Exploring the Impact of Blood Components on Myocardial and Vascular Health During Normothermic Ex-situ Heart Perfusion

by

Mitchell J. Wagner

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

Master of Science

Department of Surgery

University of Alberta

Thesis Abstract

Each year, roughly 100,000 Canadians are diagnosed with heart failure, a condition that can only be cured by heart transplantation. In recent years, there has been a plateau of heart transplantation volume, thought to be the result of declining donor sources and the bottleneck of short preservation time of procured organs. Cold storage (CS) is the standard method of preservation for solid organ transplant, but has a limited out of body time to a maximum of 4-6 hours for heart transplants. Normothermic ex-situ heart perfusion (ESHP) preserves the heart in a semi-physiologic state by enabling out-of-body perfusion of the coronary arteries with a blood-based perfusion solution (perfusate) at physiologic temperatures.

While clinical ESHP platforms have shown promise in increasing preservation times, our group and others have documented persistent decline of myocardial function whilst on the apparatus. Our group has established that perfusion of the heart on the apparatus begets an inflammatory and pro-oxidative environment. One source of inflammation could be the breakdown of red blood cells (hemolysis) over time within the blood-based perfusate. Furthermore, the field is yet to investigate optimal levels of hemoglobin, which has significance for the development of optimal perfusion solutions. A current point of contention is whether dilution of whole blood used as a perfusate in clinical scenarios with buffers to fulfill volume requirements leads to inadequate oxygen delivery, and therefore functional decline.

As presented in chapter 2, we tested the hypothesis that greater hemoglobin (Hb) concentrations could mediate increased functional preservation. We subjected hearts of Yorkshire pigs to normothermic ESHP in the working mode for a duration of 12 hours in

groups varying in perfusate composition of hemoglobin: control perfusate (low hemoglobin due to dilution of whole blood with buffer, ~4 g/dL Hb), whole blood (no dilution, ~8.5 g/dL Hb), concentrated blood (whole blood concentrated by packed red blood cells obtained through cell saver, ~12 g/dL Hb). We also tested a low hemoglobin group whereby whole blood was cut with donor plasma ('plasma' group), rather than manufactured buffer. Overall, we found that functional preservation was worse when hemoglobin concentration was high. Markers of cardiac function including cardiac index, stroke work, left ventricular dP/dT maximum and minimum were significantly reduced in the high-Hb group compared to other groups by late perfusion. This coincided with evidence of increased hemolytic burden during these perfusions, denoted by elevated concentrations of free hemoglobin, perfusate potassium, and heightened transferrin saturation.

Throughout the study conducted in chapter 2, we noticed that coronary flow rates were much lower in the new controls and in the 'plasma' group than in runs conducted historically by our group under similar conditions during ESHP. Such supraphysiologic coronary flow rates are recognized as damage endured to the endothelium. In order to avoid hemodilution via fluids provision during heart procurement, the new controls were given minimal fluids whereas historical controls were given a high degree of fluids. This led us to hypothesize that plasma proteins may play an important role in preventing supraphysiologic coronary flow rates during ESHP.

In chapter 3, we explored this hypothesis, comparing historical control ESHP runs given high amounts of crystalloid fluid to newer runs given low amounts of crystalloid fluids. We also compared the runs to the plasma group (provided a low amount of fluids during

procurement). We also trialled supplementation of the Krebs-Heinseleit buffer with high amounts of albumin with low fluids provision during procurement. Overall, we found that in groups provided low fluids, coronary flow rates were significantly lower than in the group where high fluids were provided. This was associated with increased damage to the endothelial glycocalyx, which is an endothelial structure implicated in vascular mechanosensation and regulation of vessel vasomotor tone, preceding perfusion.

The work presented in this thesis provides a variety of valuable insights into how the perfusate can be optimized during ESHP to mitigate inflammatory and oxidative stressors, such as free heme. It also preliminarily emphasizes the importance of plasma proteins as a modality to effect improved coronary vascular preservation. These may be important modalities to improve the preservation of hearts subject to prolonged ESHP and mitigate debilitating post-transplant pathology such as cardiac allograft vasculopathy.

Preface

Parts of the first chapter have been published as: **Wagner M**, Hatami S, Freed D. 2023. Thoracic organ machine perfusion: A review of concepts with a focus on reconditioning therapies. Frontiers in Transplantation. 2. doi: 10.3389/frtra.2023.1060992. This is my original work as I drafted the article with conceptual assistance of Dr. Sanaz Hatami and Dr. Darren Freed.

Otherwise, chapters 2 and 3 constitute original works which are in preparation for peer-reviewed journals, with chapter 4 acting as a summary. In these prospective manuscripts representing collaborative works within chapters 2 and 3, I was principally responsible for all data collection, analysis, and manuscript composition, with collaborating individuals providing assistance with regard to methodology, interpretation of analyses, and copy-editing. Drs. Darren Freed and Jayan Nagendran are the senior authors and assisted additionally with conceptualization of the work.

