand factors, research into normothermic perfusion was abandoned as the community heavily favoured hypothermia and the simpler method of SCS[153]. In 2003, Brasile et al. performed warm (subnormothermia; 32C) *ex situ* perfusion of canine and discarded human kidney grafts for up to 48 hours using their "exsanguinous metabolic support (EMS)" system[154].

In 2006, Hosgood and Nicholson experimented with normothermic *ex situ* perfusion of pig kidneys and proceeded to perform various pre-clinical research, culminating in their first clinical study of 18 ECD kidney transplants using NMP in 2013 with promising post-operative results[151,155,156]. NMP differs from HMP in many mechanical and physiological aspects, but also differs in goal: hypothermic preservation methods aim to preserve and evaluate, while NMP aims to not only preserve and evaluate, but also to provide an environment receptive to pre-implantation interventions.

One of the criticisms of HMP, and hypothermic preservation techniques in general, is the lack of metabolic support to maintain a normal intra-cellular environment. Hypothermia stops/significantly limits production of high energy molecules such as adenosine triphosphate (ATP), without which sodium/potassium ATPase pumps cannot function, thus allowing intra-cellular accumulation of sodium and calcium which can draw water into the cell and subsequently causing edema[153]. Hypothermia also leads to intracellular accumulation of metabolic wastes and lowered pH causing lysosomal instability[153]. NMP aims to address the lack of metabolic activity and is usually performed at normothermia, or some cases subnormothermia,

which allows the kidney to maintain metabolic activity. With intact metabolic activity comes increased risk of, and increased exposure to, warm ischemia and ischemic-reperfusion injuries in the event of device failure. Three key requirements of NMP to be addressed are: sufficient oxygen and nutrient delivery to tissues, sufficient renal activity for evaluations and intervention, and minimal vascular endothelial injury.

2.2 General Methodology of NMP and Perfusion Modes

NMP is largely performed at normothermia, which for humans is ~36.5-37.5◦C and ~38-39◦C for pigs (a popular animal transplant model), though some groups have studied subnormothermia of 20-30◦C. NMP equipment largely consists of miniaturized cardiopulmonary bypass equipment, or extracorporeal membrane oxygenation: centrifugal or roller pumps, a membrane oxygenator, a heat exchanger, a venous reservoir, filter(s), and auxiliary interventional/monitoring/control equipment. Most commercially available kidney perfusion systems only provide hypothermia, but the Kidney Assist ® system (Organ Assist, Groningen, Netherlands) offers both hypothermic and normothermic perfusion modes; however, it remains untested in pre-clinical and clinical studies.

Several important perfusion modalities may vary in NMP similar to HMP: perfusate solution composition, pump modality, temperature, and length of NMP. Because NMP is still in its infancy as a clinical protocol, the two most published groups will be the focus of this review regarding their choice and rationale for perfusion modalities: Nicholson and Hosgood in Cambridge, UK and Selzner in Toronto, Canada. Brasile et al. also produced a significant collection of literature on subnormothermic machine perfusion of kidneys, which will be examined as well.

Perfusate Solution Composition

NMP perfusate solutions differ drastically from the ionic composition of HMP perfusate solutions because they need to mimic more closely the physiological hematological environment, which is more similar to extra-cellular fluid and requires an oxygen carrier[153]. Three types of oxygen carriers exist: artificial compounds, synthetic biological compounds, and cellular (red blood cells). Artificial compounds studied for perfusate oxygenation are mostly perfluorocarbons (PFCs); synthetic biological compounds include extracellular haemoglobins, haemoglobin polymers, and invertebrate haemoglobins; and donor or banked red blood cells offer a cellular oxygen carrier like *in vivo* blood.

In 1994, Basile et al reported the use of PFC's for normothermic perfusion of organs similar to their use in oxygenating hypothermic preservation solutions[156]. Oxygent is another PFC that has been used in oxygenated perfusion of kidneys, however its cost and the availability of better alternatives make it less likely to be used and studied further[157].

Commercial cell-free haemoglobin products use extra-cellular haemoglobin to carry and unload oxygen, though they have experienced varying degrees of success. Haemoglobin based oxygen carrier-201 (HBOC 201; Biopure, Massachusetts, United States) is a tetrameric haemoglobin based molecule developed to use as an acellular blood replacement used in blood transfusions and surgical settings, however its success in clinical settings was limited and safety concerns have prevented further use[158]. Previous studies using HBOC 201 in an *ex situ* heart perfusion system found inferior preservation of myocardial function when compared to whole blood based perfusate[159]. HBOC 201 is composed of haemoglobin that is outside of the normally protected environment of erythrocytes, which may allow oxidation of ferrous iron in haemoglobin into ferric iron – increasing concentrations of methemoglobin and free radicals[159].

A more recent study comparing HBOC 201 to red blood cell concentrate showed comparable perfusion characteristics and minimal injury in an model of normothermic *ex situ* kidney perfusion[160]. Hemo2Life® (Hemarina SA, Morlaix, France) or M101 is a 3600kDa extracellular haemoglobin product composed of 156 globin and 44 non-globin moieties extracted from a marine invertebrate; M101 has a specific oxygen carrying capacity (43umol/g) lower than human haemoglobin (62umol/g)[161,162]. Hemo2Life® has been applied in several settings such as *ex situ* machine perfusion of kidneys, where it provided improved metabolic support[161] and reduced post-implantation serum creatinine but with no reported differences in injury or histology[163]. Hemo2Life® presents a promising alternative to other oxygen carriers in normothermic perfusion, but also an additive to prevent metabolic injury in hypothermic perfusion.

A simple and physiological approach to oxygen carriers is to use red blood cells (RBCs) either from the organ donor or blood bank products. Two large groups that have studied NMP (Hosgood and Nicholson; Markus Selzner) used leukocyte-depleted RBCs from donor animals, and Hosgood and Nicholson used banked RBCs for human clinical trials. Harper et al. showed leukocyte depletion in perfusate had improved *ex situ* organ perfusion haemodynamics, acid-base homeostasis, and *ex situ* creatinine clearance with no significant findings on injury[155]. Harper et al. did not report significant differences in edema or any results on inflammatory cytokines, although it is important to note that leukocyte depletion was not performed through erythrocyte washing or filtration but rather through the installation of a leukocyte filter within the circuit. In *ex situ* lung perfusion, leukocyte filter installation has been shown to be ineffective and the inclusion or removal of the filter made no significant difference in inflammation and function[164]. There is also evidence that plasma contents may provide protective antioxidant effects within an *ex situ* perfusion setting, as demonstrated by White et al. in a porcine *ex situ* heart perfusion

model[159]. Oxidative stress is a major contributor to the pathophysiology of ischemic acute kidney injury, and plasma contains a range of antioxidant molecules[20,146,165] that may increase the overall antioxidant capacity of the perfusate during kidney NMP. Plasma contains pro-inflammatory proteins further exacerbating ischemia reperfusion injury during NMP, however plasma also contains anti-inflammatory proteins such as IL-10[166] and provides oncotic pressure needed to prevent extravasation of fluid into tissue. The effects of leukocytes and plasma in normothermic *ex situ* kidney perfusion is sparsely studied, likely because it is known that leukocytes can have deleterious effects in acute kidney injury. Another reason for the preference of a washed erythrocyte or leukocyte/plasma depleted oxygen carrier is the clinical availability, as banked blood is necessary in clinical applications of kidney NMP[152].

Aside from oxygen carriers, most groups use crystalloid solutions as a primer, which largely determines the ionic composition of the perfusate aside from any blood components. In their human trials protocol, the Cambridge group currently uses only lactated ringers in addition to banked red blood cells, with added 25ml of 10% mannitol for osmotic pressure[153]. The Toronto group uses a mix of 200ml of lactated ringers and 150ml of STEEN solution, a proprietary machine perfusion solution composed of a physiological saline, dextran, and serum albumin for oncotic pressure (XVIVO Perfusion; Goteborg, Sweden)[167]. Both groups use additional sources of bicarbonate for pH buffering, insulin and nutrient solutions for metabolic support, heparin for anticoagulation, and vasodilators (Cambridge: prostacyclin; Toronto: verapamil) [152,167]. In past experiments Hosgood and Nicholson have used nitroprusside to induce urine production, though it is no longer listed in their more recent methodology[155]. Lactated ringer's solution is used by both groups to replace evaporative and urinary losses over time.

Detailed breakdown of normothermic perfusate compositions and supplemental infusions/drugs are included in table 2.

The extent of oxygenation is also variable, though most groups use a 95% oxygen and 5% carbon dioxide mixture supplying oxygenators. Adams et al. performed a study comparing 95% oxygen to 25% and 12%, while the rest was made up of 5% carbon dioxide and inert nitrogen gas[168]. They found that 95% had the highest oxygen extraction, but lowering oxygen partial pressures did not significantly reduce renal function or affect injury metrics[168]. The evidence suggests that while higher oxygen content may increase tissue oxygenation, the hyperoxemia may increase oxidative stress while lowering oxygen content to a more physiological partial pressure still provides sufficient tissue oxygenation.

Pump Pulsatility and Pressure

In NMP, the effects of perfusion pump pulsatility on vascular endothelium and tissue perfusion are similar those in HMP, however in NMP there is an added consideration that perfusates often contain cellular components, i.e. red blood cells, that may be more susceptible to the mechanical trauma of biomedical pump. Potential choices for pumps are either the traditional roller/pulsatile pumps or "atraumatic" centrifugal pumps which are supposed to have reduced haemolysis compared to previous generations of centrifugal/non-pulsatile pumps. Most of the data on red blood cell trauma by either centrifugal or roller pumps come from studies done on cardiopulmonary bypass patients, and the clinical outcomes (haemolysis, immune activation, thrombosis, and renal injury) vary significantly from study to study in both experimental studies and database analysis [169–172]. Many of these studies are not comparable as there are a variety of biomedical pumps available, and over the years each new generation of pumps may change the

degree of cellular trauma produced. Centrifugal pumps are also likely chosen because they are readily available as a part of pediatric bypass equipment.

In non-pulsatile pumps, a single stable perfusion pressure needs to be chosen, and unlike HMP where there is minimal metabolic demand, NMP will need appropriate perfusion pressures at or near physiological set point to maintain sufficient graft perfusion. The Cambridge group uses an arterial pressure of 75mmHg for human kidneys and 50mmHg in a porcine model[155]. Toronto group uses 60-80mmHg arterial pressure, starting at 75mmHg and dropping to 65mmHg after initial rewarming and reperfusion is achieved [167,173]. Brasile et al used 50mmHg of arterial pressure in their subnormothermic/hypothermic model[154]. Most groups have opted for free venous drainage into the reservoir with no venous pressure above air pressure, while Toronto group used a cannulated venous outflow with 0-3mmHg of venous pressure[173].

Perfusion Temperature and NMP Length

Temperature varies significantly more among groups studying NMP, though technically NMP would dictate perfusion at what is normothermia for the model (i.e. 36.5◦C for humans with slight variations for animal models) many groups have studied subnormothermia. Both the Cambridge and the Toronto groups have opted for the goal of normothermia at 36◦C, however Cambridge group only reached a mean temperature of 34◦C, with a range from 31.5-36◦C; this variation likely arises from variations in flow through the heater/oxygenator whose efficacy depends on reliable flow[151,174]. In addition, even if the heater can bring the perfusate to 36◦C there would still be heat loss before the perfusate reaches the kidney. With a lower end range of 31.5◦C, the Cambridge system is nearly at the subnormothermic temperature used by Brasile et al.

Another aspect regarding temperature- is the choice to either cold flush the kidney *in situ*, or to immediately connect the organ to the perfusion system. In a perfect world there would be no need for any cold ischemia; however, in clinical graft recovery settings, kidney recovery often needs to occur along side other organ recovery procedures and extra time is needed to perform back table preparations prior to start of perfusion on an NMP system. In their pre-clinical and clinical studies, the Cambridge group used either HOC or UW solution as a cold flush prior to their first period of SCS[151] while the Toronto group used HTK flush solution prior to their first period of SCS[173].

After the flush and initial SCS, the two groups used varying lengths of NMP, and have a divergence of opinion on whether NMP can be used as a prolonged storage method or as a simple intervention between SCS periods prior to implantation. The Cambridge group used NMP for only 60 minutes just before implantation while maintaining similar CIT's to the SCS only control group, and treated NMP more as a therapeutic intervention and opportunity to assess renal function rather than a prolonged preservation method[151]. The Toronto group maintained 3 hours of SCS prior to the NMP being set up, followed by a long period of NMP, and ended with a short period of SCS prior to surgical implantation in their preclinical animal model studies[173]. Kaths et al. examined different periods of NMP and concluded that prolonged NMP (16 hours after 8 hours of SCS) is preferable to brief 1 hour NMP as it can safely postpone surgical procedures and reduce cold ischemic injury to the graft[175]. Important to note were some technical difficulties encountered in prolonged NMP, including loss of perfusate volume, return of lactate accumulation after 8 hours of NMP, and haemolysis[175]. The loss of perfusate volume arises from urinary losses, leaks in the system, and perfusate collecting in an organ chamber; both leaks and undesirable pooling of perfusate can be eliminated through improved NMP system design,

leaving urinary loss as the only unavoidable depletion of perfusate volume. Urinary loss should not be a significant burden on its own, as both groups reported small volumes (Nicholson and Hosgood producing 189 ± 119ml; Kaths et al. producing 126 ± 161ml over 16 hours). Urine production should not be a metric used to compare these two settings, as a large confounding variable is the perfusate solution osmolarity.

Kaths et al. found that lactate clearance led to a steady decrease of perfusate lactate over the first 8 hours, but after 8 hours it began to accumulate again until the end of 16hours perfusion[175]. The value of perfusate lactate as a diagnostic metric may be very limited at least in the NMP systems of Toronto and Cambridge groups, as they both use Ringer's lactate as a primer solution and in perfusate replenishment thus artificially adding lactate at variable time points[152,175]. In an NMP perfusate with no lactate, it may be possible to use lactate clearance as a metric for NMP. In prolonged NMP, haemolysis is also a concern, as the use of cellular (RBC concentrate) oxygen carrier means the red blood cells may lyse through the mechanical impact of the extracorporeal circuit or simply degrade in senescence. The gradual loss of oxygen carrying and unloading capacity may lead to metabolic demands not being met in the latter half of 16 hours. One solution may be to supplement the perfusate with banked blood during prolonged NMP, or to further optimize the duration of NMP to reap maximum benefits with minimal haemolysis.

2.3 Perfusion Metrics

In normothermic machine perfusion, there are currently no established metrics for evaluating whether a graft is acceptable for transplant, though many metrics show promise in their correlation with DGF and long-term graft function. First, Hosgood and Nicholson have proposed a scoring scheme for kidney grafts undergoing their 1-hour NMP intervention during

SCS. Secondly, several proposed molecular and biochemical markers of injury have been used in pre-clinical studies showing potential to be applied in both sustained and short duration NMP. Finally, histological evaluations pre-implantation will likely continue to serve as a metric for graft viability in the clinical setting of kidney NMP, these scoring schemes include the Remuzzi score, Maryland aggregate pathology index, the Banff criteria, and hematoxylin and eosin evaluation of acute tubular necrosis.

Hosgood and Nicholson (Cambridge group) proposed a 5-point quality assessment score (QAS) based on macroscopic appearance, renal blood flow, and urine output, with 1 point being the highest quality graft and 5 points being the lowest quality graft; the scheme is to be used in their ongoing clinical trial of NMP vs SCS [152,153]. The scoring scheme evaluates macroscopic appearance from 1 point being a graft with excellent perfusion ("global" and even pink appearance), 2 points being a graft with moderate perfusion ("some" patchy or mottled appearance), and 3 points being a graft with poor perfusion ("global" patchy or mottled appearance); 1 point is added if mean renal blood flow is below 50ml/min/100g, and 1 point is added if total urine output is below 43ml/hour[152]. The Cambridge group has performed a small scale clinical study using declined DCD kidneys, in which they reported low QAS (<3) and no DGF in 4/5 transplant grafts, while the fifth had a QAS of 3 and return to dialysis post-transplant[176]. The same study found that kidneys declined for poor *in situ* perfusion, caused by thrombosis during DCD warm ischemia, can be recovered and most successfully transplanted after NMP; grafts that suffered long CIT's and poor HMP perfusion scored highest (worst) in the QAS system and were not transplanted[176].

Having a low Cambridge QAS has shown good correlation with immediate post-operative renal function, which has provided value in preparing appropriate post-operative care in the

small-scale clinical studies performed to date, however its true predictive value may be further elucidated upon conclusion of the large multi-centre study being performed by the Cambridge group. Because its application has been limited to the specific 1-hour short duration NMP intervention, the Cambridge QAS may be of limited value to intermediate to prolonged NMP proposed by the Toronto group and others. The associative values with post-implantation performance in Cambridge QAS blood flow and urine output thresholds are affected by the perfusate composition, perfusion pressure, and other parameters used in the study[176]. Although it may not be directly compatible with intermediate and prolonged NMP, the Cambridge QAS elucidates several important pieces of information: quality of perfusion (as demonstrated by qualitative measurement of macroscopic appearance and quantitative measurement of renal blood flow) can be significantly predictive of immediate post-operative renal function and NMP can improve the perfusion of grafts that suffered poor *in situ* perfusion and therefore reduce graft discard rates.

In a pre-clinical study on a subnormothermic (32◦C) canine kidney perfusion model, Brasile et al. used nitric oxide synthase (NOS) activity as a metric for vascular health[154]. Nitrate ions are a stable byproduct of NOS activity, and it was determined that nitrate ions accumulate within the perfusate over time and when NOS activity was blocked with various inhibitors (including inhibitors specifically targeting inducible isoform of NOS) the nitrate ion accumulation is abolished and significant edema and intra-renal resistance developed[154]. Brasile et al. concluded that both constitutive and inducible forms of NOS are actively producing nitric oxide and are crucial in maintaining microvascular health in the kidney graft during machine perfusion to allow for immediate return of graft function upon reimplantation[154]. Nitric oxide

synthase activity and microvascular health may be generalized as overall quality of perfusion, which can also be reflected by sufficient high renal blood flow or low intra-renal resistance.

The Toronto group used intra-renal resistance, urine production, lactate clearance, and metabolic activity (as inferred by oxygen consumption and blood gas acid-base homeostasis) as perfusion metrics, and they used aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) for kidney injury metrics[175]. Following porcine autotransplantation post-NMP, Kaths et al used serum creatinine and potassium as metrics for renal function and TUNEL staining for kidney injury histology[175].

Pre-clinical Biomarkers: Kidney Injury

In intermediate and prolonged perfusion, there is a longer period of normothermia that may allow the precipitation of more injury and inflammatory markers which has the potential to provide relevant clinical predictions of graft viability and immediate renal function. A few of the most commonly used kidney injury markers in the setting of NMP include the kidney injury molecule-1 (KIM-1 or HAVCR1), aspartate aminotransferase (AST), and neutrophil gelatinase-associated lipocalin (NGAL). Lactate dehydrogenase (LDH) released into the serum is also a reflection of general cell injury and death, and in an isolated kidney system, it may reflect generalized kidney injury as well. The use of serum LDH as a marker of renal injury in NMP may be problematic because when a cellular perfusate is used and hemolysis is present, LDH is released from hemolyzed red blood cells at concentrations 50-150 times higher than normal serum concentrations[177]. It is important to note, that in their 2010 study Moers et al. did not find significant predictive value for primary non-function in AST and LDH among other biomarkers that have been retrospectively associated with clinical renal injury and poor graft performance[135]. The lack of association with primary non-function combined with the large unmet demand for

donor grafts means that though they may be predictive of DGF or other post-operative complications, most of these markers are able to justify outright rejection of a donor graft.

Serum and urinary kidney injury molecule-1 (KIM-1), also known as t-cell immunoglobulin and mucin domain-1 (TIM-1) and hepatitis A virus cellular receptor 1 (HAVCR-1), are the most predictive protein markers for kidney injury[178,179]. KIM-1 is a transmembrane receptor, first reported by Ichimura et al. in 1998[180], that is upregulated in renal tubules. While it is undetectable in control animals or humans, urinary and serum KIM-1 are upregulated in both animal models of AKI and human cardiac surgery patients experiencing post-operative AKI[179]. Since its discovery, KIM-1 has been accepted by the United States Food and Drug Administration and the Foundation for the National Institute of Health as a marker of renal tubular injury to be used in addition to traditional markers of AKI (serum creatinine, blood urea nitrogen, and urine albumin/protein)[178,181]. Renal tubular epithelial cells expressing KIM-1 have been found to internalize apoptotic bodies of other tubular epithelial cells, potentially participating in the tissue repair process after the original renal insult[182]. KIM-1 has also been found to reduce innate inflammatory response through upregulating p85, which is a protein in the phosphoinositide 3-kinase (PI3K) pathway that suppresses NF-kB activity[183]. KIM-1 is an effective marker of tubular kidney injury in ischemic AKI, however it is also likely a reactive repair mechanism to modulate the extent of AKI due to its phagocytotic activity[182,183].