Chapter 2: **Elevation of perfusate hemoglobin exacerbates functional decline during ex-situ heart perfusion through hemolysis.** Mitchell J. Wagner, Guilherme Mainardi Aguiar da Silva, Parham Hassanzadeh, Sanaz Hatami, Xiuhua Wang, Jayan Nagendran, Darren H. Freed.

Chapter 3: **The impacts of fluids provision & plasma components on coronary functionality during ex-situ heart perfusion.** Mitchell J. Wagner, Guilherme Mainardi Aguiar da Silva, Parham Hassanzadeh, Sanaz Hatami, Xiuhua Wang, Jayan Nagendran, Darren H. Freed.

Dedications

In addition to this thesis being dedicated to the generous community of individuals outlined below who have supported me throughout my development, this thesis is dedicated to the pigs, whose sacrifice entrains me to dutifully practice excellence and learn from my failures.

“All labor that uplifts humanity has dignity and importance and should be undertaken with painstaking excellence.” – *Martin Luther King, Jr.*

Acknowledgements

There are so many people that have supported me and contributed to my learning and development over the period of this thesis.

Firstly, I would like to thank my supervisor, Dr. Darren Freed. Dr. Freed – surely, more times than I can count on my fingers I have noted to myself and others how lucky I am to work under your guidance and benefit from your sincere mentorship. Even beyond our interactions, I have observed the kind of leader you are in the operating room and laboratory, and this has been absolutely inspirational for the type of clinician-scientist I hope to be one day. I have most of all appreciated the latitude you have given me to pursue ideas relevant to our work and of my own interest. I look forward to absorbing even more of your wisdom over the upcoming years of my training – thank you for everything so far.

I would also like to thank my supervisory committee members, Dr. Jason Acker and Dr. Jayan Nagendran. Though our interactions have been infrequent, your insights during committee meetings have been invaluable in shaping my thoughts about the work I have undertaken, and I greatly appreciate each of your interest in my development.

I also sincerely thank Drs. Jennifer Conway, Dilini Vethanayagam, and Karim Samji for your mentorship during (and outside of) clinical projects over the course of grad school. Undoubtedly this has helped me grow as an aspiring clinician-scientist and academic.

I must acknowledge my colleagues in the Freed laboratory and our collaborators, whose comradery have made this experience truly special. In no particular order thank you to Dr. Sanaz Hatami, for your mentorship and all that you have taught me about machine perfusion; Dr. Guilherme Mainardi, for being a fantastic surgical role model to learn from and do these experiments alongside; Parham Hassanzadeh, for being a reliable and pleasant mentee to teach; Xiuhua Wang, for your steadfast experimental support and delightful presence in the laboratory, and many more whom I do not have the space to mention here.

To our collaborators at the Ray Rajotte Surgical Medical Research Institute: Ryan and Katie B., thank you for tirelessly accommodating our work and making the operating

room a pleasant place to conduct these experiments, even when I opted to start at 6am. Your expertise knows no bounds.

I would not have made it this far if not for my loving family. Thank you to my mom and dad, Colleen and Todd Wagner, my sister Meagan Wagner, and my grandparents, Linda and Dennis Wagner and my late grandparents Allen and Bernice Huxley for helping me become the person I am today. To know that each of you are proud of me no matter what fills me with pride and motivates me to work hard in pursuit of my goals.

I have many friends to thank (you know who you are) for encouraging me to be ambitious but also offering me an escape when it's time to recharge. From proof-reading applications to listening to me recount seemingly obscure scientific theories to celebrating victories, I sincerely appreciate everyone who has been a supportive presence.

Last but not least, I would like to thank the Patient and Family Centered Care (PFCC) team and the Cardiac Family Advisory Care Team (CFACT) at the Stollery Children's Hospital for being so supportive of my further education and development whilst I worked there part-time during my graduate studies.

Table of Contents

List of Tables & Figures

List of Tables

List of Figures

List of Abbreviations:

2,3-DPG: 2,3 diphosphoglyceric acid

ACC: American College of Cardiology

AHA: American Heart Association

AIF: Apoptosis Inducible Factor

AMP: Adenosine Monophosphate

ANOVA: Analysis of Variance

Ao: Aortic

ATP: Adenosine Triphosphate

CAMKII: Calmodulin-dependent Protein Kinase II

CAV: Cardiac Allograft Vasculopathy

CB: Concentrated Blood

CD: Cluster of Differentiation

CHOP: C/EBP Homologous Protein

CMV: Cytomegalovirus

DAMP: Damage Associated Molecular Pattern

DBD: Donation after Brain Death

DCD: Donation after Circulatory Death

dP/dT: Rate of Pressure Change

ECD: Extended Criteria Donation

ECMO: Extracorporeal Membrane Oxygenation

ESHP: Ex-Situ Heart Perfusion

EVLP: Ex-Vivo Lung Perfusion

FiO_2: Fraction of Inspired Oxygen

Hb: Hemoglobin

HFpEF: Heart Failure with Preserved Ejection Fraction

HFrEF: Heart Failure with Reduced Rejection Fraction

HMP: Hypothermic Machine Perfusion

HMGB1: High Mobility Group Box 1 Protein

HTx: Heart Transplant

ICAM-1: Intercellular Adhesion Molecule 1

IRI: Ischemia-Reperfusion Injury

ISHLT: International Society of Heart and Lung Transplantation

IU: International Unit

IL: Interleukin

KHB: Krebs-Heinseleit Buffer

LA: Left Atrium

LAP: Left Atrial Pressure

LV: Left Ventricle

LVAD: Left Ventricle Assist Device

MDA: Malondialdehyde

metHb: Methemoglobin

mPTP: Mitochondria Permeability Transition Pore

MVO_2: Myocardial Oxygen Consumption

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

NCX: Sodium Calcium Exchanger

NfKB: Nuclear Factor Kappa-Light Chain Enhancer of Activated B-cells

NHE: Sodium Hydrogen Exchanger

NMP: Normothermic Machine Perfusion

NO_x: Nitrates and Nitrites

Nrf2: Nuclear Factor Erythroid 2-related Factor 2

NWM: Non-Working Mode

NYHA: New York Heart Association

OCS: Organ Care System

OPTN: Organ Procurement and Transplantation Network

PaO_2: Arterial Oxygen Pressure

PKA: Protein Kinase A

PGD: Primary Graft Dysfunction

PL: Plasma

PPP: Pentose Phosphate Pathway

RBC: Red Blood Cell

ROS: Reactive Oxygen Species

RPM: Rotations Per Minute

SCS: Static Cold Storage

SEM: Standard Error of the Mean

TCA: Tricarboxylic Acid Cycle

TNF-alpha: Tumor Necrosis Factor-alpha

TUNEL: Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling

VCAM-1: Vascular Cell Adhesion Protein-1

WB: Whole Blood

WM: Working Mode

Chapter 1: Introduction & Literature Review.

Parts of this chapter have been adapted and published as:

Wagner M, Hatami S, Freed D. 2023. Thoracic organ machine perfusion: A review of concepts with a focus on reconditioning therapies. Frontiers in Transplantation. 2. doi: 10.3389/frtra.2023.1060992

1.1 **Heart failure**

Heart failure is a debilitating disease characterized by an inability of the heart to pump blood, leading to pulmonary and systemic congestion as it worsens[1]. Overall, the disease is a major contributor to global disease burden, with a global prevalence of 1-3% in the adult population and a 5-year mortality rate of greater than 50%[2]. Heart failure inflicts a high degree of morbidity on patients, leading to a large number of hospitalizations which constitutes a high cost of care for the condition. This is exemplified by statistics from the United States: between 2017-2020, 6.7 million adults have been diagnosed (up from 6 million from 2015-2018) with annual costs of care totalling approximately $39.2-60 billion. These costs are expected to rise alongside the increasing prevalence of heart failure, to over $70 billion by 2050[3]. In Canada specifically, a survey by the Heart and Stroke Foundation suggested a high burden of heart failure, with 750,000 people living with the disease and another 100,000 individuals being diagnosed each year, with incidence increasing year over year[4]. Therefore, even without adding the opportunity costs associated with the morbidity inflicted by heart failure, the disease constitutes a major burden to general population health and health systems across the world[5], and is only set to worsen in the coming decades at current trajectories.

Heart failure as a whole can be subdivided into two main types: heart failure with preserved ejection fraction (HFpEF) and heart failure with reduced ejection fraction (HFrEF). They can also be classified by the New York Heart Association (NYHA) classes of heart failure, which range from Class I-IV, denoting increased burden and physical limitation due to heart failure as class increases[1,6]. There is additionally the American Heart Association (AHA) and American College of Cardiology (ACC) classification utilizing stages A through D: class A denotes patients at high risk of developing heart failure, and class D denotes patients with needing a high degree of care for heart failure despite receiving treatment[7,8]. It is important to classify heart failure given that it manifests in various forms, since this provides direction for treatment.

Regardless of type or classification however, the mainstay treatment for heart failure is pharmacotherapy. It comes in a variety of forms though the main goal is to either

lessen the workload of the heart or boost contractility such that congestion can be overcome[6]. While these pharmacotherapies have proven beneficial to both morbidity and mortality due to heart failure as demonstrated by improvements of patients in various classifications, they are not a curative treatment[6]. For example, a meta-analysis of pharmacological treatment for HFrEF found that the most optimal combination of drugs across 75 trials could be estimated to extend life expectancy by 7.9 years for a 50-year old, and 5.0 years for a 70-year-old[9]. Therefore, while these therapies may reduce disease burden in the short term and mildly improve life expectancy, they do not halt progression and thus are not a long-term solution.

Heart transplantation, on the other hand, constitutes a longer-term solution that is applicable in non-responders to therapy. Patients with advanced heart failure (NYHA class IV, or class D) are considered given the inability of mainstay treatments to improve disease[10]. Though, it is not without its own limitations, discussed in later sections of this chapter.

1.2 **Heart transplantation**

It wasn't until the sixties that heart transplantation took a leap forward into the clinical setting, after decades of animal research. In one of the most publicized medical events of the 20th century, Christiaan Barnard performed the world's first human to human heart transplant in 1967[11–14]. One year later in 1968, D.A. Cooley performed the first heart-lung transplant[11,15,16]. Though the recipients of such transplants quickly experienced complications, these monumental events brought to life the past decades of experimentation and demonstrated the potential of transplantation as a therapeutic option when others were exhausted. The immune system presented itself as a barrier to the success of these patients. It wasn't until chemical immunosuppression, as opposed to sublethal irradiation, emerged as an approach that would vastly increase the one-year survival of transplant patients. This technique began with the use of drugs like 6-mercaptopurine and azathioprine combined with steroids before cyclosporine was approved for clinical practice in 1984[11,14,17,18].

Currently, the International Thoracic Organ Transplant Registry reports that upwards of 115,000 heart transplants occurred worldwide between 1990-2015, and that median survival from most recent data was almost 15 years following transplant[19]. Publicly available data from the Organ Procurement and Transplantation Network (OPTN) reports that for the United States from 1988-2023, 92,000 heart transplants have been performed[20]. This data emphasizes how far transplantation has come in terms of safety, efficacy, and acceptance amongst health centers – heart transplantation is now considered a "gold standard" of treating late-stage heart failure[21–23].

While heart transplantation can confer the greatest yield to survival and morbidity in those with advanced heart failure, the supply of hearts is dwarfed by the number of individuals that could potentially benefit. Even within those where transplant is indicated, supply is limited: the International Society for Heart and Lung Transplant (ISHLT) reported in 2019 that heart transplants done across North America and Europe has plateaued at roughly 4500-5000 per year in recent decades. This is despite there being 7000-8000 people on the waitlist in the USA alone at any given moment[16,22,23]. Further, the number of individuals with NYHA class IV heart failure was estimated to range from 15,600 to 156,000 people[24]. Thus, a current challenge of heart transplantation (and essentially all other transplantable organs), is reconciling organ demand with the availability of organs[17,25]. Although organ supply may be unable to satisfy all demand, the world currently operates at below its potential transplant volumes due to a lack of optimization of the transplantation process[26]. From organ procurement to preservation, there are a variety of constraints on organ supply that can be tackled to improve the delivery and quality of hearts for transplant to individuals with severe heart failure. Until such bottlenecks are widened, individuals wait with severe illness and even die on the waitlist before they can get the transplant they need[27].

1.2.1 **Donor organ shortages: donor types, outcomes, and impacts on transplant volume**

The organ donor can be classified into three categories: donation after circulatory death (DCD), donation after brain death (DBD), and marginal or extended criteria

donors (ECD). DCD entails organ procurement following the cease of all cardiorespiratory activity, with an ethically mandated standoff period of 2-5 minutes to ensure that autoresuscitation does not occur[28]. Thus, procurement from circulatory death donors is associated with a greater degree of organ damage associated with ischemia in comparison to DBD donors, in which the persistence of cardiorespiratory mechanisms in the brain and clinical management can facilitate preservation of organs before procurement[29]. In the early days of transplantation, organs for transplant were procured from DCD donors, though DBD quickly overshadowed DCD donation[14,23,30]. In the current era, as donor demand is being significantly outpaced by donor supply, efforts to consider organs from suboptimal sources have resurged to boost transplant volumes.

It has been long appreciated that decreased ischemic times both during procurement and storage lead to a lower risk of primary graft dysfunction post transplant, being demonstrated in clinical studies[31–33]. For this reason, as well as management practices which can stabilize inflammatory and hormonal processes within the DBD donor that may be exacerbated in the DCD donor[34], organs from DBD donors are regarded as optimal[35,36]. However, improvements in road and vehicle safety, as well as improved management of conditions that result in brain death continue to decrease availability of donors from this source[21,36]. This is exemplified by data from Transport Canada: between 2002 and 2019 (in order to exclude pandemic years from our consideration), the number of collisions resulting in serious injuries (eg. traumatic brain injury) roughly halved from approximately 16,000 to 9000 per year[37]. Improvements in medicine have also impacted the DBD pool, demonstrated by the primary cause of brain death shifting from traumatic brain injury to brain hypoxia. Additionally, median age, and incidence of co-morbidities in donors have also increased, signalling a decrease in numbers but also quality of organs from this source[14,21,30].

The push to widen the donor pool, as well as a growing sentiment that traditional donation criteria restrict organ supply, has prompted a liberalization of traditional donor criteria, introducing the ‘marginal’ or ‘extended criteria’ donor (ECD)[25,38]. Liberalization of such criteria, in regard to heart transplantation, can vary between centres but typically entails accepting organs which have endured greater ischemic times, come from older

donors, have reduced ventricular performance (ejection fraction <50%), valvular disease present, focal lesions of the coronary artery present, and ventricular hypertrophy[39,40]. To provide an idea of the extra organ supply such criteria can provide, the ISHLT reported that between 2005 and 2018, an additional 20,000 lung transplants were undertaken due to such widening of traditional criteria[41]. A study of marginal heart donation found similarly that marginal donations could yield a 37% increase in transplant volume with similar risk-adjusted mortality to standard donation[39]. Other studies have reported similar numbers, with another study citing that between 2010-2021, 32% of heart donations met marginal criteria[42].