Aspartate aminotransferase (AST) is largely associated with *in vivo* liver injury, however it is also present in renal parenchyma cells and its release into the serum and urine can be reflective of renal parenchymal injury while in an isolated kidney NMP system[135]. AST was found to show some correlation with DGF at the end of HMP[135]. No conclusive studies have emerged showing

its relevancy in NMP, however it is likely to produce a more significant difference between poorly-perfused grafts and well-perfused grafts due to increased metabolism in normothermia.

In surgical models of acute kidney injury, NGAL was found to be predictive of acute kidney injury and correlated with sustained post-operative kidney injury and loss of function[184–186]. NGAL is produced and released in the renal proximal tubules after ischemic and antigenic (pathogenic antibody induced nephritis) insult[185,187], and drastic increases in both serum (up to 300 fold increase) and urine (up to 1000 fold increase) makes it a useful early detection metric for ischemic AKI[188]. Similar to KIM-1, NGAL may be a reactive defense mechanism against AKI, as it may also be able to modulate further extension of acute kidney injury and participate in the stimulation of recovery and proliferation[188]. In a rat model, intravenous NGAL injection reduced post-ischemic tubular cell death and increase tubular cell proliferation, suggesting potential use of exogenous NGAL as a therapeutic intervention[189]. Conversely, there is also mounting *in vitro* evidence that NGAL may exert profibrotic influences and may in fact negatively impact recovery from ischemic injury for both renal and cardiovascular systems[188]. In a review of NGAL's effects on cardiovascular and renal health, Buonofine et al. concluded that NGAL participates in various cardio-renal pathophysiologies, specifically through the proliferation/death related ERK1/2 pathway and the inflammatory/fibrotic related NF-kB pathway[188].

Pre-clinical Biomarkers: Inflammatory

Although human leukocyte antigen matching and immunosuppressive therapy have significantly reduced the risk of immune rejection of a transplanted kidney graft, allograft recipients still experience increased inflammation when compared to the control population which is associated with an increase in risk of rejection and graft failure[190–192]. There are many

inflammation-related biomarkers, the three discussed here are serum interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α), and c-reactive protein (CRP). Aside from serum cytokines, ischemia-reperfusion injury can also activate innate immune response through toll-like receptors, with toll-like receptor-2 and 4 (TLR-2, TLR4) being specifically identified.

IL-6 is a cytokine produced traditionally through NF-kB activation by Pathogen-Recognition Receptors (such as toll-like receptors and NOD-like receptors) in response to pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs)[193,194]. PAMPs arise from pathogen infection of the tissue, while DAMPs can arise from sterile damage to tissue and cells as well as unregulated cell death. IL-6 is mainly a pro-inflammatory cytokine that acts on cellular receptors to produce a range of immunogenic effects such as B-cell differentiation, hematopoiesis, induction of acute phase response[193], and potentially increased vascular permeability to facilitate neutrophil infiltration[195]. IL-6 knockout mice are significantly more resistant to HgCl2 induced AKI, where neutrophils and T-lymphocytes facilitated IL-6 dependent AKI development; neutrophil depletion was found to reduce the severity of HgCl2 induced AKI[196]. The traditional IL-6 and IL-6 receptor mechanism leads to activation of various pro-inflammatory pathways. However, IL-6 membrane receptors only exist in renal podocytes and leukocytes such as monocytes and neutrophils [196,197]. Therefore, in a leukocyte depleted/reduced environment such as *ex situ* kidney perfusion, plasma IL-6 may have a renal protective effect against AKI through trans-signalling processes with soluble IL-6 receptors (sIL-6R)[194,196].

Clinically, post-operative serum IL-6 has predictive value for acute kidney injury (AKI) and overall mortality after transplants and non-transplant surgeries[192,198–200]. IL-6 has also been found to directly increase chemotaxis and increase leukocyte infiltration through increasing

vascular permeability, further participating in extension of the initial acute kidney injury[195,201,202]. Serum IL-6 strongly reflects AKI and its associated inflammatory and fibrotic actions, and it is also associated with negative post-operative outcomes, making it a strong candidate for a biomarker during NMP that may reflect the ischemic AKI experienced during recovery surgery.

Tumour necrosis factor-alpha (TNF-α) is a proinflammatory cytokine that is associated with both hyper/hypotensive renal effects depending on receptor activation *in vivo*[203]. In renal transplant patients, serum TNF-α is significantly higher in both acute and chronic rejection groups compared to stable transplant recipients; while TNF-alpha is also higher in transplant recipients compared to healthy controls[190]. Outside of transplant, serum TNF-α is significantly increased in patients experiencing acute renal failure[204]. Both serum IL-6 and TNF-α have been independently associated with all-cause mortality in renal transplant patients with a functioning graft, potentially demonstrating non-rejection/graft failure related risks due to transplant-related inflammation[192]. Molnar et al. theorized that the increase in long-term mortality was caused by two groups of factors: energetics (insulin resistance, protein energy wasting, and loss of appetite) and cardiovascular effects (atherosclerosis and oxidative stress related endothelial dysfunction)[192]. Serum IL-6 and TNF- α can be valuable metrics for initiation of inflammatory cascades and infer kidney injury during normothermic machine perfusion.

C-reactive protein (CRP) is a serum protein produced systemically in hepatocytes and local tissues such as endothelium, macrophages, and lymphocytes; CRP is an acute phase protein that reflects generation inflammation and increases up to a 1000 fold at the site of inflammation[205]. Increase in post-operative CRP expression accompanies and exacerbates proinflammatory cytokine production such as IL-6[205], it is also associated with negative cardiovascular outcomes, deterioration of graft function, and all-cause mortality in renal transplant recipients[206,207].

Because of its systemic applications and general nature as an indicator of inflammation and infection[205], CRP may not be the ideal biomarker for renal inflammatory injury within the NMP setting. Outside of renal transplantation, there is significant research into CRP's role in cardiovascular disease as an independent risk factor[208–210]. A high-sensitivity CRP test is often used to screen for cardiovascular disease risk, reflecting vascular inflammation, potential for atherosclerosis, stroke, and all-cause mortality[210]. CRP is more likely associated with general cardiovascular health instead of acute kidney injury, however it has potential to reflect a generally inflammatory state[205,211].

In ischemia-reperfusion and surgery related acute kidney injuries, necrotic cell death releases damage associated molecular patterns (DAMPs), which can activate the innate immune system normally activated by pathogenic antigens[212]. The activation of the innate immune system causes increased cytokine release through the NF-kB pathway and it has been linked to increased risks of solid organ transplant rejection[212]. Kim et al. found that TLR-2 and TLR-4 expression increased significantly in mice renal tubules, and the increase in expression was detectable within 1 to 5 days[213]. Upregulation of tissue expression of toll-like receptors and their downstream mechanisms may reflect increased activation of the innate immune system and post-operative inflammatory injuries.

Histological Metrics:

There are many different but overlapping systems for histological evaluation of graft viability, which have all demonstrated degrees of predictiveness for end-points such as immediate and long-term graft function, acute and chronic rejection, and graft survival. Two types of scoring schemes/systems prior to graft implantation exist: one is donor-based, pre-

recovery evaluation, and the other is graft-based where grafts are evaluated on histology pre-implantation.

For donor-based risk assessments, the most well known and currently used system is the Kidney Donor Profile Index (KDPI), which is based on the Kidney Donor Risk Index (KDRI) and its relative risk compared to a reference population of other kidneys recovered for transplant[214]. The KDPI was developed originally in the United States and offers a comparison of expected graft longevity against a reference population of all recovered kidneys in the United States. The donor characteristics used in KDPI include: age, height, weight, ethnicity, history of hypertension, history of diabetes, cause of death, serum creatinine, hepatitis C virus status, and DCD status; full KDPI parameters also include non-donor exclusive characteristics such as human leukocyte antigen matching status, CIT, and transplant procedure type[214]. KDPI takes into consideration the interdependence of its various factors, which means individual parameters should not be the deciding factor in a dichotomous system of rejecting or accepting a recovered kidney; although widely adopted, a significant limitation is its low predictive value (c=0.6) as stated by OPTN in their guideline[214].

KDPI is also used in Canada, however with different effectiveness observed and reported in different provinces. A DCD kidney, multi-center study in British Columbia found no improvement of the predictiveness performance when compared to simply using donor age alone[215]. When evaluating different methods of DCD donor evaluation and graft prediction for kidney transplants in Alberta, it was found that KDPI did not perform significantly better than methods with fewer (5 instead of 15) parameters[216]. An Ontario study assessing KDPI performance in DCD donors found a significant increase in predictive performance when compared against using only donor age[217]. Most likely the ability to generalize KDPI to non-US

centres depends on the use of a local reference population and assessment of its value locally prior to implementation (retro-active studies). However, the use of KDPI in any setting will need refinement through testing the individual performance of its many donor parameters. Because KDPI is based on pre-recovery parameters, it is not as relevant to evaluation of kidney grafts during NMP, though its lack of consistent predictive value does increase the necessity for more precise evaluative schemes such as the following pre-implantation histopathology scoring systems.

Pre-implantation, graft-based evaluation systems are currently based only on pathohistology, as machine perfusion characteristics still need to be incorporated after larger scale clinical studies on predictive values of various biomarkers. The US national agency Organ Procurement and Transplantation Network (OPTN) recommends preimplantation biopsies to be performed on all donor kidneys with a donor KDPI score of >85%[218]. Three of the most often used systems include: the Remuzzi score[219], Maryland aggregate pathology index[220], and the chronic Banff score[218,221].

The Remuzzi score is a histological evaluation system designed to evaluate whether marginal kidneys from a donor with age over 60 could be safely transplanted either in single or dual transplants with reasonable post-implantation function[219]. The Remuzzi score is based on glomerulosclerosis, tubular atrophy, interstitial fibrosis, and vascular narrowing each with 3 possible points; with a final score of 0-3 the kidney is deemed to have mild baseline injury and may be considered for single transplant, with a final score of 4-6 moderate injury is indicated and may be considered for double transplant, while a final score of 7-12 is considered to have severe baseline injuries and should not be transplanted at all[219]. The Remuzzi score does not offer any predictive values in the NMP setting and its clinical value is limited to the use of kidneys from

donors with over 60 years of age, however its histological criteria for macroscopic evaluation of nephron injury may be valuable in constructing a predictive score system for kidney NMP.

The Maryland Aggregate Pathology Index (MAPI) is a scoring system out of 15 based on glomerular pathology and vascular pathology; 2 points for above 15% glomerulosclerosis, 4 points for any periglomerular fibrosis, 4 points for any arteriolar hyalinosis, 3 points for any arterial scarring, and 2 points for arterial wall-to-lumen ratio greater than 0.5[220]. MAPI predicts 90% graft survival rate for scores of 0-7, 63% from 8-11, and 53% from 12 to 15[220]. MAPI offers predictive values specifically based on graft survival and its components may be useful for development of a system for NMP evaluation, however one component, the arterial wall-to-lumen ratio, is difficult to measure and perform with limited success in implementation[220,221].

Finally, the Banff Classification of Renal Allograft Pathology is a diagnostic framework created by a consensus working group. The Banff system includes a preimplantation biopsy score, as well as post-implantation biopsy score systems that may offer useful parameters for the development of a kidney NMP histological evaluation system[218,222]. The Banff preimplantation criteria evaluate grafts semi-quantitatively based on glomerular pathology, tubular atrophy and necrosis, interstitial fibrosis and inflammation, and vascular pathology (intimal fibrosis, arteriolar hyalinosis)[218]. The system does not offer independent predictive values that lead to conclusive decisions on accepting or rejecting a graft, however it does point to strong correlations between diagnostic parameters and post-implantation outcomes. The most common limitation among all of the above graft evaluation systems is a strong sample selection bias towards successfully transplanted grafts/clinically acceptable donor grafts, therefore offering limited clinical predictive power[218,221].

Functional Metrics: GFR and Clearance

Aside from the previously described metrics, a more traditional method of evaluating graft function is estimated glomerular filtration rate, or eGFR. A limitation is that eGFR based on serum creatinine (or inulin) requires a stable serum concentration of creatinine that is difficult to achieve in an NMP system; eGFR also comes with various patient modifiers that are not directly translatable in an animal model or in an isolated kidney perfusion system. A rough estimate of creatine (or inulin) clearance may be used to reflect GFR, as the perfusionist may add a known amount of creatinine, collect serum samples, and quantify its presence over time. The equation used to estimate GFR based on collected samples is: urinary creatine * urine flow / perfusate serum creatinine[155,223]. Another limitation with using GFR as a metric is that urine flow may be drastically changed by variations in perfusion methodology (as seen with several current NMP research groups) such as the use of diuretics and variations in perfusate osmotic/oncotic pressures due to composition. However, GFR and serum creatinine are good metrics in a transplant model for evaluating post-operative function of an implanted graft, along with other accepted clinical metrics such as blood urea nitrogen, urinary protein, and glucose presence.

2.4 Perfusion Interventions

Because of its relatively novel nature, there are currently no clinically applied therapeutic interventions for kidney NMP. Due to the isolated nature of a kidney NMP system, the application of interventions can be more concentrated and targeted than what is otherwise safe or possible within the *in vivo* system. Proposed interventions largely fall into three categories: cell therapy, gene therapy, or pharmaceutical therapy. Some proposed therapies have been tested in animal models of kidney NMP and transplants, while others have been tested in non-kidney models of NMP but may have application in kidney NMP. Another interpretation of NMP, is that

the perfusion itself can be considered as an intervention, and therefore different parameters of the perfusion can be considered treatments[224–226].

Cell therapy in the setting of kidney NMP is focused on the use of mesenchymal stem cells (MSC's) for its immunomodulatory effects as well as its tissue repair effects[225]. MSC's are non-hematopoietic, multipotent progenitor cells found within the bone marrow that can assist in tissue repair and proliferation, but also modulate immune response within the host by converting immune cells into regulatory cells[227]. In a rat model of DCD kidney transplantation, the donor kidneys were subjected to 60 minutes of subnormothermic NMP during which MSC's were injected; MSC treated grafts had significantly increased post-operative survival and function[228]. Iwai et al. also showed that a LacZ transgene expressed in the injected MSC's migrated into the injured renal tubules and were no longer visible after 24 hours, which demonstrates that the beneficial effects of MSC therapy does not depend upon persistence of multipotent MSCs[228]. Several studies have supported the immunomodulatory effects of MSC's in animal models, yet other studies have found MSC's to take on a pro-inflammatory phenotype and worsen graft survival[227]. Casiraghi et al. found that the localization of MSC's and the timing of its application (with pre-transplant preferable over post-transplant) surrounding the transplant and associated ischemia reperfusion injury determines its effects – either taking on a pro-inflammatory phenotype or modulating immune activation[229]. Although Casiraghi et al. found improved immunomodulatory effects of MSC therapy when applied pre-transplant, its effects are reliant upon *in vivo* localization within the donor body and interactions with non-renal tissues, therefore its applicability to an isolated kidney NMP setting must be re-evaluated[229]. There are still concerns about potential for tumour formation, risk of embolism, and increased thrombosis with

systemic injection of MSCs[230,231], which can be ameliorated if used in the *ex situ* setting of kidney NMP.

Gene therapy is another promising category of therapeutic interventions applicable during kidney NMP. A significant challenge facing renal gene therapy was lower rate of transfection efficiency, however in an *ex situ* system, the isolated kidney may be perfused with vector media for longer periods of time[226,232]. For example, Alport syndrome is a genetic disease that has no specific treatment currently, however as explored in the hypothermic review in chapter 2, the application of gene therapy in *ex situ* kidney perfusion has shown promising developments in pre-clinical studies. Another application of gene therapy is the use of small interference RNA (siRNA), which interferes with the expression of pro-inflammatory moieties or other molecules that may be deleterious to the graft during and after NMP[226]. siRNA was used in a recent study on liver HMP and NMP to silence the genes of pro-apoptotic and pro-inflammatory NF-kB pathway; importantly, the study found effective uptake and gene silencing activities in both HMP and NMP[233]. Gene therapy has been explored in *ex situ* organ perfusion settings for more established *ex situ* organ perfusion systems in lungs and livers to various degrees of success, while gene therapy in *ex situ* kidney perfusion has not advanced to the same degree due to the current clinical standard being hypothermic preservation[99].

Pharmaceutical therapy within the setting of NMP is similar to its application in HMP discussed in chapter 2 (such as thrombalexin), with the exception being improved circulation and potential uptake due to hemodynamics during perfusion, and the inability to recirculate the urine means it is necessary to take into consideration the breakdown and excretion of the agent in NMP.

Another perspective on the potential beneficial effects of NMP on transplant graft quality, is a so called "re-conditioning" effect, whereby the period of *ex situ* normothermic perfusion simulates the *in vivo* environment and prepares the kidney for reperfusion in the recipient[223]. "Controlled oxygenated rewarming" (COR) is the gradual increase of temperature and pressure during oxygenated NMP within animal models of kidney NMP[223]. Von Horn et al. found COR treatments led to improved post-implantation aerobic metabolism and renal function[223]. Similar methods of controlled rewarming were applied by Mahboub et al., whose group found reduced renal injury markers, heat shock protein, and improved metabolic activity during rat kidney NMP[234]. An immunological perspective of graft conditioning centres around the saturation of immunological response (i.e. proinflammatory cytokines, leukocyte mobilization) during *ex situ* perfusion of the graft, and the removal of these humoral factors upon flush-out prior to implantation[224]. Graft preservation/conditioning in a NMP system may be considered an intervention in itself due to the various parameters that may be manipulated to improve transplant outcome. Overall NMP provides a stable and isolated system that mimics *in vivo* environments, allowing novel therapeutic opportunities pre-implantation such as cell, gene, and pharmaceutical therapies.

2.5 Future Direction:

Ex situ Normothermic Machine Perfusion of kidney grafts provides a uniquely isolated environment for preservation/treatment of kidney grafts pre-implantation. However, the technique's clinical application is still limited. To improve the effectiveness and applicability of kidney NMP, large scale clinical studies are needed to validate its effectiveness against current hypothermic gold standards and to elucidate the predictive value of various metrics applied during NMP. Once there is more widespread clinical application of kidney NMP, different

interventions such as cell and gene therapy can be tested in clinical trials to validate their effects found in animal studies.

NMP will continue to face obstacles in its technical application when compared against past hypothermic methods, these include more complicated machinery, greater metabolic demand and risk of warm ischemic injury, and greater demand on perfusion machine operators. These obstacles may be overcome through both automation engineering and validation of a consolidated set of perfusion parameters in standardized protocols (perfusate composition, perfusion pressure, supplemental infusions). NMP has already demonstrated significant clinical advantage compared to hypothermic methods in small scale studies in the past decade, and there is still potential for significant improvements.

3) Goals and Hypotheses:

It is necessary to select one of the two temperature modes for this project studying kidney machine perfusion, therefore the first goal was to establish a metabolically intact machine perfusion system that allows investigation of evaluation metrics. The hypothesis is that kidney normothermic machine perfusion offers an intact metabolic state and improved tissue perfusion.

Because there is no current consensus on optimal perfusate, it is necessary to optimize components of the perfusate. One component is the use of an oncotic agent, where the traditional agent was albumin but dextran-40 is a colloid alternative that may have anti-inflammatory effects. The hypothesis is that in kidney normothermic machine perfusion, dextran-40 has an anti-inflammatory effect when replacing bovine serum albumin as the oncotic agent.

Finally, kidney normothermic machine perfusion requires an oxygen carrier to provide effective oxygen carrying and unloading in the metabolically active tissue. A commonly used

oxygen carrier is red blood cell concentrate, however there is evidence that whole blood may have protective effects in an *ex situ* perfusion setting. The hypothesis is that during kidney normothermic machine perfusion, whole blood perfusates provide improved protection of the kidney compared to red blood cell concentrates.

3) Aims:

3.1 Hypothermic Machine Perfusion (HMP) and Normothermic Machine Perfusion (NMP):

To study *ex situ* kidney preservation, HMP and NMP protocols were established. A porcine model was used for both HMP and NMP because of its anatomical similarity to humans, and because it is a pre-established pre-clinical model for research into kidney transplantation and preservation[152,173]. The goal of this study is to investigate the differences in perfusion hemodynamics and biochemistry when a kidney is perfused in NMP or HMP. During hypothermic machine perfusion, intra-renal resistance (inverse of renal blood flow at a set arterial pressure) has often been used as a metric for quality of tissue perfusion and it has been associated with worse immediate post-operative function and 1-year graft survival[136]. An important role of the kidneys within normal physiology is to regulate the composition of blood through urinary excretion, which often depends upon active transport systems within the renal tubules. Due to the differences in perfusates used in HMP and NMP systems, ionic compositions will not be quantitatively comparable, but may still bring qualitative insight. Severe hypothermia within HMP (4◦-8◦ Celsius) significantly reduces metabolic activity and often leads to metabolic depletion, which will likely stop or reduce active transport processes necessary to regulate excretion and reabsorption of ions from the urine[235]. In similar fashion, the upregulation of inflammatory moieties is also likely prevented during HMP, making comparisons of serum markers incomparable to an NMP system which maintains active metabolism.