Along the same line, DCD donation has been met with renewed interest. Despite the increased ischemic burden on top of ischemia during cold storage due to the mandatory 'standoff' period, it has been reported that an additional 149 suitable heart donors could have been utilized over 2011-2013 at an institution in the United Kingdom if DCD hearts were considered, an unrealized 30% per year increase in cardiac transplants at that centre[43]. Furthermore, the same group showed that DCD hearts were non-inferior to DBD hearts based on 30-day survival[44]. A more recent study comparing 6 month and 1 year outcomes of recipients of DCD hearts reported no significant differences in comparison to DBD hearts in terms of 1 year survival, primary graft dysfunction, treated rejection, or cardiac allograft vasculopathy[45]. Even if DCD heart donation may perhaps only yield a short-term solution to patients on the waiting list, they represent a promising extra source of donor organs. Transplant centers within North America have already begun to make use of this extra donor source: within Canada as of 2021, 26% of locally retrieved organs were DCD status[46]. However, DCD hearts are currently not utilized in Canada despite similar conclusions that they can increase donor availability and therefore cut wait times[47].

1.2.2 **Donor organ shortages: pathophysiology of the procurement and transport process**

A second source of limitation on organ supply is the succession of pathophysiological insults experienced by such organs which are associated with donor

morbidities, as well as procurement and storage of the organs. These insults inevitably limit the period with which the organs can stay viable before they are donated and threaten primary graft dysfunction, which can necessitate a replacement for the newly received graft[48]. A short viability time following procurement limits utilization rates, makes highly matched donation difficult, and restricts donation to local donors[21].

The experienced onslaught begins before the organ is even procured. Both DBD, and to a greater degree DCD donation, are associated with the induction of a catecholamine storm. Catecholamines have been studied for their cardiotoxic effects, especially in models of stroke whereby transient elevations in catecholamines following stroke present a similar threat as Cushing's syndrome during brain death, which also precipitates a catecholamine storm[49,50]. Catecholamines have been associated with acute structural damage to the thoracic donor organs[23,34,51], demonstrated by the induction of apoptotic and necrotic damage to the myocardium, and compromise of the endothelium[52,53]. The cellular toxicity that yields apoptotic and necrotic damage due to catecholamine storm follows similar mechanisms as ischemia reperfusion injury (IRI) within the myocardium[49]: both epinephrine and norepinephrine act through a variety of mechanisms to induce intracellular calcium overload, which is a hallmark cellular pathology of IRI (discussed below). Acute levels of catecholamines yield a stimulation of G-proteins (Gs subtype), yielding the activation of protein kinase A (PKA) via the induction of cyclic AMP production by adenylate cyclase. PKA acts pathologically to phosphorylate key proteins involved in calcium cycling in the myocardium to precipitate elevated intracellular calcium: this includes L-type calcium channels, sarcoplasmic ryanodine receptors, phospholamban, troponin and myosin binding protein C (whereby phosphorylation weakens calcium affinity)[54]. The net affect is cytosolic calcium overload that translates into increased intramitochondrial calcium levels, which upsets mitochondrial membrane potential, prompts mitochondrial transition pore (mPTP) opening, and yields damaging oxidative stress[54,55]. Following these events, cells are prompted to undergo apoptosis.

During procurement, all organs undergo a period of global ischemia, followed by reperfusion injury upon donation. The cellular pathophysiology and result of IRI has

been well reviewed[23,48,56–58]. In brief, a lack of oxygen supply to the procured organ necessitates anaerobic metabolism, quickly depleting the cells of ATP needed to maintain ionic homeostasis whilst also producing acidifying hydrogen ions[23,48]. In light of the acidifying cytosol, sodium hydrogen exchangers (NHE) trade hydrogen ions for sodium ions, which are then acted on by the sodium calcium exchanger (NCX) to then move calcium in to maintain sodium at a low intracellular concentration. The net effect is accumulating calcium in the cytosol, which is exacerbated by sarcoplasmic reticulum failure. As ATP dwindles within the cell, calcium reuptake following contraction is impaired whilst ryanodine receptor activity is increased[59,60]. Supraphysiological calcium within the cytosol is pathological to the cell in a variety of ways. First, as mentioned above, it drives uptake of calcium into the mitochondrial matrix, upsetting mitochondrial membrane potential and triggering mPTP opening, an initiative process of cell death and ATP loss[61]. Second, it can activate calmodulin-dependent protein kinase II alpha subunit (CAMKII), which contributes to worsening calcium overload by phosphorylating sarcoplasmic reticulum proteins and activating inflammation via NfKB[62]. Third, it activates calpain, a cysteine protease which non-specifically cleaves a variety of proteins to influence mPTP opening, apoptosis, inflammasome activation, and autophagy[63]. Therefore, ionic homeostasis of great importance given the constellation of cellular processes affected by its dysregulation.