3.2 Normothermic Machine Perfusion: Oncotic Agents

Unlike traditional hypothermic methods, Normothermic Machine Perfusion (NMP) maintains the cellular metabolism at normothermia by pumping an oxygenated physiological perfusate, thus removing the need for extended periods of cold ischemia[16,153]. Kidney NMP perfusates are mainly composed of an oxygen carrier and a crystalloid solution with an added source of oncotic pressure, such as albumin, to reduce the potential for tissue edema. Dextran-40 is a volume expander, an anti-thrombotic, and a component in some extra-corporeal circulation perfusates, however it also provides oncotic pressure and could be used to replace albumin in normothermic organ perfusion. This study investigates the effectiveness of dextran-40 as a replacement for albumin in Kidney NMP through various metrics for inflammation, renal injury, and perfusion dynamics.

Albumin is a globular protein approximately 66kDa in size that is synthesized by the liver, and it is the most common protein within blood accounting for approximately 50-60% of human plasma protein and 80-85% of the plasma osmotic or oncotic pressure[236]. Albumin has been considered as a treatment for hypovolemic patients, but aside from limited evidence of improvement in sepsis, albumin showed no improvement over crystalloids[237]. Albumin is, however, used as an oncotic agent alongside dextran-40 in *ex situ* organ perfusion solutions such as the STEEN Solution® (XVIVO Perfusion, Goteborg, Sweden)[152,159,238]. Bovine serum albumin was previously used in porcine models of *ex situ* lung perfusion[239].

Dextran is a complex branched glucose polymer produced naturally by microorganisms but can be produced synthetically by a non-pathogenic organism *Leuconostoc mesenteroides*[240]. Dextran has been used as a volume expander dating back to the early 20th century, when it was first used as a non-antigenic, easily cleared, and hyperoncotic solution able to prevent

hypovolemic shock in patients[241]. Since its early adoption as an antithrombotic agent during surgery[242], the use of dextran has been significantly reduced due to its side effects such as renal dysfunction and excess bleeding due to coagulopathy[236]. Dextran is commercially available in 40, 60, and 70 kDa, and continues to see limited use as an oncotic and antithrombotic agent[240,242]. Dextran-40 has been used in settings similar to kidney NMP as both a protective agent and an oncotic agent, therefore it is likely that dextran-40 may be used to replace bovine serum albumin in a porcine model of kidney normothermic machine perfusion. Due to its anti-inflammatory properties, dextran-40 may reduce inflammatory biomarkers during kidney perfusion and provide comparable preservation.

Metrics based on perfusion dynamics can reflect the function and preservation of machine perfused grafts, including intra-renal resistance (or the inverse of renal blood flow at controlled temperature and pressure) and perfusate biochemistry. Intra-renal resistance or renal blood flow have been used as a metric for quality of machine perfusion and delivery of oxygen/nutrients to the renal tissue in NMP[151,175] and have been included in the Quality Assessment Score for NMP kidney evaluation developed in Cambridge[152]. Blood gas analysis of NMP perfusate may reveal many biochemical metrics reflecting the overall function of the graft; these metrics include perfusate pH, bicarbonate, and lactate. In normal physiology, kidneys are responsible for the excretion of acids and production/reabsorption of bicarbonate in response to blood pH[243]. Lack of lactate clearance may indicate reduced renal function, though many NMP systems use exogenous lactated ringers as fluid replacement and may reduce the effectiveness of perfusate lactate as a marker.

Markers of inflammatory cytokines IL-6 and TNF-α are associated with worsened long term post-operative graft function and delayed graft function[190,192]. Due to the normothermic and

metabolically active nature of NMP, it is possible to evaluate these inflammatory markers prior to transplantation, and they can be used to evaluate effectiveness of different perfusate compositions. Increased serum concentrations of IL-10 and TNF-α have been found to be positively associated with acute kidney injury clinically, in non-transplant post-operative settings[199,204] and renal transplant recipients[190,200]. Graft rejection has been associated with increased serum concentrations of TNF-α, which may also increase vascular permeability to allow leukocyte infiltration into the tissue[190]. Tissue expression of TLR-4 is also used as a metric of the activation of the innate immune system[213].

This study will also investigate serum concentrations of kidney injury molecule-1 (KIM-1), which may indicate early kidney injury within hours of organ retrieval in the setting of normothermic machine perfusion system[179]. Because KIM-1 expression is first upregulated in the renal epithelium prior to release into urine and blood, tissue expression levels of KIM-1 may also reveal development of acute tubular injury[178].

3.3 Normothermic Machine Perfusion: Oxygen Carriers

Normothermic machine perfusion (NMP) provides *ex situ* oxygenation and tissue perfusion of the donor graft at normothermia, thus maintaining an active metabolism and offers the possibility of evaluation and intervention pre-implantation. Due to the active metabolic state, kidneys in NMP require more efficient delivery of oxygen to the tissue, which can be met by the addition of oxygen carriers in the perfusate and an oxygenator in the NMP circuit. Many different oxygen carriers have been proposed[156,158,162], however the most commonly used oxygen carrier is still washed or filtered erythrocyte[152,173]. The rationale behind the removal of other blood components is the detrimental effects of leukocytes and pro-inflammatory cytokines within the plasma[155,190,192]. Leukocytes have been implicated in extending the acute kidney injury

through stimulating production of inflammatory cytokines, killing of tubular epithelial cells, secretion of pro-fibrotic factors, and other pathophysiologies[244,245]. Although uncommon in kidney NMP, the use of donor whole blood in *ex situ* perfusion of other organs has shown improvements in preservation over red blood cells alone[159,246,247].

This study aims to investigate the effects of using donor whole blood compared to washed donor erythrocytes in kidney normothermic machine perfusion. The hemodynamic and biochemistry during perfusion will be used as a reflection of both quality of graft perfusion and graft function. Perfusate inflammatory cytokines, kidney injury biomarkers, and post-perfusion histological evaluation will be used to evaluate graft preservation.

Chapter 3: Methods

Animal Ethics:

Animals used in the study of *ex vivo* organ perfusion experiments were cared for humanely and the use of the porcine animal model was approved by the Institutional Animal Care and Use Committees. Heart, Lung, Limb, Kidney, and blood samples were recovered for research. University of Alberta Animal Use Protocol #00000943.

Porcine kidney recovery:

Female adolescent Yorkshire-Landrace pigs of approximately 2 months age were selected between weights of 35-50kg. The animals were anaesthetized with ketamine and maintained through 1-3% isoflurane, with orotracheal intubation and mechanical ventilation support. Lactated ringer was administered through a central venous cannula. Heparin was administered at 1000units/kg. Midline dissection was made down the abdomen exposing renal artery and both kidneys. One kidney was selected for machine perfusion (preference given to left kidney for easy of isolation of the hilum) and the other for *in vivo* biopsy sample. Renal artery, vein, and the ureter were isolated and were severed upon euthanasia, with an extra cuff of the aorta taken with the renal artery for ease of cannulation. In the even of abnormalities in vasculature (e.g.: multiple renal arteries coming directly from the hilum) the contralateral kidney was selected for machine perfusion instead. *In vivo* urine samples were obtained directly from the bladder and *in vivo* blood samples were obtained from the central venous cannula.

In some experiments static cold storage (SCS) controls were used to obtain histological control samples. Contralateral kidneys were collected for control samples and were either immediately taken for samples (*in vivo*) after recovery or assigned to the static cold storage

(SCS) control. Static cold storage control kidneys were flushed with a 0-4◦ Celsius modified University of Wisconsin (UW) solution (analogous to commercially available Kidney Perfusion Solution-1 or KPS-1®; Organ Recovery Systems, Illinois, United States) pushed by gravity at 100cmH_2O, after flush solution exiting the kidney was clear and the kidney was no longer patchy in appearance the kidney is stored in cold modified UW solution for 12 hours. Kidneys procured for HMP were also flushed with the modified UW solution prior to perfusion.

Kidney Hypothermic Machine Perfusion (HMP) System setup and operation:

A diagram of the system is included in Figure 1. The Kidney HMP system was comprised of a kidney holder draining into a venous reservoir and filter. The perfusate was pumped through a non-occlusive centrifugal pump (Biomedicus 540, Medtronic, Minnesota, United States) into a heat exchanger that drew heat from the perfusate. The heat exchanger cooled the perfusate to a temperature of 4◦-8◦ Celsius. The perfusate was then pumped into the renal artery through an arterial cannula. Pressure was monitored at the level of the arterial cannula and controlled by the computer with variable pump RPM; temperature was monitored through a needle probe inserted into the renal parenchyma and at the heat exchanger. Sampling ports connected from the arterial side of the heat exchanger and drained back into the venous reservoir. The cannulated ureter drained into a sample tube and was collected every hour.

The system was primed with 600ml of a modified University of Wisconsin solution (analogous to Kidney Perfusion Solution-1or KPS-1®, Organ Recovery Systems, Illinois, United States), and cooled in the circuit prior to the start of perfusion. Upon connection of the renal artery to the arterial cannula, perfusion began at ~30mmHg increasing 5mmHg per 5 minutes until a stable perfusion pressure of 60mmHg. Within the first hour of HMP stable graft

temperature and pressure were reached. HMP system was operated for 12 hours with bi-hourly perfusate sample collection.

Normothermic Machine Perfusion system setup and priming:

A diagram of the system is included in Figure 2. The normothermic machine perfusion system used adapted neonatal bypass equipment and consisted mainly of a venous reservoir, centrifugal pump, oxygenator and heat exchanger, and various tubing. A filtered venous reservoir collected venous return from the kidney holder, where the kidney was placed on a silicone holder allowing free venous drainage. The perfusate flowed from the venous reservoir to the computer controlled Biomedicus® centrifugal pump (Medtronic, Minnesota, United States), which then pumped the perfusate through the Quadrox-i® neonatal oxygenator and heat exchanger (Maquet, Rastatt, Germany) and into the arterial line.

A computer system controlled the pressure through manipulating the revolutions per minute of the pump. Pressure was monitored by a pressure sensor module connected to the renal arterial line near the cannula. Flow was monitored by a TS410 tubing flow module (Transonic, New York, United States) attached to the renal arterial line nearby the connection to the pressure sensor. Temperature was monitored through a needle probe inserted into the renal parenchyma and at the heater. The computer received information on arterial flow and pressure and graft temperature. Temperature was set at 30◦ Celsius prior to mounting of the graft. Gas supplied to the oxygenator and heat exchanger unit was mixed with approximately 500ml/min of 30% oxygen and 70% medical air and 50ml/min of carbon dioxide (adjusted to reach physiologic partial pressures of oxygen and carbon dioxide). A sample port line runs from arterial side of the oxygenator and heater back into the venous reservoir; infusions enter after the sample port.

Normothermic Machine Perfusion Perfusate Composition: Dextran vs Albumin

The circuit was primed with 300ml of a modified Krebs-Henseleit buffer added with 8% w/v bovine serum albumin (albumin group) or 8% w/v dextran-40 (dextran group). After blood collection during surgery, 300ml of donor whole blood was added to the circuit.

Normothermic Machine Perfusion Perfusate Composition: Whole blood vs Red blood cell concentrate

In the experiment on oxygen carriers, the circuit was primed with 300ml of a modified Krebs-Henseleit buffer added with 8% w/v bovine serum albumin. After blood collection during surgery, either 300ml of donor whole blood (whole blood group) or 150ml of saline washed donor erythrocytes (RBC group) was added to the circuit. The volume of the buffer was adjusted to 450ml for red blood cell concentrates because only 150ml of red blood cell concentrates was used (instead of 300ml of whole blood) to maintain similar final haemoglobin concentrations and total perfusate volume.

Kidney Normothermic Machine Perfusion:

After graft recovery, the kidney was placed onto the holder and the renal artery was cannulated to begin perfusion. Initial temperature was set at 30◦ Celsius and initial pressure was set at 30mmHg. Upon start of perfusion, temperature at the water heater was increased to 38.5◦ Celsius immediately to allow time to warm up, and the kidney graft temperature arrived at 36-37◦ Celsius within the first hour of perfusion. Renal arterial pressure was increased approximately 5mmHg per every 10 minutes over the first hour and reached 60mmHg at the end of the first hour. The ureter was cannulated and drained into a collection tube. Glucose was infused at a variable rate to maintain approximately 5mmol/L; insulin was infused at 2IU/hour; priming Krebs-Henseleit buffer was infused at variable rate to maintain reservoir volume.

Oxygen, carbon dioxide, and medical air were mixed to provide initial perfusate blood gas of 7.3-7.5pH and pO2 of 100-150mmHg and pCO2 of 20-40mmHg.

Perfusion Dynamics: Intra-Renal Resistance (IRR):

Perfusion dynamics are measured by Intra-Renal Resistance (IRR), which is calculated by the equation: IRR (mmHg/ml*min) = perfusion pressure (mmHg) / renal arterial blood flow (ml/min); IRR can be represented by peripheral resistance units (PRU). Perfusion pressure is measured by a pressure sensor connected to the renal arterial line via a Luer lock T-connector close to the renal artery and perfusion flow rate is measured by the flow sensor module on the arterial line tubing.

Perfusate and Urine Analysis:

Perfusate samples were collected hourly for blood gas analysis with an automated blood gas analyzer machine ABL800 Flex® (Radiometer, Brønshøj, Denmark), and bihourly for serum samples. Urine samples were collected and analyzed bihourly. Serum and urinary samples were analyzed for biomarkers and cytokines using enzyme-linked immunosorbent assays (ELISA) kits according to the manufacturer protocols. Interleukin 6 (IL-6), interleukin 10 (IL-10), and tumour necrosis factor α (TNF-α) ELISA assays were performed with DuoSet® ELISA kits (R&D Systems, Biotechne; Minneapolis, USA); Kidney Injury Molecule-1 ELISA assays were performed with ELISA kits from MyBioSource (San Diego, USA). Colorimetric quantifications of samples were performed using Synergy™ H4 Hybrid Microplate reader (Biotek®; Agilent Technologies, Santa Clara, USA) using settings recommended in individual ELISA kits.

Tissue Western blot analysis:

Tissue samples collected prior to perfusion (contralateral from the perfused kidney) and collected from the NMP perfused kidney at the end of perfusion were initially flash frozen with liquid nitrogen. Mechanical protein homogenization methods were used to obtain tissue lysate

samples to be used in Western blot antibody analysis for tissue expression of toll-like receptor-4 and kidney injury molecule-1. The gels were run at 75 volts until the dye has nearly reached the bottom of the gel (10-20%) left and transferred onto a polyvinylidene fluoride membrane (Millipore; Massachusetts, USA) for detection and secondary antibodies. For Western blot detection of toll-like receptor-4, mono-clonal primary antibodies (mouse anti-pig) were used at 1:1000 dilution (Santa Cruz Biotechnologies; SC-293072) and visualized using horseradish peroxidase-conjugated secondary horse anti-mouse antibodies (Cell Signaling Technology; CST-7076s) at 1:2000 dilution. For Western blot detection of kidney injury molecule-1, poly-clonal primary antibodies (rabbit anti-pig) were used (MyBiosource; MBS 2032350) at 1:1000 dilution and visualized using HRP-conjugated secondary goat anti-rabbit antibodies (Santa Cruz Biotechnologies; SC-2054) at 1:2000 dilution.

To visualize the bands, the Western blots were captured using Carestream 4000 mm Pro imaging station (Carestream Health ©; New Haven, USA). Bands representing target molecules were digitally quantified using standard ImageJ software (National Institute of Health public domain software) under default settings, and individual bands were normalized to the corresponding control, β-actin bands on the same blots.

Histology Scoring:

Tissue samples were collected and preserved in 10% neutral buffered paraformaldehyde at the end of perfusion for both groups, and control samples of the contralateral kidney were collected and preserved either immediately after recovery or after 12 hours of cold static preservation using a UW solution. Preserved tissues were embedded in paraffin then slides were prepared with a Periodic-Acid Schiff stain. A renal pathologist evaluated the slides for acute

tubular necrosis, brush border loss, vacuolization, positive stained droplets, cellular cast formation, interstitial edema, and interstitial inflammation.

Acute tubular necrosis was identified by dislocation and shedding of tubular epithelial cells or nuclear pyknosis or drop-out. Brush border loss was identified by the flattening of the apical tubular epithelial border and the apparent enlargement of the tubular lumen. Vacuolization was identified by the presence of clear vacuoles within tubular cells. Positively stained droplets within the tubular lumen were visible at 200x. Cellular cast formation appeared as groups of dislocated epithelial cells within the tubular lumen. Interstitial edema was identified by expanded interstitial spaces. Interstitial inflammation was identified as infiltration of leukocytes and any lesions. Within each sample the score was an average of individual scores from 10 representative 20x magnification fields; each field was scored as a percentage of total number of renal structures observed with the target histological feature.

Statistics:

All statistical analyses were performed using the IBM SPSS® software (IBM, New York, United States). Student's t-test or independent samples t-test was performed for comparing means of continuous variables between groups. Two-way repeated measures ANOVA was performed (with Fisher's Least Significant Difference post-test) for comparing differences within groups over time. Mann-Whitney U test was performed for percentage score rankings of histological scores. Statistical significance was indicated for $p<0.05$.

Chapter 4: Experimental Results

1) Hypothermic Machine Perfusion and Normothermic Machine Perfusion:

Perfusion Dynamics and Characteristics

During perfusion, NMP perfused kidneys experienced a significant drop in intra-renal resistance (IRR) within the first two hours, then maintained a steady IRR of 0.5-0.7 mmHg/ml*min (peripheral resistance units, PRU's). IRR of both HMP and NMP groups over 11 hours of perfusion are shown in Figure 3 as means $\pm$ standard error of means in PRU's. However, HMP perfused kidneys did not experience the same decrease, but rather a slight increase after the first two hours and significantly higher IRR (2.5-3.9 PRU's) over the course of the perfusion with large variance between trials. Prior to the start of perfusion, cold ischemia times during graft procurement may have varied from trial to trial, though most were mounted and perfused within 30 minutes after exsanguination.

Perfusate and Urine Composition:

Perfusate and urine compositions of samples from HMP, NMP, and *in vivo* blood are shown in table 3 as means $\pm$ standard error of means; the values shown for pH, potassium, sodium, calcium, chloride, osmolarity, and glucose are averaged values over 12 hours of perfusion. Perfusate compositions of *in vivo* blood and NMP were insignificantly different in for most parameters except for sodium and calcium, while perfusate compositions of HMP were significantly different from *in vivo* blood in all parameters except osmolarity. *In vivo* and NMP urine samples both had similarly low pH, low osmolarity, and low glucose. Compared to *in vivo*, urine produced during HMP had higher potassium and similar sodium and calcium, it had significantly higher pH (7.22 vs 6.94 in NMP and 6.42 *in vivo*; as determined by student t-test, $p<0.05$ for both comparisons), higher osmolarity, and significantly higher glucose (8.8mmol vs

1.99mmol in NMP and 0.95 mmol *in vivo*; as determined by student t-test, $p<0.05$ for both comparisons). The urine produced during HMP was not significantly different in all observed parameters when compared against HMP perfusate.

2) Normothermic Machine Perfusion: Oncotic Agent

Perfusion Dynamics and Characteristics

Both albumin and dextran-based perfusates were successfully perfused in the NMP system for 12 hours at 38.5◦ Celsius and 60mmHg arterial pressure and demonstrated stable renal blood flow over the duration of perfusion. Intra-renal resistance (IRR) of albumin and dextran-based perfusate groups are presented in Figure 4 as means $\pm$ standard error of means, with units of $mmHg*min*ml^{-1}$ or PRU. Within first hour of perfusion, the kidneys had a larger variance in their mean IRR due to individual variability, reaching mean IRR's of 0.75 PRU $\pm$ 0.19 PRU for albumin group and 0.79 PRU $\pm$ 0.40 PRU for the dextran group. However, in the second hour of perfusion both groups experienced stable perfusion, reaching mean IRR's of 0.29 $\pm$ 0.07 PRU for the albumin group and 0.32 $\pm$ 0.06 PRU for the dextran group. Perfusion IRR did not differ significantly between the two groups. Within the first hour of NMP, all kidneys had a uniformly pink and perfused appearance without any patchy dark spots.

Initial venous return out of all kidneys were dark and blood gas analysis showed high lactate, potassium, and low pH. After the first hour of perfusion, venous return became much more physiological, with slightly elevated lactate, potassium, and reduced oxygen saturation, glucose, and pH.

Inflammatory Cytokines: TNF-α and IL-6

Serum inflammatory cytokines tumour necrosis factor- α (TNF-α) and interleukin-6 (IL-6) were quantified in serum samples of albumin and dextran-based perfusate over different time points of the kidney NMP perfusions, and mean concentrations $\pm$ standard error of mean are

presented in pg/ml in Figures 5 and 6 respectively for each cytokine. Both perfusate groups had undetected serum concentrations of TNF-α in the *in vivo* blood sample and perfusate samples from the first two hours of perfusion, however serum TNF-α, increased to 978pg/ml for albumin-based perfusate group and 793pg/ml for dextran-based perfusate group. Towards end of the perfusion, serum concentrations of TNF-α became undetectable for dextran-based perfusates while serum concentrations of TNF-α remained at 547pg/ml for albumin-based perfusates. Serum concentrations of TNF-α was significantly higher in albumin-based perfusate at the 9^{th} (T9) and 11^{th} hour (T11) (student's t-test, $p<0.01$).

For both albumin and dextran-based perfusate groups, serum concentrations of IL-6 were undetectable in *in vivo* blood samples and perfusate samples of the first two hours of perfusion, but from the third hourly perfusate sample, serum IL-6 continued to increase in concentration over time for both groups (repeated measures ANOVA; $p<0.05$). From the third hour (T3) serum IL-6 reached 306pg/ml for the albumin group and 318pg/ml for the dextran group, increasing to 5237pg/ml for the albumin group and 2452pg/ml for the dextran group at the 11^{th} hour (T11). Serum concentrations of IL-6 is significantly higher in albumin-based perfusate when compared to dextran-based perfusate at the 9^{th} (T9) and 11^{th} (T11) hours (student's t-test, $p<0.05$).

Kidney Injury Marker: Kidney Injury Molecule-1

The serum concentrations of kidney injury molecule-1 (KIM-1) was quantified in serum samples of albumin and dextran-based perfusate groups over different time points during perfusion. Serum concentrations are presented as means $\pm$ standard errors of means in pg/ml in Figure 7. Serum concentrations of KIM-1 were not significantly different between the two groups.

Tissue Expression: TLR-4 and KIM-1:

Tissue expression of toll-like receptor-4 (TLR-4) and kidney injury molecule-1 (KIM-1) were quantified through Western blot performed on protein extract samples collected from the NMP-perfused kidneys at the end of perfusion. Mean tissue expressions of TLR-4 and KIM-1 are presented in Figure 8 and 9 as relative optical density comparing albumin and dextran-based perfusate groups. Tissue expression of TLR-4 was found to be significantly increased in albumin-based perfusate group when compared against dextran-based perfusate group (as determined by student's t-test, $p<0.01$). While tissue expression of KIM-1 was found to be insignificantly different between the two perfusate groups, both groups showed significantly lower tissue expressions of KIM-1 compared to tissue samples collected from contralateral kidney prior to kidney NMP (as determined by student's t-test, $p<0.01$).

Histology:

Histological evaluation of the tissue was performed using metrics reflecting acute tubular injury. In Figure 10 the scores of the following metrics were shown as average percentage scores $\pm$ standard error of means: acute tubular necrosis, tubular epithelial brush border loss, and tubular epithelial vacuolization. All samples had negligible presence of abnormal or loss of glomeruli or other glomerular pathologies. All samples had negligible presence of the following features: positively stained droplets, cellular cast formations, interstitial edema, and interstitial inflammation. No significant differences were found in most categories between the dextran group and the albumin group. The scores for vacuolization of both experimental groups were significantly higher when compared against samples collected prior to perfusion, with no significance between the dextran and the albumin groups. The scores for brush border loss were not significantly different between the dextran group and the albumin group, though dextran

showed a trend of higher scores compared to albumin (with p=0.61, approaching statistical significance).

3) Normothermic Machine Perfusion: Oxygen Carrier

Perfusion Dynamics and Characteristics

The perfusion intra-renal resistance (IRR) of both the whole blood group (WB) and red blood cell concentrate group (RBC) are presented in Figure 11 as means + standard errors of means, with units of mmHg*min*ml^{-1} or peripheral resistance units (PRU). Both groups experienced a rapid decline in IRR within the first hour of perfusion. After which they both experienced stable perfusion IRR (0.05-0.15 PRU) and had uniformly pink macroscopic appearance. There was no statistical significance between the IRR's of the two groups.

Perfusion parameters were stable, though both groups experienced moderate perfusate alkalosis and free water loss which developed over the twelve hours, with no statistical significance between groups. At the start of perfusion, the RBC group had a perfusate pH of 7.31$\pm$0.16 and the WB group had a perfusate pH of 7.47$\pm$0.08, at the end of perfusion the RBC group had a perfusate pH of 7.62$\pm$0.04 and the WB group had a perfusate pH of 7.59$\pm$0.04. The slight alkalosis was reflected by a steady increase of bicarbonate concentrations within the perfusate: at the start of perfusion, perfusate bicarbonate concentrations were 12.08$\pm$2.74mmol/L for the RBC group and 16.25$\pm$5.42mmol/L for the WB group, and at the end of perfusion, perfusate bicarbonate concentrations were 49.8+3.87mmol/L for the RBC group and 40.68+1.85mmol/L for the WB group. The free water loss found in perfusates is reflected by the significantly increased osmolarity at the end of perfusion (340.8$\pm$3.1 mOsm/kg H_2O for the WB group and 359.05$\pm$14.1 mOsm/kg H_2O) compared to the beginning of perfusion (301.9$\pm$3.2 mOsm/kg H_2O for the WB group and 309.0$\pm$5.3 mOsm/kg H_2O) as determined by student t-test $p<0.01$.

Perfusate lactate did not increase significantly over the duration of the perfusion for either of the two groups and it was not significantly different between groups at the end of perfusion. Perfusate lactate concentrations at the start of perfusion were 1.33±0.10mmol/L for RBC group and 1.67±0.29mmol/L for WB group; perfusate lactate concentrations at the end of perfusion were 1.83±0.25mmol/L for RBC group and 1.37±0.22mmol/L.

Urine production was low but present up to the 5th hour of perfusion but declined to <5ml/hr after the 5th hour likely due to the free water loss previously described.

Inflammatory Cytokines: TNF-α, IL-6, and IL-10

ELISA quantified serum concentrations of IL-6, TNF-α, and IL-10 of both RBC and WB groups over time are shown in Figures 12, 13, and 14 respectively. Serum concentrations of TNF-α and IL-10 were significantly higher in the WB group when compared to the RBC group. Serum TNF-α found in the WB group was significantly higher than the RBC group at the 7th, 9th, and 11th hours ($p<0.05$), with a trend towards statistical significance at 5th hour ($p=0.06$). Serum IL-10 found in the WB group was significantly higher than the RBC group at the 3rd, 5th, 7th, 9th, and 11th hours ($p<0.05$). While IL-6 was only significantly higher in WB group at the 11th hour ($p<0.05$). Both IL-6 and IL-10 increased significantly from the start of perfusion. TNF-α concentrations showed significant decrease after the 3rd hour for both groups (repeated measures ANOVA of 3rd, 5th, 7th, 9th, 11th hours; $p<0.05$), but the TNF-α concentrations for both groups at the end of perfusion remained higher than their respective concentrations at the start of the perfusion.

Kidney Injury Markers: KIM-1, NGAL, and AST

Perfusate injury marker KIM-1 of RBC and WB groups are shown in Figure 15, from *in vivo* blood sample to the 11th hour of perfusion. Perfusate KIM-1 did not demonstrate significant

increase from *in vivo* for either RBC or WB groups, though RBC group demonstrated a trend of higher mean concentrations when compared against WB group. The perfusate KIM-1 concentration of the RBC in the 11th hour was significantly higher than the perfusate KIM-1 concentration of the WB group in the 11th hour ($p<0.05$).

Perfusate injury marker NGAL was not detectable within the perfusate, for all time points in both groups, and the data was not shown; the kit was rated for lowest sensitivity of 0.01ng/ml. Perfusate injury marker aspartate aminotransferase (AST) did not demonstrate significant increase over time for either the RBC or WB groups. There were also no significant differences between groups.

Histology

Histological features found in the WB and the RBC groups were scored and shown in Figure 16 as mean scores $\pm$ standard errors of means. Vacuolization was the only category that showed statistically significant increase from *in vivo* samples; the WB group was scored for 28$\pm$7.8% and the RBC group was scored for 26$\pm$3.7%, with no difference between groups. Brush border loss was scored at 7$\pm$3.8% and 4$\pm$4% for the WB group and the RBC group respectively. Acute tubular necrosis was scored at 4$\pm$2.9% and 0% for the WB group and the RBC group respectively. Other categories were evaluated but demonstrated no significant findings and were not shown, these include positively stained droplets, cellular casts in the tubular lumen, interstitial edema, and interstitial inflammation.

Chapter 5: Discussion

1) Hypothermic Machine Perfusion and Normothermic Machine Perfusion:

This preliminary study examined a porcine model of *ex situ*, hypothermic and normothermic machine perfusion of kidney grafts. The perfusate used for kidney hypothermic machine perfusion (HMP) in this study was chosen because the modified University of Wisconsin solution, KPS-1, is widely used in clinical transplant settings and can be readily produced using research grade materials[81]. Although there were concerns about the nephrotoxicity of hydroxyethyl starch (HES), they were mostly found in clinical acute care situations[59,248]. Large systematic studies showed inconclusive results, and therefore the concern may not be applicable to an isolated, hypothermic kidney perfusion system[59]. The use of only 5% HES does not induce excessive oncotic pressure and any nephrotoxic effects would be minimal in a hypothermic environment.

The perfusate used for kidney normothermic machine perfusion (NMP) in this study was an oxygenated Krebs-Henseleit buffer modified to mimic extracellular fluid, maintain oncotic pressure with 8% w/v bovine serum albumin, and provide metabolic support through glucose and insulin. The use of an extracellular ionic composition is to simulate the *in vivo* environment to allow for physiologic renal function (excretion and reabsorption of molecules in the perfusate) to take place and be observed. Whole blood was chosen to minimize alterations from the *in vivo* environment and simplify the methods. Significant hemolysis was observed in initial iterations of the NMP system set up. However, after reducing the impacts of blood on non-biological surfaces and changing to a softer arterial cannula, hemolysis was reduced sufficiently for free haemoglobin to be no longer detectable in most serum supernatants.

Hemodynamics of the two systems were drastically different with significantly higher intra-renal resistance (IRR) observed in hypothermia than in normothermia. This hemodynamic difference was likely due to the significant vasoconstrictive effects of hypothermia on renal vasculature[235], but may also be due to differences in viscosity as the HES component of the HMP solution has often been implicated in reduced perfusion flow[43,45,48]. Because of the significant reduction of metabolic demands and waste production during severe hypothermia, the restricted renal perfusate flow may not be detrimental; in fact, higher initial flush rate in renal allografts undergoing static cold storage has been implicated in microvascular injury[49].

Low IRR and high renal blood flow observed in NMP reflects the macroscopic, qualitative observations that normothermic perfused kidneys had uniformly pink appearance, indicating thorough tissue reperfusion after the ischemic renal insult during graft recovery. Stable IRR without vasospasms even in absence of vasodilators used by other kidney NMP studies[152,173] confirmed that kidney NMP may be performed with minimal vasodilator intervention.

Urine and perfusate composition analysis strongly suggest a lack of metabolic activity within the hypothermic perfusion system, as the urine produced during HMP was all but indistinguishable from the perfusate. During hypothermic preservation, metabolic depletion may stop ionic transport necessary to concentrate urine, but may also cause cellular edema[20]. The significant biochemical differences between urine and perfusate composition in NMP also reflect the active renal function occurring during NMP. Urine pH is decreased (reflecting renal function in acid-base balance), potassium is increased (excretion of excess potassium), and glucose is decreased (glucose should not be found in the urine unless it exceeds the reabsorption capacity of the tubules)[249]. Notably urine osmolarity is quite low, potentially reflecting a lack of vasopressin action in the isolated kidney system. Urine osmolarity is also low *in vivo*, however that may

reflect the large amount of volume replacement (1L of lactated ringer crystalloid solution) during surgery. These results highlighted the isolated nature of the kidney NMP system, where the kidney graft is removed from systemic humoral and neural factors that normally modulate renal function.

In this initial experience with kidney *ex situ* perfusion at normothermia and hypothermia, a significant limitation is the lack of systemic influences such as metabolic wastes, hormones, and renal autonomic nervous input. Without a constant steady state supply of metabolic wastes from systemic circulation, it is difficult to evaluate traditional renal function metrics such as blood urea nitrogen or serum creatinine without exogenous addition of creatinine or other markers such as inulin. Because of the isolated nature of kidney NMP, there is no influence from systemic hormones such as aldosterone, natriuretic peptides, antidiuretic hormone, etc., potentially abolishing normal physiological responses to ionic imbalances. Without systemic influence, most of the ionic reabsorption and excretion activity may be explained by either local paracrine feedback or electrochemical gradients and existing ionic transports.

This preliminary study established both an HMP and an NMP system using a porcine kidney model and offered insight into the differences in hemodynamics and renal function between the two systems. The equipment used were mostly adapted from neonatal bypass circuits. To further optimize the NMP system, it may be necessary to alter different components of the perfusate and their effects on perfusate metrics such as inflammatory and injury markers. Histological evaluation and score comparison will also aid in comparing different perfusate components, though in this experimental model some of the clinically established criteria may not be applicable[221]. The study demonstrated that HMP does not offer a sufficiently metabolically active environment to manifest physiologic renal function or detectable

inflammatory/injury metrics; therefore, a normothermic *ex situ* system could offer more insights into kidney graft preservation, evaluation, and intervention for the purposes of transplantation.

2) Normothermic Machine Perfusion: Oncotic Agent

Normothermic machine perfusion (NMP) is a novel method of kidney graft preservation that has shown improvements over traditional hypothermic methods in pre-clinical studies and small-scale clinical studies. Because the clinical application of NMP is still in its infancy, there is no current consensus on optimal perfusate composition. In our porcine model of prolonged kidney NMP, albumin was replaced by dextran-40 as the main source of oncotic pressure in the perfusate. We found that the dextran-based perfusate performed equally well against the albumin-based perfusate in perfusion hemodynamics and kidney injury markers, however, dextran-based perfusate demonstrated significantly reduced inflammatory markers.

In the production of perfusates with 8% dextran-40 as a replacement for 8% bovine serum albumin as a source of oncotic pressure, the addition of dextran-40 to perfusates produced comparable increases in oncotic pressure per gram as albumin (averaging 30-35mmHg). Over time in 12 hours of perfusion, the dextran-based perfusate lost oncotic pressure at different rates depending on urine production, likely caused by the excretion of lower molecular sized dextran through urine. The excretion of dextran likely draws water with it into urine through "colloid osmotic diuresis", therefore the increased urine output is most likely a physical effect of dextran excretion instead of improved renal function[250]. Matheson et al. also noted that physiologic doses of vasopressin abolished the diuretic effects of dextran without preventing excretion of dextran as the urine was still similarly hyper-oncotic[250]. There are continued concerns about clinical use of dextran and other colloids, however evidence is inconclusive and often come from intensive

care situations where hyperoncotic nature of colloid use was more significant than the colloids themselves[251].

Dextran-40 is a component of the STEEN Solution™ where it is marketed as a protective agent for the endothelium and a colloid agent alongside albumin (XVIVO Perfusion, Goteborg, Sweden). STEEN Solution™ is considered a gold standard for *ex situ* lung perfusion and has been used to prime the kidney NMP system by the Toronto group[167]. Within STEEN Solution®, dextran-40 is considered a vascular protective, anti-inflammatory agent that also prevents edema by providing oncotic pressure[238]. Dextran-40 is also an important protective component in a hypothermic lung preservation fluid marketed as Perfadex® (XVIVO Perfusion, Goteborg, Sweden). Perfadex® is a low potassium, high sodium, and high chloride solution with 5% w/v dextran-40 providing oncotic pressure[252]. Perfadex® has shown comparable performance in *ex situ* lung preservation[252–255]. Although there is commercial application of dextran-40 in organ perfusion solutions, there is no recent research on its effects in kidney NMP.

Post-transplant renal thrombosis is a significant concern for early graft survival, as it accounts for 2-7% of early graft loss[256,257]. The risk of post-operative thrombosis may be increased by mechanical/technical factors, pre-existing vascular factors, or even the use of immunosuppressive drugs[256–258]. The risk of renal vein thrombosis may be post-operative, but the use of dextran-40 during perfusion may also assist in preventing thrombotic risks post-implantation by reducing microvascular thrombosis during NMP. Evaluating the two groups based on intra-renal resistance (IRR), we found both groups had stable and low IRR throughout perfusion, and no significant differences were found between the groups. Any anti-thrombotic effects of dextran-40 during perfusion may be masked by the anti-thrombotic effects of heparin used in both dextran and albumin perfusates. To explore potential anti-thrombotic effects of

dextran-40 during NMP, further study in a transplantation model will be necessary. Dextran-40 demonstrated equivalent and stable IRR, a metric often used to represent effectiveness of renal tissue perfusion in kidney NMP.

Lower perfusate concentrations of inflammatory markers IL-6 and TNF-α in dextran-based perfusate group demonstrated a reduced inflammatory effect of dextran-40 as an oncotic source. Increased serum inflammatory cytokine is associated with poorer post-operative outcome in transplant recipients[192,204]. Dextran may be exerting its effects through reducing leukocyte infiltration into renal tissue, as previous research has shown dextran-40's ability to inhibit the adhesion of t-lymphocytes to endothelial cells *in vitro*[259]. During ischemia-reperfusion injury leukocytes have been implicated in increased production of pro-inflammatory and pro-fibrotic factors, extending the initial ischemic acute kidney injury[244,245]. Further investigation of potential anti-inflammatory effects of dextran-40 may require both wider comparisons with other oncotic agents (to eliminate potential pro-inflammatory effects of bovine serum albumin) and studies into its *in vitro* interactions with cultured endothelial cells.

We did not find significant differences in markers of acute kidney injury as measured by perfusate marker KIM-1, tissue expressions of biomarkers, and histology. However, tissue expression of KIM-1 was significantly lower in both groups when compared to samples collected immediately after procurement. This reduction in tissue expression of KIM-1 may reflect the wash-out of KIM-1 molecules into the perfusate after initiation of kidney NMP, as we saw a trend indicating increasing perfusate KIM-1 for both groups from the start of perfusion.

Many pre-implantation graft biopsy criteria are targeted at chronic kidney injuries and pathologies more often found in expanded criteria donors who may have chronic cardiovascular diseases or older age[218,221]. However, these chronic injuries will not be present in the younger

animals from our porcine model of adolescent pigs and cannot develop within the relatively short timeframe of our kidney perfusion. This is confirmed by the lack of glomerular loss or other glomerular pathologies such as glomerulosclerosis and inflammatory lesions. Therefore, we have opted to investigate acute tubular necrosis (ATN) and ATN related injuries that may develop within the relatively short time frame. The acute tubular injuries we investigated are non-specific and may be reversible, however they do reflect features that may contribute to the eventual development of irreversible ATN.

Histological scores for vacuolization did increase significantly post-NMP for both perfusate groups, which is likely due to the reversible process of pinocytosis of colloid molecules such as dextran and albumin into the tubular epithelial cells[57]. The increase in epithelial vacuolization is likely similar to an observed phenomenon called osmotic nephrosis, which can be reversible when the causative agent (i.e. dextran or albumin) is removed from the perfusate through a flush after NMP[54,57]. The presence of osmotic nephrosis may be a cause for concern over a longer period, as it may cause less reversible tubular injury, but this phenomenon should dissipate once the kidney graft is flushed prior to implantation.

An increase in brush border loss was observed with dextran-40 perfused samples, with histological scores approaching significance when compared against albumin perfused samples ($p=0.06$). The dislocation or flattening of tubular epithelial cells is an indicator of mild tubular injury used in the Banff criteria[218], though it may be a reversible process[260]. Brush border loss may reduce tubular epithelial function of ionic exchange as it reduces the surface area[261]. The observed trend towards an increase in brush border loss may bring statistical significance with an increase in the number of trials.

Although we replaced the entire albumin content of the perfusate with dextran in our study, it may be valuable to manipulate the different proportions of albumin and dextran in the perfusate to achieve optimal perfusate composition. Dextran may be able to provide endothelial protection and anti-inflammatory effects at a significantly decreased concentration, while albumin or another oncotic agent acts as the main source of oncotic pressure to protect against fluid extravasation. Reducing the concentration of dextran may also reduce any potential nephrotoxicity associated with the observed brush border loss. Further optimization of perfusate solutions are necessary to protect against different pathophysiologies of acute kidney injury during normothermic machine perfusion of kidneys.