High osmolarity within the cells can also lead to the formation of edema, which further impairs function[44,64]. Reperfusion, while the only antidote for ischemia mediated pathology, only serves to briefly exacerbate its effects: the washout of formed proton gradients across the cell membrane during ischemia leads to further increased calcium intake. Reintroduction of oxygen to the cells, which have now built-up anaerobic metabolites and depleted their antioxidants, generates more deleterious ROS[23,56–58].

While the effects of IRI are well documented within the myocardium, it seems less attention has been paid to the effects of ischemia-reperfusion on the endothelium. This is especially important given that cardiac allograft vasculopathy (CAV) remains a leading long-term cause of death and re-transplantation following heart transplant[19]. CAV refers to microvascular pathology that is mediated following transplantation,

whereby eventual thickening of the vasculature from the epicardial vessels to the microvascular bed is a hallmark feature[65]. Similar to atherosclerosis, this can result in eventual occlusion of the vessel with downstream ischemia taking place[66]. Treatment of CAV similarly to an atherosclerotic lesion can help explain why statin therapy is beneficial to mitigate progression, though this seems to be challenged in children and adolescents[67,68]. Along similar lines, post-transplant hypertension is also associated with incidence of CAV, with there being evidence that antihypertensives may slow development[67,69].

Perhaps it is true that pathology within the endothelium brought by ischemia-reperfusion injury primes long term vasculopathy and rejection, given that the endothelium interfaces with the recipient hematopoietic system. Indeed, IRI related to the transplant process has been identified as a risk factor for CAV[65], with studies in mice suggesting that oxidative stress and inflammation arising from ischemia-reperfusion injury contribute to initiating the immune response that gives way to CAV[70–72]. One such study by Fukunaga *et al*. in 2020 showed that in a mouse model of CAV, knockout of Nrf2 (a regulator of cellular antioxidant responses) precipitated greater graft dysfunction and coronary luminal narrowing, whereas agonism of Nrf2 with sulforaphane helped protect against such effects[71]. When this evidence is considered in parallel with studies that show that immune recruitment and activation in the context of allograft injury follows complement deposition or endothelial cytokine release[72,73], it appears that endothelial cell activation due to either IRI or donor specific factors may lead to the induction of chronic inflammation. This is reinforced by serum samples studied in pediatric heart transplant (HTx) recipients: patients receiving HTx had increased levels of circulating IL-6, VCAM-1, ICAM-1, and thrombomodulin, with individuals with increased intima-media thickness (a marker for CAV) having further raised von Willebrand factor[74]. Therefore, ways to mitigate endothelial activation throughout the transplant process and during follow-up may be valuable ways to hamper incidence and progression of CAV.

1.3 **Introduction to ex-situ organ preservation**

1.3.1 **Static cold storage & its limitations**

The ability to preserve organs for an extended period following procurement without loss of function represents the holy grail of organ preservation and has been the subject of many investigations in recent years. Static cold storage (SCS), a mainstay of organ transplantation, stores organs in a non-functioning manner at 4°C. The provision of hypothermia to the ischemic organ is done to 1) slow rates of metabolism which fuel processes that degrade vital cellular proteins, and 2) to slow the lysis of lysosomes containing autolytic enzymes[58]. Though SCS is inexpensive and requires minimal effort and technical expertise, hearts may only be preserved for a maximum of 6 hours before the risk of primary graft dysfunction post-transplant becomes intolerable. This is despite the delivery of a cardioplegic solution that reduces oxygen consumption[21,30,75,76]. Similarly, lungs which have been stored in this manner are used given that the preservation period is no more than 8 hours, though retrospective study has showed no significant difference in outcomes when preserved by SCS for 12 hours[77]. The slowed onset of anaerobic metabolism via lessened metabolic requirements of the lungs and lingering oxygen within the alveoli can perhaps explain the lengthened period of viability in comparison to the heart during SCS. This can be taken as a suggestion that maintaining oxygen delivery to organs during storage can increase viability time; indeed, this is a valuable strategy discussed below. Nevertheless, given the cold ischaemic stress that thoracic organs are subject to during SCS, the method is not suitable for extended criteria nor DCD donations; their inability to tolerate further damage during cold storage and an inability to evaluate the functionality of the organ during storage present further limitations to this method[23,78].

1.3.2 **Historical context of ex-situ heart perfusion**

Preliminary studies by Wicomb *et al.* and Hassanein *et al.* in the 80's and late 90s respectively signaled a growing appreciation that preservation of the donor heart

entailed keeping the organ supplied with continuous oxygenation as well as nutritional and ionic support[79–81]. These initial studies, contrasting in their approach, exemplify the two main frontiers of heart machine perfusion: hypothermic machine perfusion (HMP), whereby the heart is kept at low temperatures in a non-working mode (Langendorff perfusion); or normothermic machine perfusion (NMP), whereby perfusion is undertaken at temperatures close to 37℃ in either non-working or working modes. As with clinical protocols developed for the lungs, there is a variety of experimental and clinical approaches that are accompanied by a plethora of formulations of the perfusion circuit and perfusate composition and come with their own advantages and disadvantages. For ESHP, HMP was first investigated as an upgrade to static cold storage, with NMP being explored later in the history of the field of heart machine perfusion.