There are several limitations to this study, including a relatively small sample size and a lack of a transplant model. In the future, it will be necessary to test the efficacy of a dextran-40 based perfusate in a transplant model, as the quality of graft preservation can only be fully measured by post-operative serum creatinine and other renal function measurements. It is also important to note that the albumin used in this experiment is of high-quality cell culture grade bovine serum albumin, because porcine serum albumin is not available in similarly refined quality readily accessible for research use. The feasibility of using dextran to replace human serum albumin in perfusates used for human kidney NMP remains untested, and similarly different ratios of dextran to albumin within a mixture warrants further testing. A future study to further elucidate the mechanism by which dextran exerts its anti-inflammatory effects may be to use dextran in a leukocyte depleted perfusate. The endothelial protection theory proposed by Termeer et al. postulates that dextran-40 prevents leukocyte adhesion and infiltration, therefore in a leukocyte depleted environment any anti-inflammatory effects of dextran-40 would be unrelated to leukocyte adhesion and infiltration[259].

This study demonstrated the effective normothermic *ex situ* perfusion of porcine kidneys using a dextran-40 based perfusate, and it showed dextran-40's ability to modulate inflammatory response of the kidney during perfusion. Hemodynamics and injury metrics between dextran-40 and the traditional oncotic agent bovine serum albumin were comparable. Because of concerns over its potential nephrotoxicity in some acute care applications, dextran-40 has not been studied in kidney preservation. However, if dextran-40's anti-inflammatory effects can be safely applied in an isolated kidney NMP system with optimized dosing, it may improve post-implantation outcomes.

3) Normothermic Machine Perfusion: Oxygen Carrier

Most kidney NMP studies used red blood cell concentrate as a cellular oxygen carrier, due to either better availability of washed erythrocytes or research indicating the deleterious effects of leukocytes[155,192,245,262]. However, there is a return of clinical interest in the limited and controlled use of O-negative, leukocyte-reduced stored whole blood for traumatic transfusions mainly for its hemostatic effects[263]. Increases in clinical interest in stored whole blood products may increase availability for ABO-matched whole blood in organ perfusion. Within the setting of organ perfusion, White et al. found that whole blood based perfusate provided improvement in myocardial function in normothermic *ex situ* perfusion of porcine hearts, and postulated the improvement may be due to plasma's antioxidant abilities[159]. In 2017, Church et al. found the use of a cross-circulation technique improved myocardial function in normothermic *ex situ* perfusion of porcine hearts; the cross-circulation of live animal whole blood or plasma showed equal performance[247]. Church et al. suggested two potential mechanisms of improvement: one is the therapeutic effects of plasma alone, and the other is the exchange of wastes and metabolites across between the perfusate and the live animal blood or plasma[247]. With increasing evidence of

therapeutic benefits of plasma, we investigated the effects of replacing washed erythrocytes with donor whole blood in a normothermic *ex situ* kidney perfusion system.

The main difference in composition between the two groups is the absence of leukocytes and plasma within the red blood cell concentrate (RBC) group. The hemodynamic and perfusion results demonstrated equivalency between the red blood cell concentrate (RBC) and whole blood (WB) groups. Within this system, donor whole blood perfusates demonstrated equivalent intra-renal resistance, perfusate acid-base homeostasis, and perfusate lactate. When inflammatory markers were examined, it was unsurprising that the perfusates of the WB group had significantly higher TNF-α than the RBC group and a trend towards increased IL-6. This increased presence of pro-inflammatory cytokines was likely due to the increased presence of leukocytes in the whole blood-based perfusate. However, there was also significant increased perfusate concentration of the anti-inflammatory/immunomodulatory IL-10 in the WB group compared to the RBC group. IL-10 is immunomodulatory (reducing further transcription of IL-6 and TNF-α)[166,264] and may have protective effects against renal ischemia-reperfusion injury. In a recent study, Sakai et al. found that IL-10 knockout mice demonstrated worsened *in vivo* renal function and increased expression of apoptotic factors and kidney injury molecule-1 (KIM-1)[265]. The effect of increased perfusate IL-10 concentration on future IL-6 and TNF-α production might be elucidated by increasing the length of the perfusion or applying exogenous IL-10 at the start of perfusion. The corresponding increase in anti-inflammatory IL-10 and a lack of histological evidence of inflammation suggest that although statistically significant, the increase in TNF-α may not cause or reflect physiologically relevant inflammatory injury to the kidney.

Perfusate injury marker KIM-1 of the RBC group showed a trend towards significant increase over the WB group. By increasing the number of trials and the length of perfusion,

future experiments may be able to elucidate any divergence in perfusate KIM-1 and histological scores. However, within this preliminary study of limited sample size, the data seems to suggest equivalent level of kidney injury in the WB group compared to the RBC group. There was no significance in perfusate injury markers NGAL and AST. The urinary concentrations of all three injury markers may be more revealing as other studies have found larger increases in these injury markers in the urine[178,179,188].

Histological scores were compared, and no statistically significant differences were found in any of the features. Both the WB group and the RBC group experienced increased vacuolization after perfusion as compared to *in vivo* samples collected prior to perfusion, this is likely due to the same reversible process of pinocytosis or uptake of the colloid used in the perfusate, in this case, bovine serum albumin. A lack of significant difference between histological scores of the WB group and the RBC group demonstrates equivalent safety of their use as the oxygen carrier in the kidney NMP system. However, it is worth investigating the relationship between tubular epithelial vacuolization and colloids used in the kidney NMP setting, as although it is reversible, the acute tubular injury may lead to permanent necrosis if the injury process continues. An interesting comparison may be drawn between the histological scores of the whole blood group in this experiment to those of the albumin group from the previous experiment on oncotic agents. The injury scores for brush border loss show a trend lower in the latter experiment's whole blood perfusate group (7+3.7%) compared to the earlier experiment's whole blood perfusate group (34$\pm$28%). Although the composition of these two perfusate groups were the same (Krebs-Henseleit buffer with 8% w/v bovine serum albumin and equal proportion of donor whole blood), the potential improvement in injury score may reflect improvements in perfusion techniques and organ recovery.

This study found that donor whole blood and washed donor erythrocytes performed equally well in perfusion, inflammatory, and injury metrics within a porcine normothermic *ex situ* kidney perfusion system. Along with the return of interest in the use of stored whole blood in certain clinical situations, there may be a role for the use of donor or stored whole blood in kidney NMP as well. The use of autologous donor whole blood is also feasible within a similar kidney NMP system, as the perfusate volumes may be relatively low, only requiring 300ml of whole blood; the volume of whole blood required may be further reduced with optimization of the perfusion circuit. Although a concern may be the deleterious effects of serum proteins within autologous donor blood, if the kidney is a marginal donor kidney and the donor has multiple comorbidities.

To further refine the use of whole blood, it may be useful to deplete leukocytes while saving the plasma component of whole blood, or simply add fresh frozen plasma to washed erythrocytes. Because other studies that supported the use of whole blood have suggested that the therapeutic effects are likely due to antioxidant capacity of plasma components[247]. As previously discussed, dextran-40 may also have anti-inflammatory effects, which is thought to be exerted through the prevention of leukocyte adhesion and infiltration. If the deleterious effects of whole blood are caused by leukocyte presence, it may be useful to investigate the effects of dextran-40 in both whole blood and washed erythrocyte perfusates. To continue the investigation of this study, it may be valuable to examine the antioxidant capacity of both perfusates and post-implantation renal function within a transplant model.

4) General Improvements to Perfusion:

There are several possible improvements to the normothermic *ex situ* kidney perfusion system used in this study: reduction of free water loss, optimization of perfusion pressures and

temperature, and optimization of a nutrient solution. The progressive free water loss, as reflected by increasing osmolarity observed, causes reduced urine output and an artificial increase in ionic concentrations within the perfusate (hypernatremia). Two urine-related metrics were rendered less effective by the decrease in urine production in the second half of perfusion: glomerular filtration rate and urinary injury markers. To improve the feasibility of these metrics, it is important to not only increase urine output, but also to maintain stable perfusate osmolarity throughout perfusion. Because the system is not entirely closed, there are also significant evaporative losses during perfusion. To address this issue, the setup may be improved by sealing off openings and creating curvatures in the container to allow better return of condensation back into the reservoir. Another method is to provide an infusion of a low osmolarity version of the Krebs-Henseleit buffer used in priming (for example a 1:2 dilution with injection water). The use of a vasodilator or diuretic seen in other studies[152,155] may be useful in increasing urinary output, however it may further exacerbate the water loss issue in the perfusate if it has not been addressed. Perfusate osmolarity and oncotic pressure need to be optimized, as although water loss and increased osmolarity causes many of the above problems, if the perfusate is too dilute it may cause tissue and cellular edema.

Perfusion pressure and temperature may need further optimization, as previously discussed in the dextran-albumin experiment, the hypernatremia developed over the course of perfusion may be caused by a lack of pressure natriuresis. The Toronto group uses a protocol of initial pressure of 75mmHg dropping to 65mmHg for the rest of their porcine kidney perfusions[173], while the Cambridge group uses a constant renal arterial pressure of 75mmHg in their human trials[152].

The use of solutions like parenteral nutrition in kidney NMP is common in both Cambridge and Toronto groups, however there is little research into the actual benefits of their use in a short time span of 1 hour (in the Cambridge group's protocol) and up to 12 hours (in the Toronto group's extended NMP protocol). Within the system used in this study, only glucose and insulin were used with no apparent detriment to preservation and perfusion. However, the dosing and timing of insulin infusion should be optimized, as it may be inducing a hypokalemic state (<3.5mmol/L) in the initial few hours of perfusion.

5) Limitations of the Model

There are several limitations to this study, including small sample size and the lack of initial kidney injury. The sample size was only 4 for most of the groups, which meant several metrics only showed a trend approaching statistical significance, which may become more apparent with a larger sample size such as 6 or 8. Initial kidney graft injury may allow the studies to better elucidate any reparative effects in the different perfusion techniques and perfusate components. This may be achieved by using a "donation after circulatory death" model by creating an artificially increased warm ischemic period where the renal vasculature is clamped for up to 30 minutes before retrieval and subsequent mounting to the NMP system. However, in this current model, there is already up to 15 minutes of anoxic warm ischemia between exsanguination and start of perfusion that may result in moderate acute kidney injury. There is also up to 15 minutes of partially hypoxic warm ischemia prior to kidney retrieval, due to surgical blood loss, hypotension, and blood collection for future use. In our kidney injury molecule-1 (KIM-1) assays, *in vivo* blood sample also showed detectable levels of KIM-1, even though KIM-1 should be undetectable within normal serum[179]. The multi-organ procurement surgery-induced acute kidney injury may be quantified by comparing samples of *in vivo* histology and blood samples collected prior to major surgical injuries (blood loss and

hypotension caused by the procurement procedures of other organs) and tissue and blood samples collected after the regular multi-organ procurement surgery. The use of discarded human kidney grafts may also be effective in studying the effects of kidney NMP on chronic glomerular pathophysiologies.

Another limitation in this porcine model is the absence of chronic histological alterations/injuries usually only found in expanded criteria donors. Within the clinically used pre-implantation histological scoring systems, the features most strongly associated with worse post-operative recipient outcomes are glomerular loss or injuries[218,221]. These glomerular pathologies were absent in all the sample groups, which made the comparison to clinical experience in extended criteria donation difficult. Existing inflammatory lesions and chronic injuries were also absent within our porcine model, as it is unlikely to be found in healthy adolescent pigs. The examination of discarded human kidney grafts may also shed light on any changes to chronic histological features caused by perfusion.

Chapter 6: Conclusion and Summary

The preliminary experiment on hypothermic and normothermic machine perfusion systems established a basic protocol on the preservation of porcine kidneys in this thesis. The first experiment demonstrated the value of using a normothermic system for investigating renal function and that metabolic activity may be required to manifest and evaluate ischemia-reperfusion injuries. The two kidney NMP experiments described here demonstrated the equivalent usage of dextran-40 and albumin as oncotic agents, and the equivalent usage of red blood cell concentrate and whole blood as cellular oxygen carriers. Through the examination of inflammatory markers, the discussions point to the investigation of a whole blood perfusate containing a mixture of albumin and dextran-40 as oncotic agents. Dextran-40 may be able to exert an immunomodulatory role against the increased leukocyte presence in the whole blood perfusate, while the plasma moieties may exert renal protective effects. The optimization of a kidney NMP perfusate may translate to improved clinical preservation of marginal kidney grafts, and the further clinical implementation of kidney NMP may increase both quality and quantity of available donor kidney grafts.

Kidney transplantation is still the gold standard treatment for end-stage renal disease, and with ever rising demand for donor grafts, there is increased interest and experimentation in the normothermic *ex situ* perfusion of kidney grafts. Normothermic perfusion not only provides preservation of the kidney graft, but also an opportunity to evaluate and apply relevant interventions to the perfused graft. With future large-scale clinical studies of normothermic kidney perfusion, it may be possible to find perfusion metrics and biomarkers that are predictive of immediate and long-term renal graft function and survival.

Tables:

Table 1: Electrolyte and biochemical composition of cold preservation solutions.

Name of Solution	**Euro-Collins (EC)**	**Sack's**	**University of Wisconsin (UW)**	**Histidine-Tryptophan-Ketoglutarate (HTK)**	**Celsior**	**Hyperosmolar Citrate (HOC)**	**Institut Georges Lopez-1 (IGL-1)**
pH	7.30	7.3	7.40	7.20	7.30	-	7.4
Sodium	10	10	30	15	100	84	120
Potassium	115	115	120	9	15	84	25
Chloride	15	15	20	32	42	-	20
Calcium	-	-	-	0.0015	-	0.25	-
Bicarbonate	10	10	-	-	-	10	-
Glucose	195	-	-	-	-	-	-
Impermeants	-	Mannitol 50	Lactobionate 100	Mannitol 38	Lactobionate 80; Mannitol 60	Citrate 80; Mannitol 185	Lactobionate 100
Antioxidants	-	-	Allopurinol 1; Glutathione 3	Tryptophan 2	Glutathione 3	-	Allopurinol 1; Glutathione 3
Osmolarity	406	410	320	310	255	400	320

Table 2: Breakdowns of Perfusate Composition for Cambridge and Toronto Groups

Component	Nicholson and Hosgood; Human	Selzner; Porcine
Oxygen Carrier	1 Unit of banked, matched **RBC** (294±14 ml)	Non-matched donor **RBC** (125ml; double washed from whole blood)
Crystalloid Solution (s)	**Lactated ringer**: 300-400ml	**Lactated ringer**: 200ml STEEN: 150ml
Oncotic/Colloid Osmotic Agent (s)	**Mannitol** 10%: 25ml	(**STEEN**: 150ml contains dextran and human serum albumin)
Vasodilator	**Prostacyclin** (0.5mg, **infused** at 4ml/h)	**Verapamil** (0.25mg/h)
Buffer	Sodium Bicarbonate (within infusion)	**Sodium bicarbonate** 8.4%, 8ml **Water** (27ml)
Glucose and Insulin	**Glucose** 5% at 7ml/h (Insulin mixed into nutrient infusion)	Glucose (mixed in with nutrition solution) **Insulin** 5IU/h
Nutrients and other infusions	1L **Nutriflex** solution at 20ml/hr (lipids, amino acids, vitamins, and ~144g/L glucose in a 2090mosm/L solution) With added: 25ml 8.4% **sodium bicarbonate** **Insulin** 100IU **Multivitamins** (unspecified conc.)	**Nutrition** (Amino acids and **glucose** of variable concentration). The infusion of nutrition solution is controlled to maintain 5-15mmol/L glucose.
Pharmaceutical agents	8mg Dexamethasone (corticosteroid) 2000IU Heparin (anticoagulant)	1000IU Heparin (Anticoagulant)

Table 3. Perfusate and urine blood gas analysis data for *in vivo* blood sample, kidney normothermic machine perfusion (averaged values over time) and kidney hypothermic machine perfusion (averaged values over time). Student's t-tests were performed between pH and glucose of *in vivo,* warm (NMP), and cold (HMP) perfusates, with significant differences found between HMP and other groups in pH and glucose ($p<0.05$).

Perfusate	*In Vivo*	Warm (NEVKP)	Cold (HEVKP)
pH	**7.45** ± 0.06	**7.45** ± 0.04	**7.34** ± 0.10
K (mmol/L)	**5.47** ± 0.59	**5.28** ± 0.45	**24** ± 1.20
Na (mmol/L)	**137.67** ± 2.33	**145.80** ± 2.02	**95** ± 2.01
Ca (mmol/L)	**1.16** ± 0.03	**0.93** ± 0.06	**0.42** ± 0.04
Cl (mmol/L)	**103.67** ± 1.76	**106.55** ± 2.27	**8.1** ± 4.01
Osm (mmol/kg)	**279.43** ± 5.17	**303.19** ± 6.04	**260** ± 9.2
Glucose (mmol/L)	**4.00** ± 0.55	**5.29** ± 0.63	**9.70** ± 0.45
Urine	***In Vivo***	**Warm (NEVKP)**	**Cold (HEVKP)**
pH	**6.42** ± 0.02	**6.94** ± 0.06	**7.22** ± 0.14
K (mmol/L)	**20.9** ± 4.10	**15.1** ± 1.77	**22.2** ± 1.9
Na (mmol/L)	**73.8** ± 25.36	**102.4** ± 4.80	**92.1** ± 2.01
Ca (mmol/L)	**0.39** ± 0.12	**0.79** ± 0.04	**0.41** ± 0.12
Cl (mmol/L)	**68** ± 30.56	**69.9** ± 6.63	**4.2** ± 2.42
Osm (mmol/kg)	**98.4** ± 33.66	**104.1** ± 14.35	**246** ± 6.3
Glucose (mmol/L)	**0.95** ± 0.13	**1.99** ± 0.63	**8.80** ± 0.62

Figures:

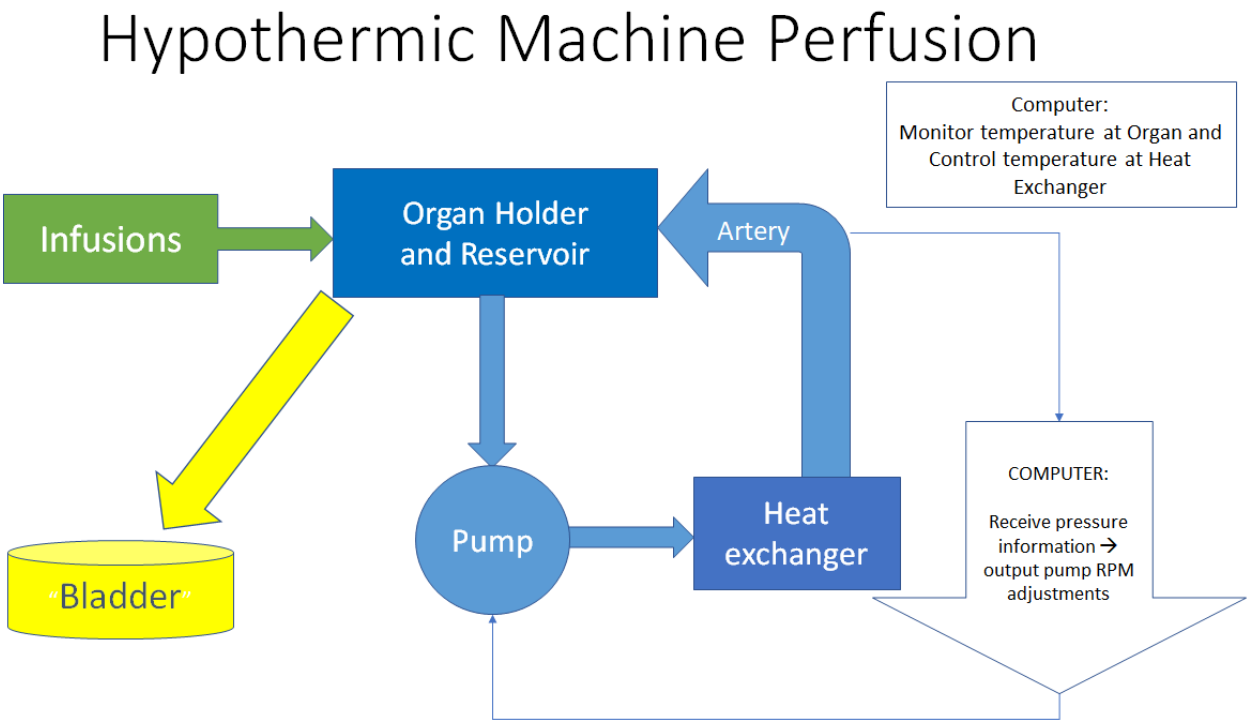


Figure 1. Diagram of the Hypothermic Machine Perfusion (HMP) system. The perfusate was pumped through the system as indicated by the light blue arrows, infusions entered through ports depicted by the green box and arrow, and urine was collected in the tubes depicted as "bladder" in yellow.

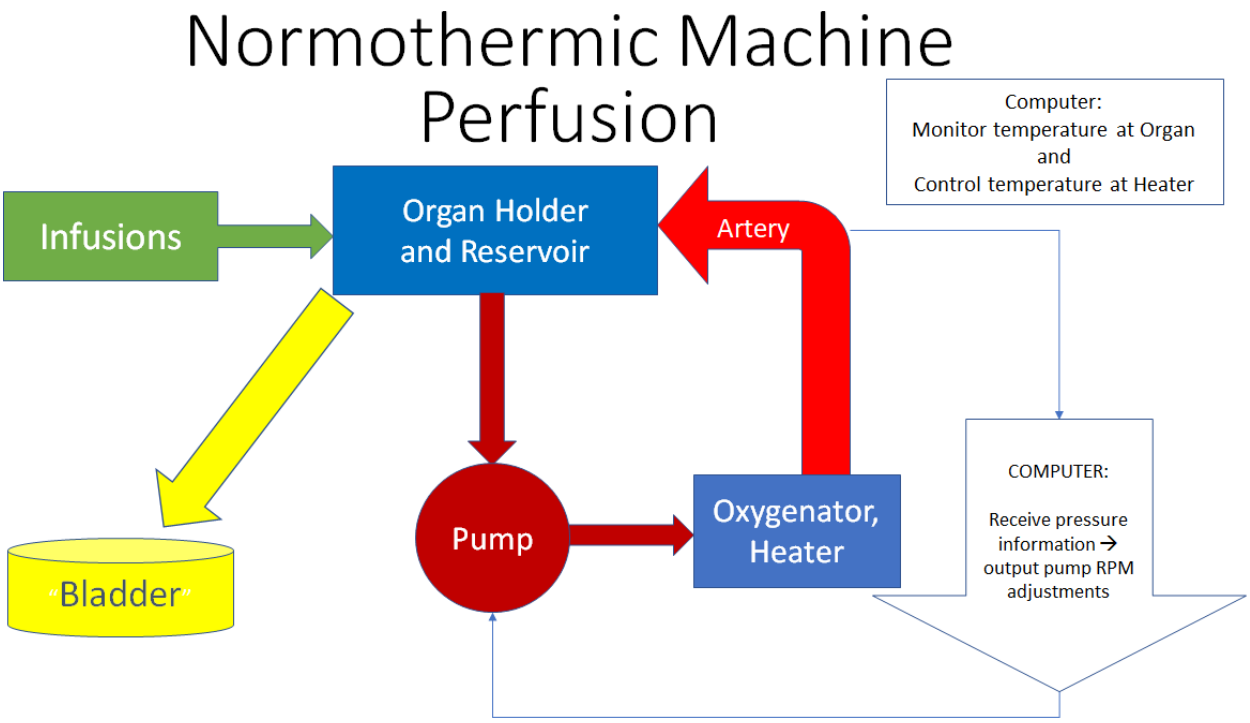


Figure 2. Diagram of the Normothermic Machine Perfusion (NMP) system. The perfusate was pumped through the system as indicated by the red arrows, infusions entered through ports depicted by the green box and arrow, and urine was collected in the tubes depicted as "bladder" in yellow.

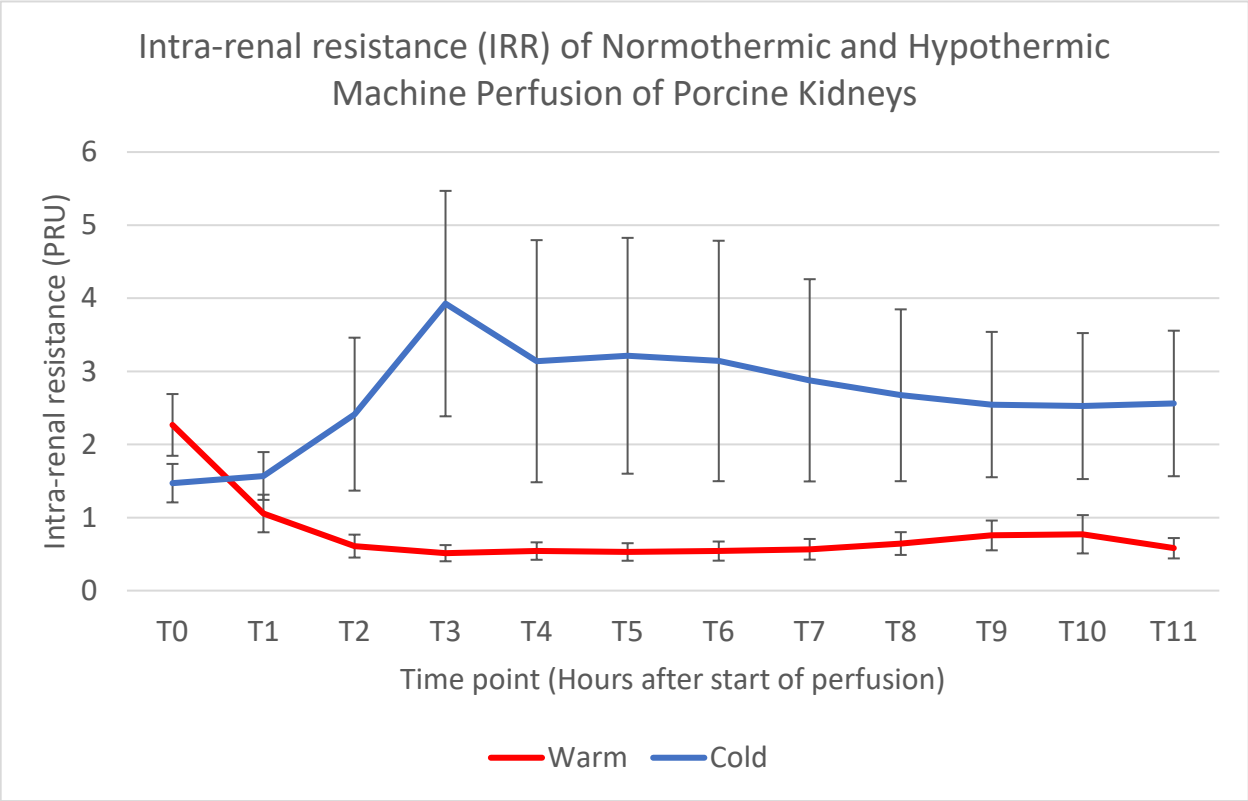


Figure 3. Intra-renal resistance (IRR) of normothermic machine perfusion (warm) compared to hypothermic machine perfusion (cold) over time (first hour = T1, nth hour = Tn). Values are in peripheral resistance units (PRU) or arterial pressure divided by renal blood flow ($mmHg*min*ml^{-1}$). Statistical significance between groups were found using repeated measures ANOVA, $p<0.05$; Student's t-tests were performed between T4 to T11, finding significant differences between groups $p<0.05$.

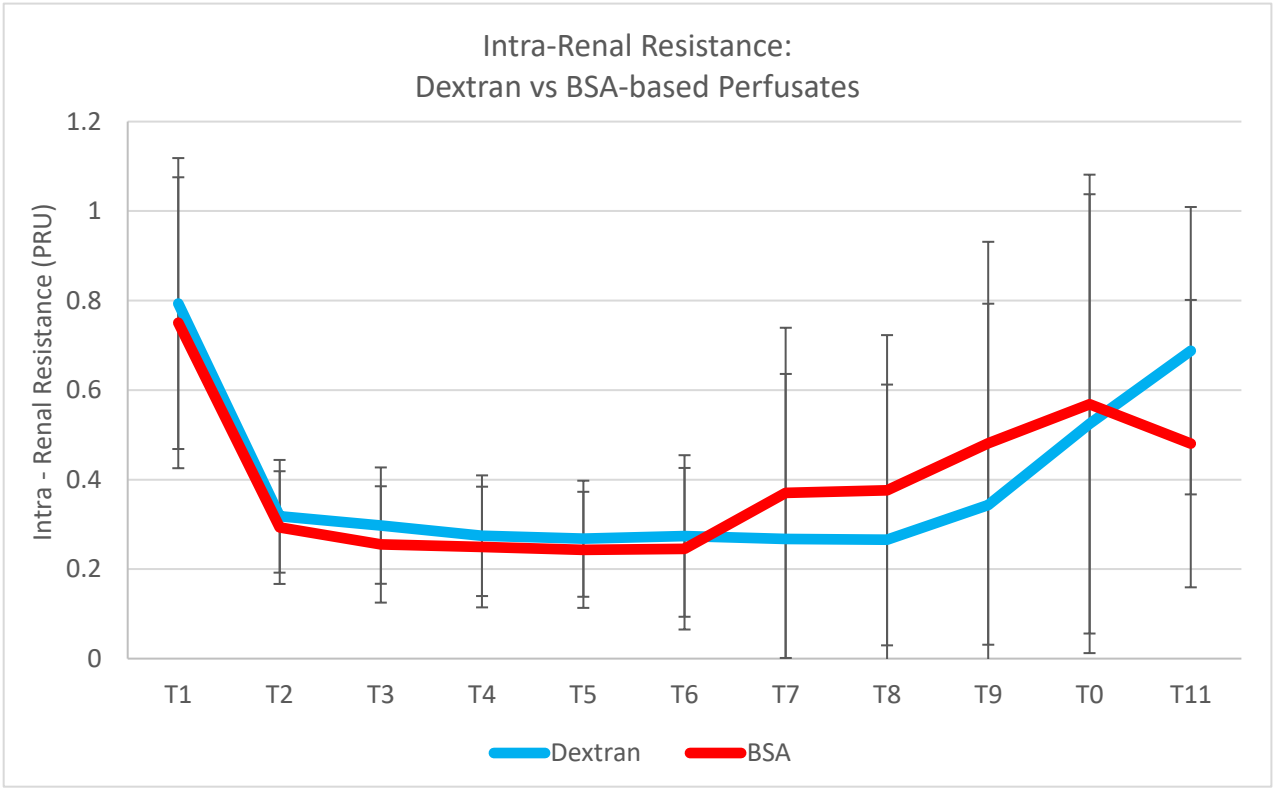


Figure 4. Intra-renal resistance (IRR) for the dextran group and the albumin group (first hour = T1, nth hour = Tn), presented as mean IRR ± standard error of means. Values are in peripheral resistance units (PRU) or arterial pressure divided by renal blood flow ($mmHg*min*ml^{-1}$). Repeated measures ANOVA was used for between group comparisons, with no significant differences found ($p>0.05$).

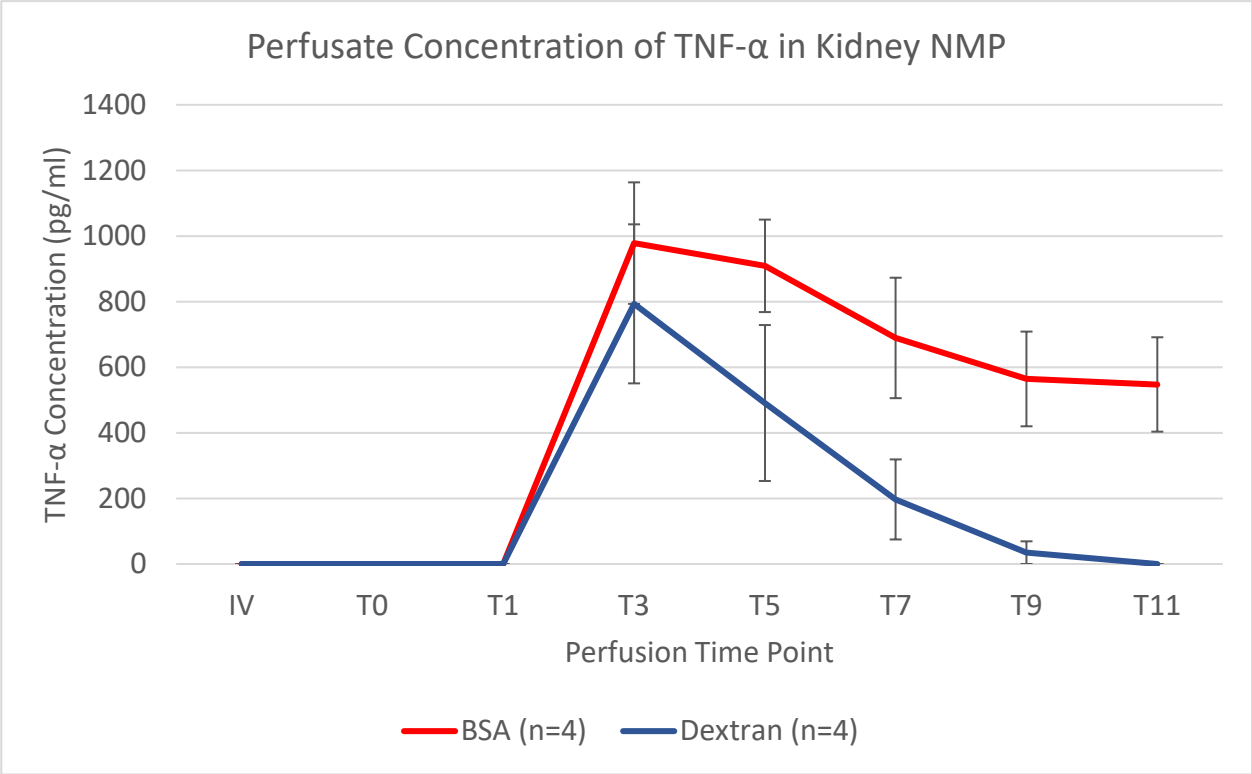


Figure 5. Perfusate concentrations of tumour necrosis factor-α (TNF-α) for dextran-based perfusate groups and albumin-based perfusate groups for samples collected at each hour of perfusion (first hour = T1, nth hour = Tn), presented as mean concentrations ± standard error of means, with units of pg/ml. Repeated measures ANOVA was used for between group comparisons, with no significant differences found until T9; within group analysis of time points between T3 and T11 (mid to end of perfusion) showed significant change over time only in the dextran group ($p<0.05$). At T9 and T11, Student's t-test found significant difference between the BSA and the dextran group ($p<0.05$).

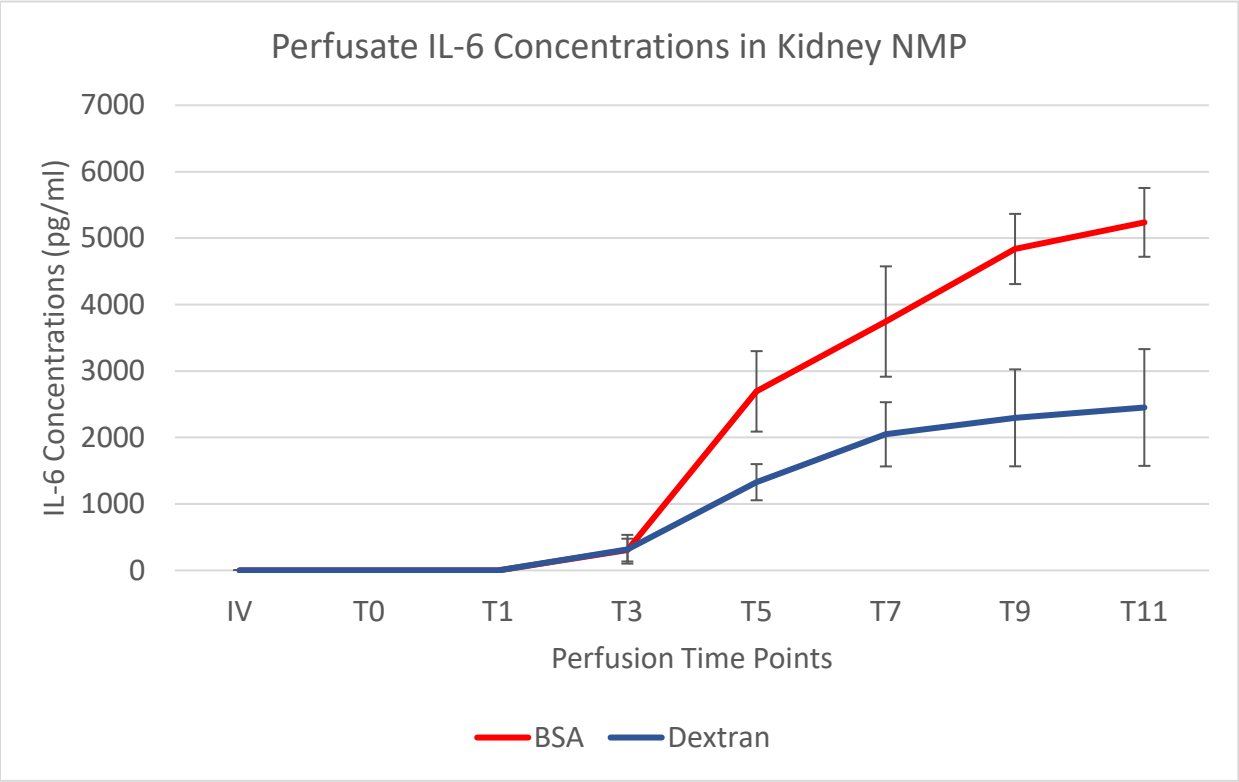


Figure 6. Perfusate concentrations of interleukin-6 (IL-6) for dextran-based perfusate groups and albumin-based perfusate groups for samples collected at each hour of perfusion (first hour = T1, nth hour = Tn), presented as mean concentrations ± standard error of means, with units of pg/ml. Repeated measures ANOVA was used for between group comparisons, with no significant differences found between groups; within group analysis of time points between T3 and T11 (mid to end of perfusion) showed significant change over time in both groups ($p<0.05$). At T9 and T11, Student's t-test found significant difference between the BSA and the dextran group ($p<0.05$).