1.3.3 **Hypothermic machine perfusion of the donor heart.**

Generally, HMP keeps the ex-situ heart in a non-beating state, similar to SCS, typically at temperatures between 4-8℃. HMP quickly gained appreciation by investigators as an improvement over SCS, given that the heart could be maintained with more physiologic delivery of nutritional requirements and washout of metabolic wastes while still providing myocardial cooling[82]. Wicomb *et al*. demonstrated the potential for HMP to preserve donor hearts in the early 1980s. The group showed that pig hearts retained a level of cardiac output and stroke volume comparable to those of freshly excised hearts, being significantly preserved compared to hearts kept on classic SCS after 24h of preservation time[80]. It was also noted in a canine model that oxidative stress as measured by 8-oxoG (a marker of ROS mediated DNA damage) was lessened during HMP[83]. Further investigation showed that in comparison to SCS, HMP was shown to better preserve endothelial structure as marked by reduced levels of endothelin-1 (ET-1, a marker of endothelial damage), as well as a lack of microscopic ultrastructural damage observed after 4 hours of perfusion in comparison to SCS[82,84]. Better ultrastructural characteristics were also shown in a comparison of hypothermic perfusion methods[85]. Perfusion of porcine hearts for 4 hours resulted in lower lactate levels, reduced AMP/ATP ratios, and higher phosphocreatine/creatine ratio in addition to

preserved functional parameters, suggesting that HMP provides superior metabolic outcomes to SCS[86].

Despite a plethora of evidence of superiority over SCS, the uptake of HMP into clinical use at first was limited for a few reasons. Formation of edema due to perfusion is a concern of this technique, given the fact that many studies have reported this[80,83,85,87]. The formation of edema can compromise myocardial perfusion by increasing coronary vascular resistance, exposing cardiomyocytes to varying levels of oxygenation[83]. Edema has also been associated with worsened diastolic function[22]. The episode of cold ischemia-reperfusion imposed by HMP may be unacceptable for some extended criteria or DCD donor organs, which are increasingly being utilized to meet organ demand[22,23]. Another concern is that HMP, as with NMP, is a much more expensive and technically challenging method with regard to the mechanics of the circuit and perfusate. This was exemplified in some studies of HMP. Fitton *et al*. reported that only 60% of hearts being perfused by HMP were able to be successfully weaned from bypass[83]. In a study by Wicomb *et al*, 6/10 hearts utilizing modified Krebs solution were functionally nonviable due to a complication with the perfusate, whereby the cause would be almost impossible to verify concretely due to the multiplicity of ingredients in the perfusate[79]. Thus, the risk of edema and the introduction of a host of variables through HMP represented a great risk to reliability not mirrored by SCS, perhaps adding resistance to clinical uptake. A final disadvantage of HMP is a lack of continuous functional evaluation. Though some apparatuses may allow hearts to be transitioned into working mode for functional evaluation, most rely on periodic assessment by an intraventricular catheter, preventing real-time evaluation of the myocardial function. Further, measurements of function at low temperature or even after being rewarmed may not be entirely comparable to a heart kept at normothermia throughout preservation. This is an important consideration, given the fragile nature of extended criteria and DCD organs being increasingly utilized to widen the donor pool, which require close monitoring and management.

1.4 **Normothermic machine perfusion of the donor heart**

1.4.1 **Normothermic machine perfusion vs. Hypothermic machine perfusion**

In comparison to HMP, NMP keeps the heart in a semi-physiologic state at ~37ºC. The provision of a working mode in which the left ventricle pumps against a physiological workload (afterload), has been achieved by our group, enabling functional parameters to be measured in real time by varying pump flow[88]. However, clinical ESHP protocol such as the TransMedics Organ Care System™ (OCS) protocol perfuse the heart in a non-working, unloaded state which precludes direct functional assessment[89]. Hassanein and colleagues were among the first groups to publish on ex-situ heart perfusion at normothermia using a blood-based perfusate, finding that in comparison to SCS, hearts perfused at normothermia benefitted from preserved contractile, metabolic, and vasomotor function, with decreased edema formation over 12 hours[81]. Theoretically, NMP enables better preservation of donor organs than SCS, or even HMP as applicable to ESHP, by subverting the period of cold ischemia which perpetuates time dependent reperfusion injury and potential metabolic dysfunction at low temperatures. This allows better preservation of organs, especially those from extended criteria or DCD donors[90].