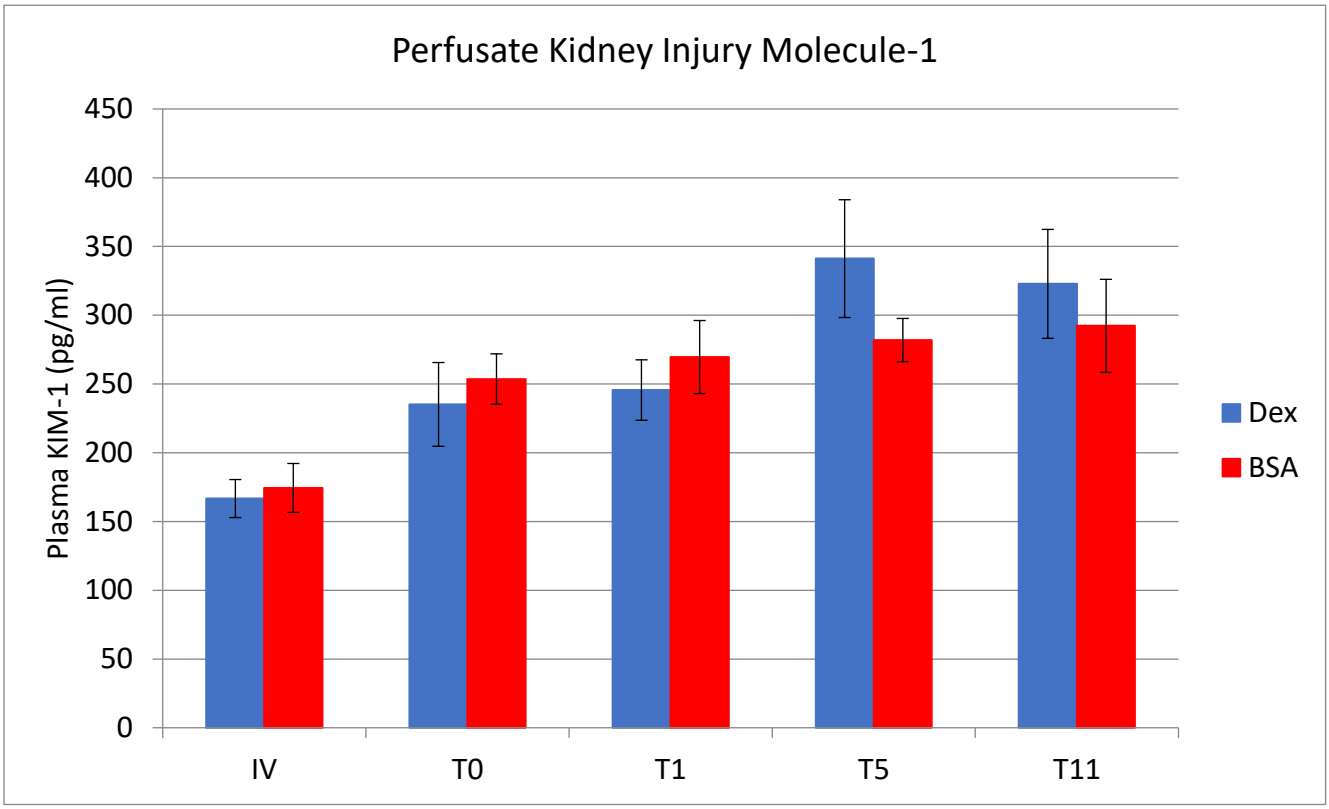
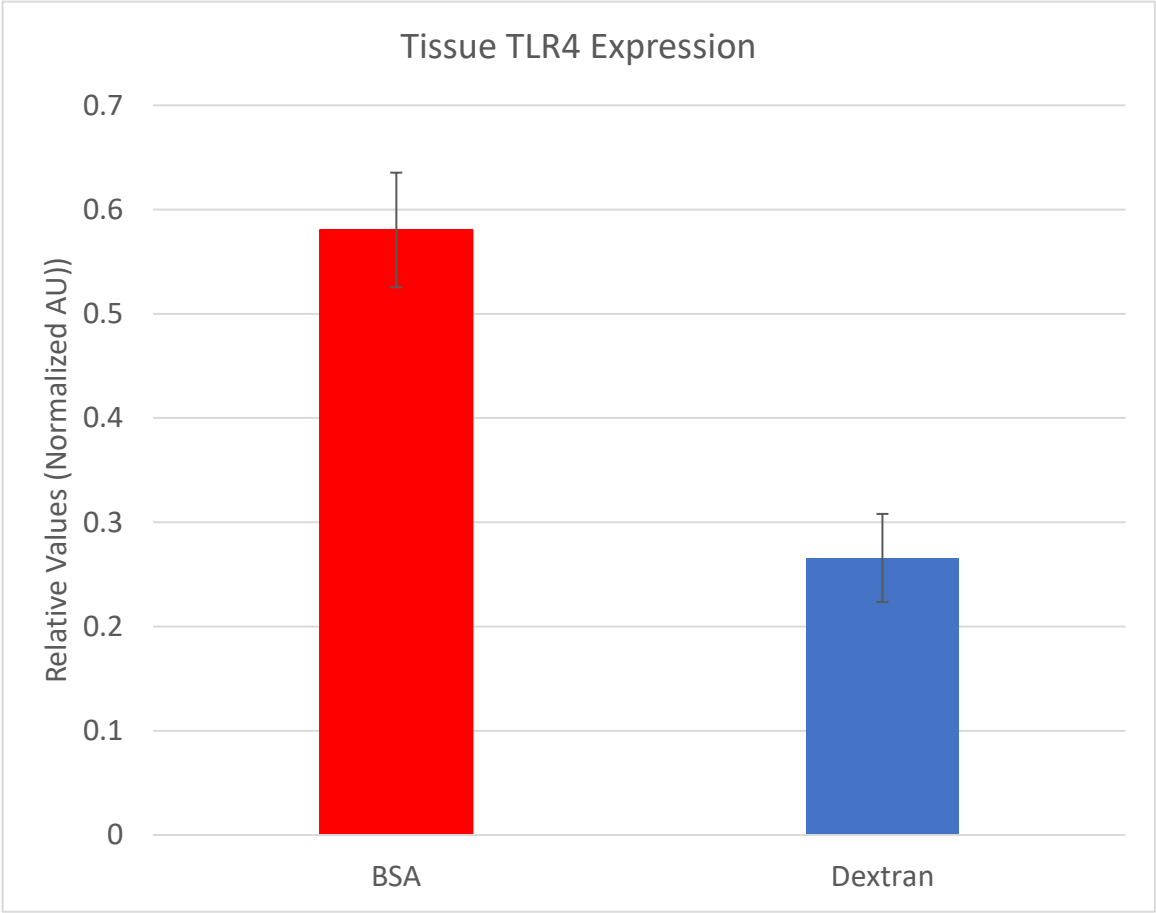


Figure 7. Serum and perfusate concentrations of kidney injury molecule-1 (KIM-1) for dextran-based perfusate groups and albumin-based perfusate groups for samples collected at *in vivo* (IV), start of perfusion (T0), first hour (T1), 5th hour (T5), and 11th hour (T11), presented as mean concentrations ± standard error of means, with units of pg/ml. Student's t-tests were performed between the two groups at each time point, with no statistically significant differences ($p>0.05$).



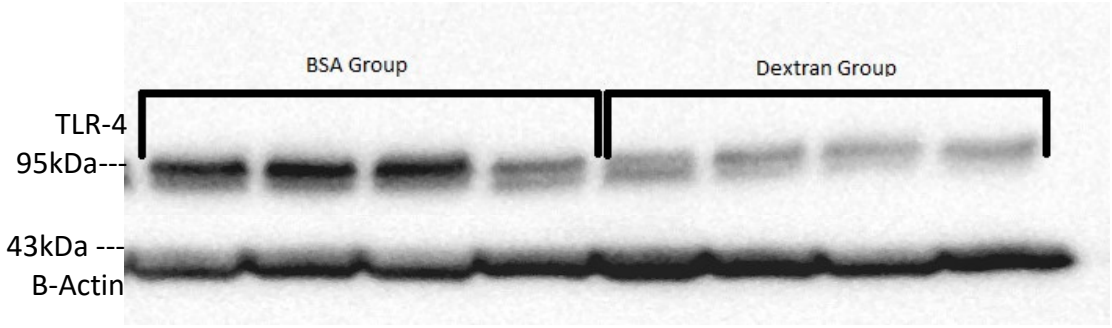


Figure 8. Tissue toll-like receptor-4 (TLR-4) expression for dextran-based perfusate groups and albumin-based perfusate groups quantified through western blot technique of samples collected at the end of kidney NMP, presented as mean optical densities (based on mean optical density) ± standard error of means. Values were normalized to mean optical densities of *in vivo* samples (collected prior to perfusion); units are arbitrary units (AU) for optical density. Mean values between the two groups were compared using Student's t-test, significant difference was found ($p<0.01$).

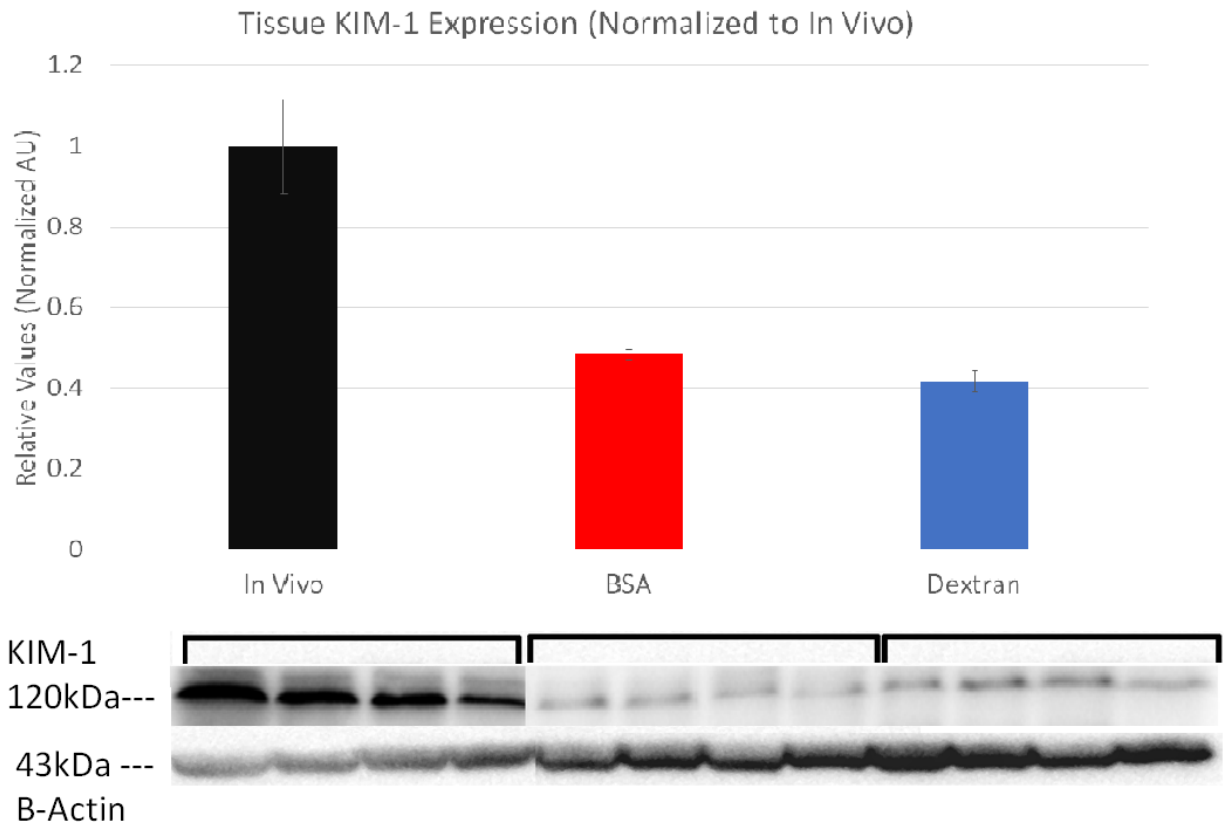


Figure 9. Tissue kidney injury molecule-1 (KIM-1) expression for dextran-based perfusate groups and albumin-based perfusate groups quantified through western blot technique of samples collected at the end of kidney NMP. Values presented are mean optical densities (based on mean optical density) + standard error of means. Values were normalized to mean optical densities of *in vivo* samples collected prior to perfusion; units are arbitrary units (AU) for optical density. Mean values between the two groups were compared using Student's t-test, significant difference was found between the BSA group and the *in vivo* and between the Dextran group and the *in vivo* group ($p<0.01$).

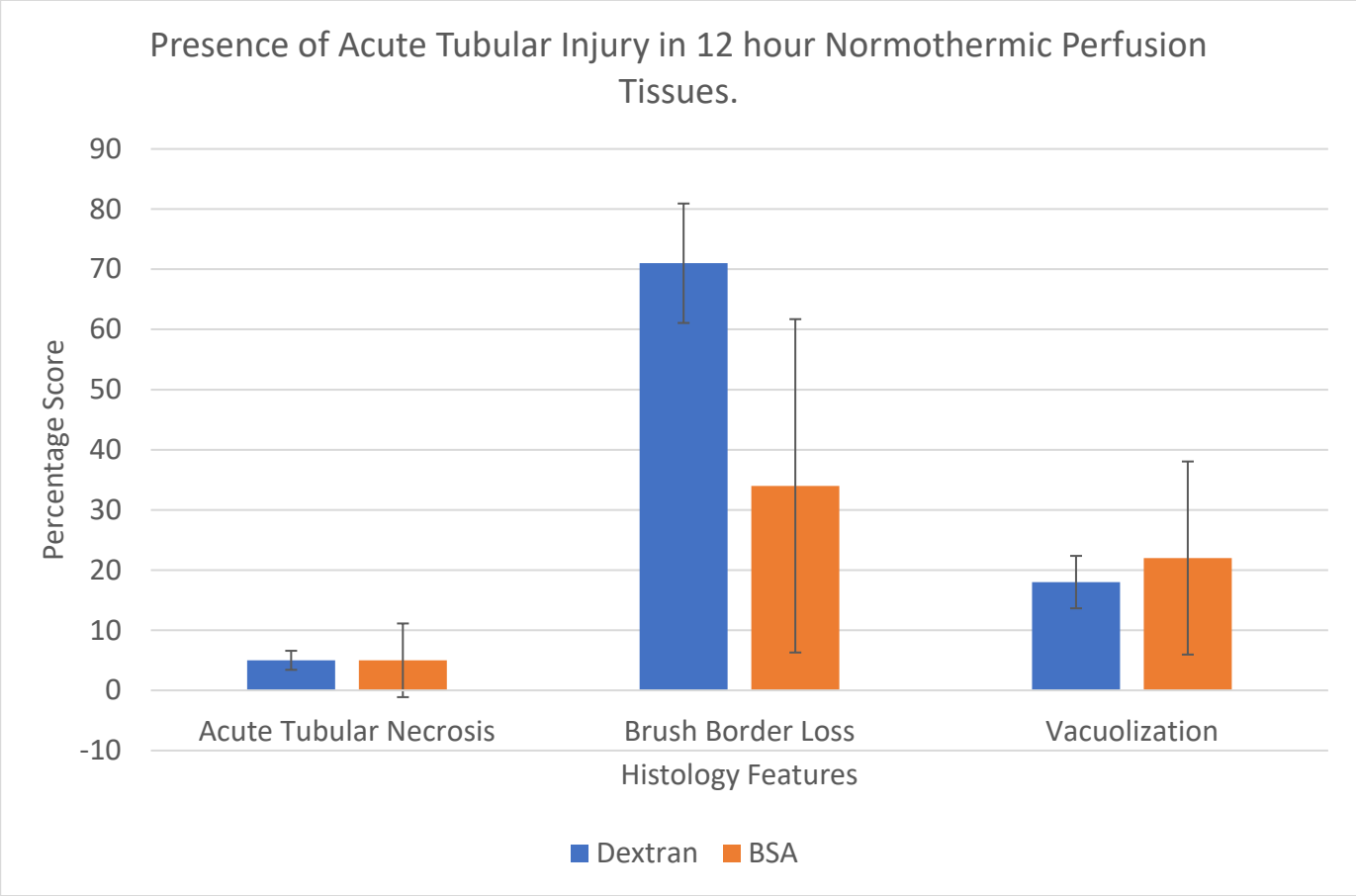


Figure 10. Histological scores of acute tubular necrosis, tubular epithelium brush border loss, and tubular epithelial cell vacuolization from the dextran group and the albumin group, presented as averaged percentage scores for each feature and error bars indicating standard error of means. Not shown are the categories for the presence of positively stained droplets and cellular cast formations within the tubular lumen, interstitial edema, and interstitial inflammation, as their presence was negligible in all samples. Histology scores were compared between the groups using the Whitney-Mann U-Test, with no statistically significant differences between groups ($p>0.05$); for Brush Border Loss, the difference approached statistical significance ($p=0.61$).

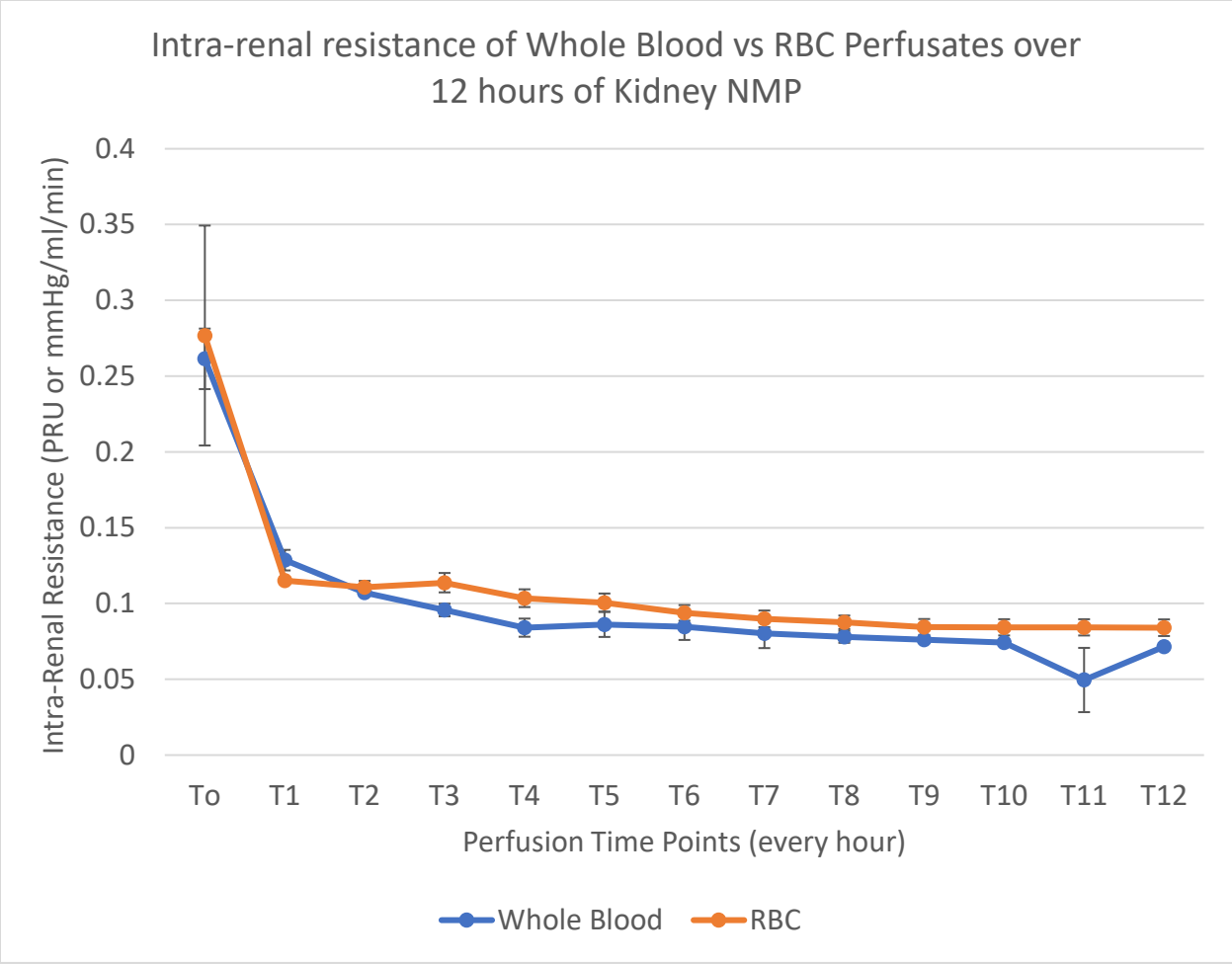


Figure 11. Intra-renal resistance (IRR) of the whole blood group and the red blood cell concentrate group over 12 hours of kidney normothermic machine perfusion (NMP) shown in peripheral resistance units (PRU) or millimeters of mercury per millilitres per minute ($mmHg*min*ml^{-1}$). Values are presented as means ± standard error of means. Repeated measures ANOVA was used to test between group differences, no statistically significant differences were found ($p>0.05$).

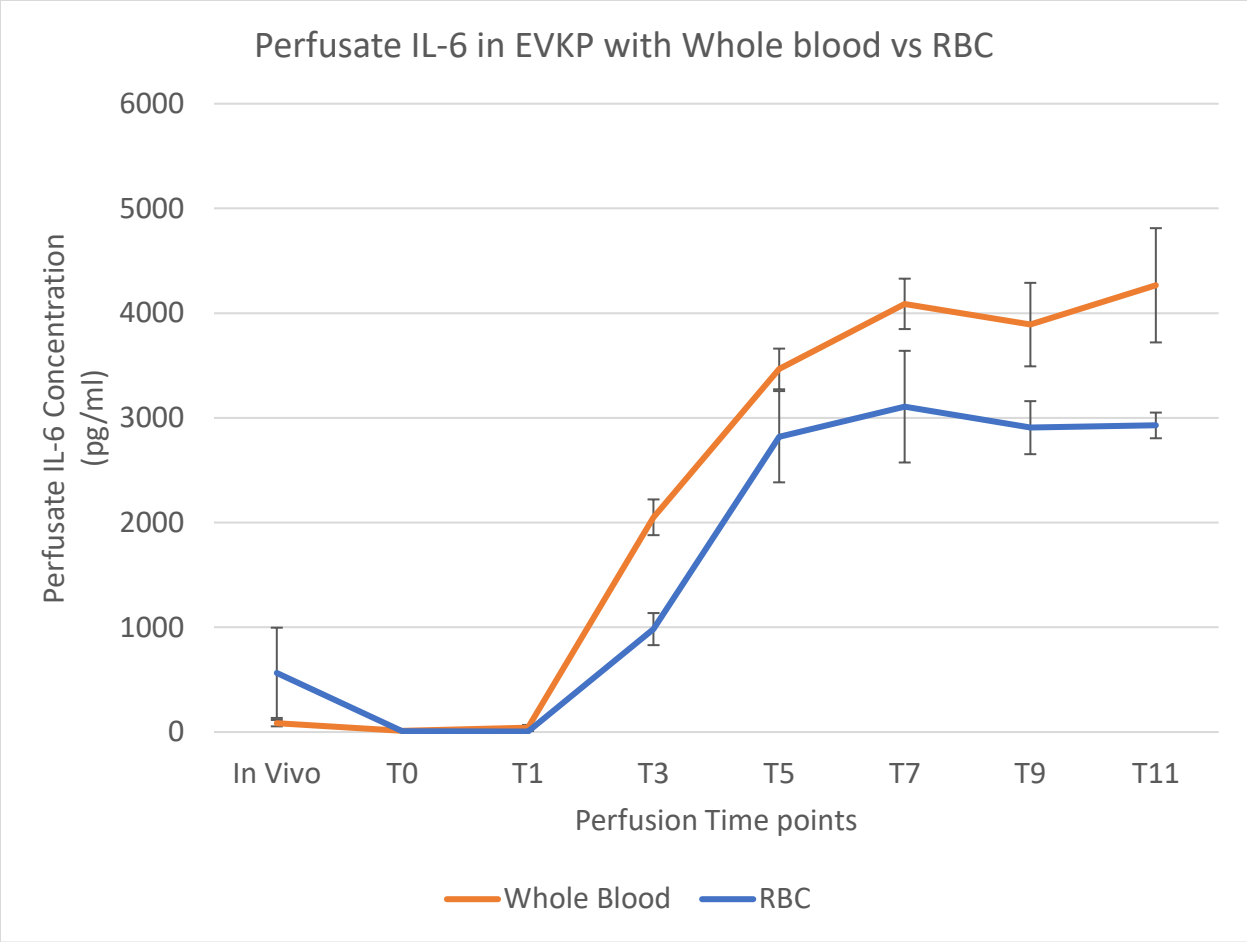


Figure 12. Perfusate concentrations of interleukin-6 (IL-6) in the whole blood and the red blood cell concentrate group shown in pg/ml. Values presented are means ± standard errors of means. Repeated measures ANOVA was used to test between group differences, no statistically significant differences were found ($p>0.05$) except for the 11th time point (T11) where there was statistically significant difference ($p<0.05$).

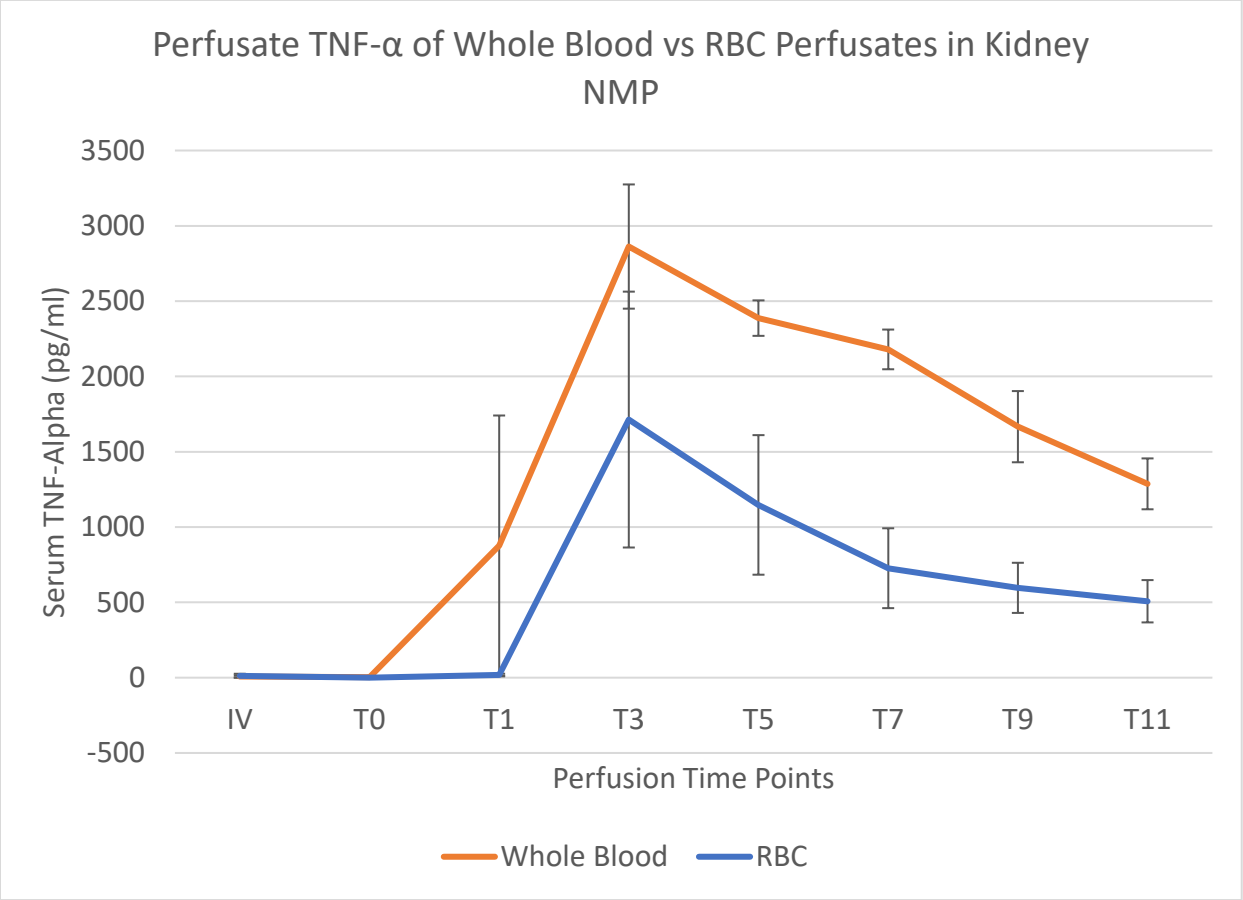


Figure 13. Perfusate concentrations of tumour necrosis factor-α (TNF-α) in the whole blood and the red blood cell concentrate group shown in pg/ml. Values presented are means ± standard errors of means. Repeated measures ANOVA was used to test between group differences at time points T5, T7, T9, and T11, statistically significant differences were found at T7, T9, and T11 ($p<0.05$).

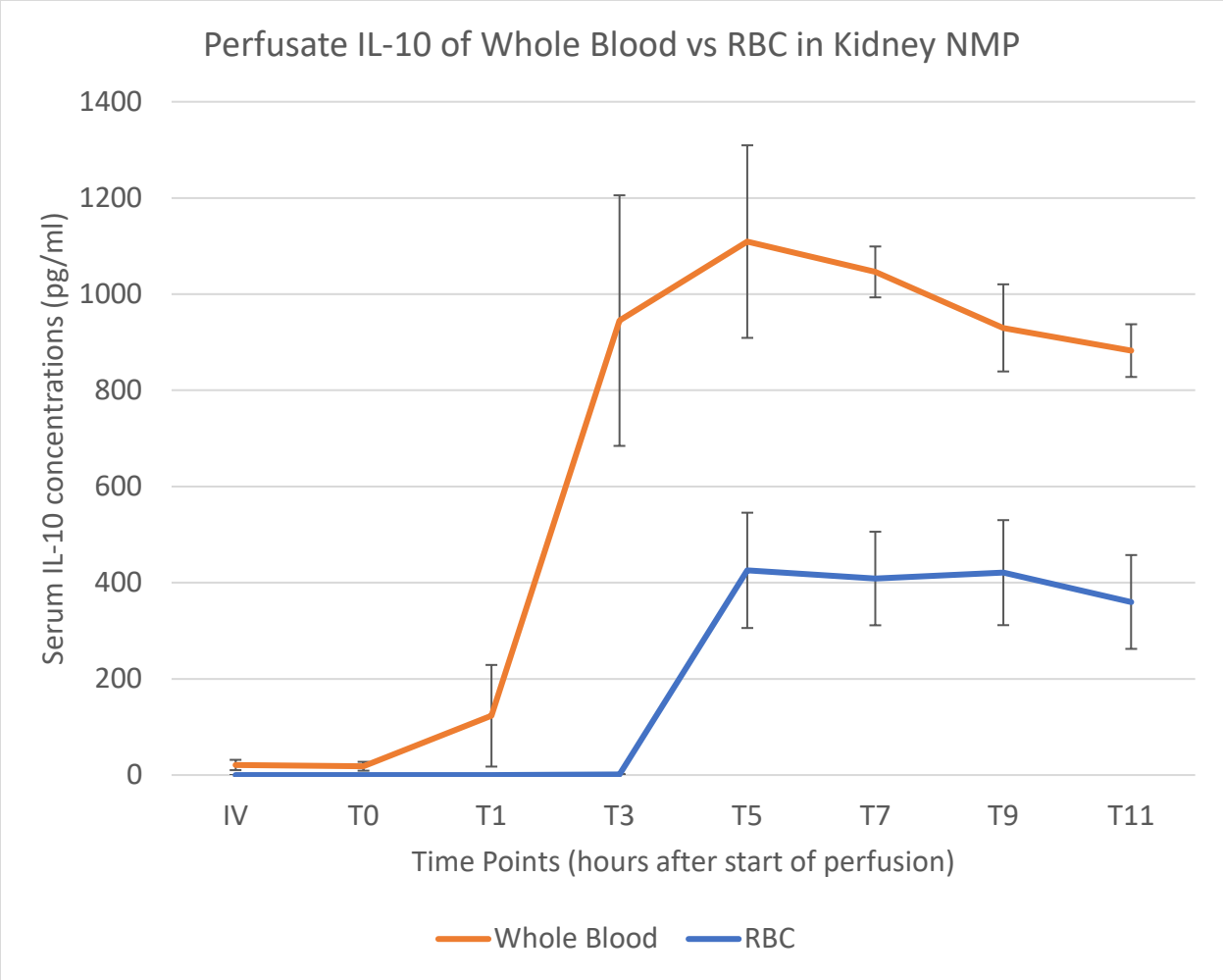


Figure 14. Perfusate concentrations of interleukin-10 (IL-10) in the whole blood and the red blood cell concentrate group shown in pg/ml. Values presented are means ± standard errors of means. Repeated measures ANOVA was used to test between group differences at time points T3, T5, T7, T9, and T11, statistically significant differences were found at all tested points ($p<0.05$).

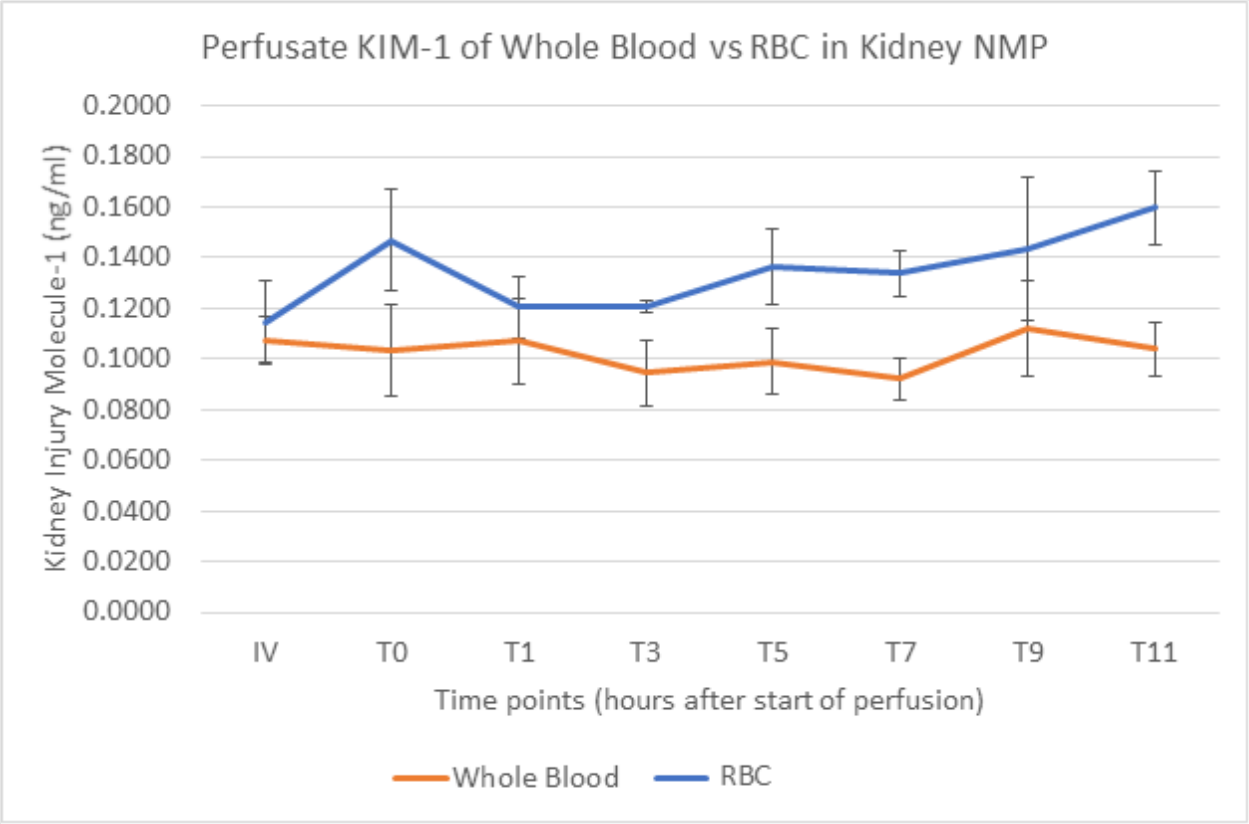


Figure 15. Perfusate concentrations of kidney injury molecule-1 (KIM-1) in the whole blood and the red blood cell concentrate group shown in ng/ml. Values presented are means ± standard errors of means. Repeated measures ANOVA was used to test between group differences, no statistically significant differences were found in time points until the 11th hour ($p>0.05$). At T11 or the 11th hour, there was statistically significant difference between the mean values ($p<0.05$).

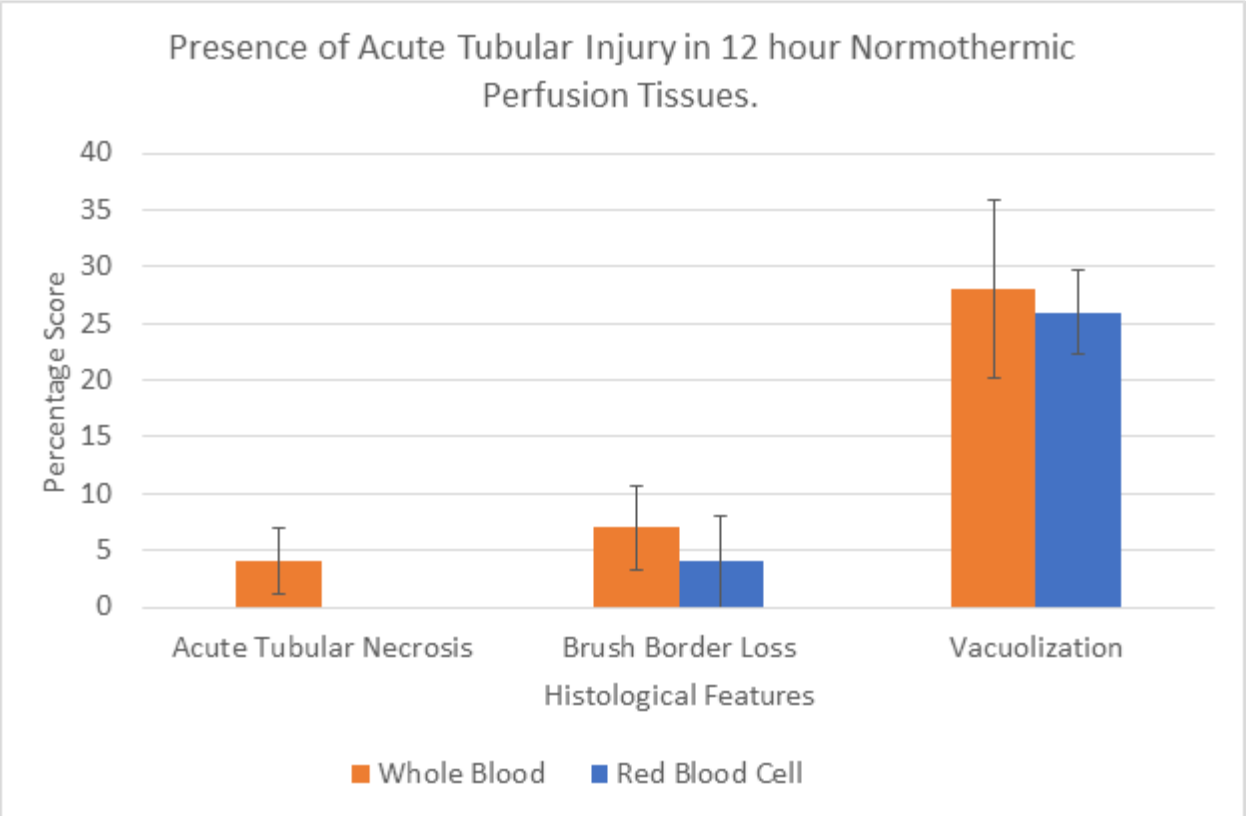


Figure 16. Histological scores of acute tubular necrosis, tubular epithelium brush border loss, and tubular epithelial cell vacuolization from the whole blood group and the red blood cell concentrate group. Values are presented as averaged percentage scores for each feature and error bars indicating standard error of means. Not shown are the categories for the presence of positively stained droplets and cellular cast formations within the tubular lumen, interstitial edema, and interstitial inflammation, as their presence was negligible in all samples. Whitney-Mann U-Tests were performed between the whole blood and red blood cell concentrate groups on percentage histology scores, no statistically significant differences were found ($p>0.05$).

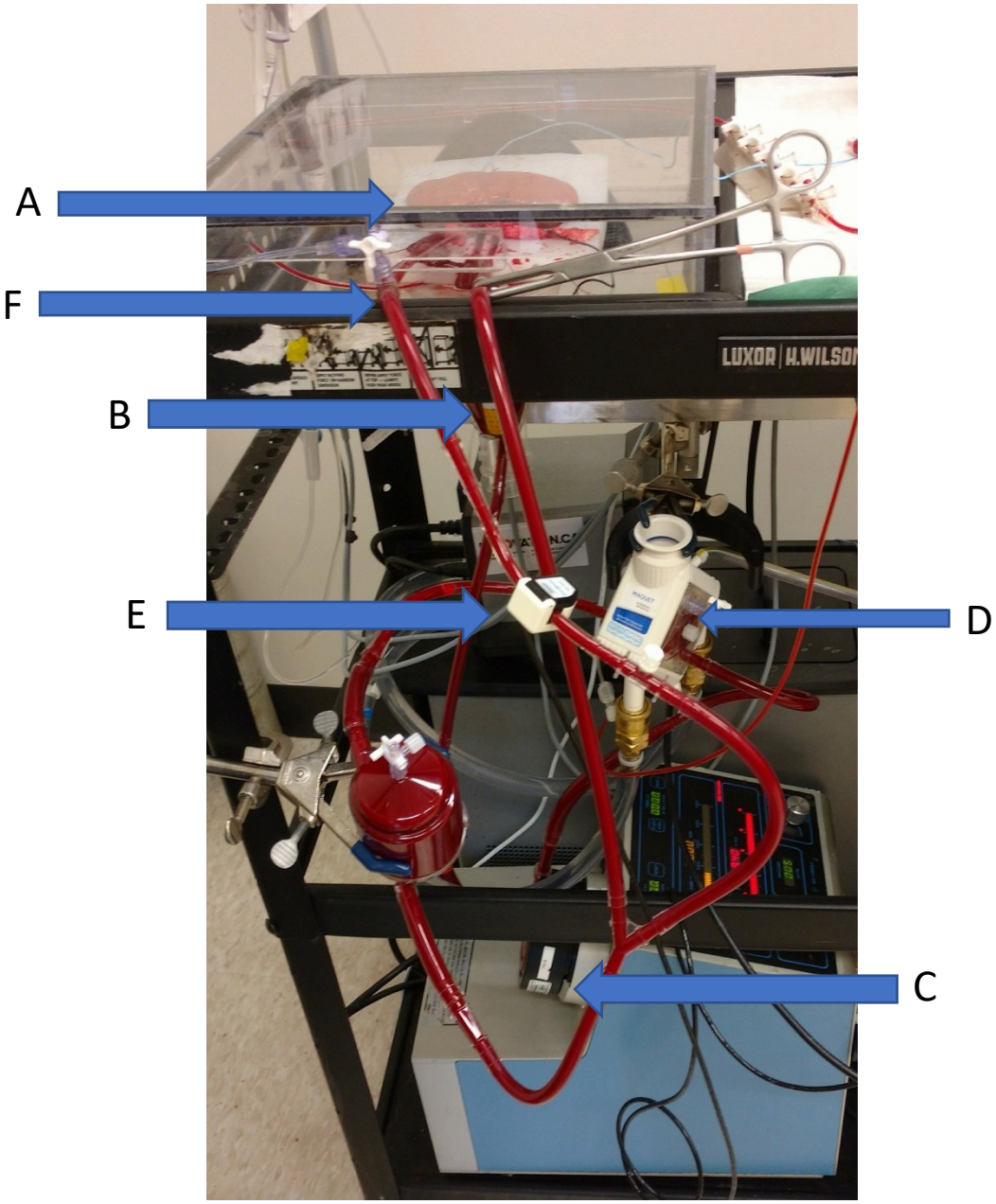


Figure 17. Labeled image of main components of the experimental set up showing A) the kidney sitting in its silicone holder, B) the venous reservoir where the venous outflow drains into, C) the centrifugal pump head, D) the oxygenator and heater, E) arterial flow module, and F) arterial catheter.

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