Beyond the ischemic insults that accompany procurement, NMP enables a suite of benefits that may be harder to realize on an HMP platform. NMP is more compatible with blood based perfusates in comparison to HMP. This compatibility is due to the fact that in hypothermic settings, blood must be diluted to prevent coagulopathies resulting from increased viscosity at low temperatures. Furthermore, the dissociation of oxygen from hemoglobin is attenuated at low temperatures, becoming indissociable at 12ºC[21,91]. Blood is thought to offer a more physiologic delivery of oxygen via red blood cells (RBCs) and has the additional nutritional and oncotic benefits of plasma components in comparison to synthetic solutions used in the setting of HMP[21,92]. Near physiological metabolism at higher temperatures enables the prospect of dynamic, responsive management via pharmacological agents and hormonal support, an especially pertinent benefit for suboptimal donor organs[21,93]. Normothermia, while complimentary to

responsive management, also lends itself to reconditioning approaches working as intended given that gene expression profiles and the functioning of enzymes or proteins can be altered at cold temperatures. Increased temperatures also make it a better platform for delivery of novel gene therapies, whereby efficient viral delivery of the target gene is temperature dependent[94]. Multiple groups have demonstrated that an ESHP setup at normothermia is able to facilitate thorough transgene delivery and expression throughout the heart using a luciferase expression system leveraging a cytomegalovirus (CMV) promoter[94,95]. This reconditioning modality has been explored to a greater extent on the ex-vivo lung perfusion platform, with studies experimenting with IL-10 transgene[96–98]; such studies are conducted at normothermia for this reason.

1.4.2 **The clinical evidence for normothermic ex-situ heart perfusion.**

NMP has been generally regarded as the most optimal temperature for which out of body preservation can be affected, with a suite of theoretical benefits compared to other perfusion temperatures as mentioned. Practically speaking, the provision of machine perfusion to the clinical sphere has also transformed transplant capabilities, with an abundance of clinical trial results published to demonstrate its practicality in effecting greater organ utilization. The only currently clinically available normothermic ESHP apparatus, the TransMedics OCS, has gone through an abundance of clinical study: non-randomized PROCEED I and PROTECT I trials were conducted in the USA and Europe respectively, comparing NMP using the OCS platform with traditional SCS. After, the PROCEED II prospective, multi-center, randomized trial established non-inferiority of the OCS heart platform with SCS[89,99]. Findings reported from the PROCEED II trial found that in comparison to hearts stored using SCS, there was no significant difference in adverse cardiac related events or 30-day graft survival, though there was a significantly increased out of body preservation time (5.4 hours on OCS vs. 3.2 hours of SCS)[89,100]. While the designation of study endpoints in this trial precludes insight of the myocardial protection effected by NMP, it at least demonstrates its efficacy as a vehicle to effect greater transport distance of procured hearts while decreasing the period of cold ischemia, therefore benefitting organ utilization.

It was originally thought that lactate >5 mmol/L by the end of the run could provide sensitive and specific prediction of outcomes following perfusion[101], however it was pointed out that a low lactate level does not necessarily preclude a high risk heart, as demonstrated by a case study by Stamp and colleagues[102,103]. Experimental study by our own group has suggested a very strong correlation of functional parameters with myocardial viability during NMP rather than lactate, however this would require the OCS to support the excised heart in a loaded, working mode, which is a current limitation[21]. Regardless, more optimal predictors for graft performance following clinical machine perfusion that can be assessed before transplantation are warranted.

The OCS Heart EXPAND trial, which was a single arm study to assess the use of OCS within ECD heart transplantation also demonstrated the ability of the OCS to widen the heart donor pool[104]. They reported that an additional 75/93 (81%) ECD hearts were successfully transplanted, with a mean OCS perfusion time of 6.35 hours and 30-day and 6-month survival rates at 94.7% and 88% respectively[104]. This is very favourable to the prospect of widening the donor pool, given that most ECD hearts would not be able to tolerate six hours of SCS. Further to widening the donor pool, additional study has been conducted for DCD heart transplantation as well: a randomized controlled trial comparing DCD transplantation using OCS preservation with DBD hearts preserved with SCS showed a high utilization rate (89%), with survival rates for DCD transplantees and grafts trending higher than control up to 1 year[105]. This echoes earlier studies carried out utilizing normothermic regional perfusion by Messer and colleagues, which showed that DCD heart transplants did not significantly differ in terms of survival at 1 year, length of hospital stay, or adverse events related to the allograft[106]. Therefore, there exists strong evidence to suggest that NMP is a powerful technique to not only increase the distance between potential donor-recipient pairs, but also to unlock extra supply of hearts via ECD or DCD transplants while retaining comparable patient/graft survival outcomes.

These are not the only benefits to be realized, as the OCS platform has also proven beneficial to complex congenital patients and left ventricular assist device (LVAD) recipients. Patients with multiple VAD implantations or congenital patients with

complex cardiac anatomy pose a challenge for surgeons to navigate delicate mediastinal dissection quickly to explant the recipient's original heart, therefore increasing the amount of time the donor organ must wait on ice before implantation. However, with the OCS providing continuous metabolic support, performing surgeons can take their time to perform careful dissection without worrying about deleterious time-related cold ischemia pathology to the incoming organ[107–109].

1.5 **A deep dive into functional decline during normothermic ex-situ heart perfusion.**

Despite this potential, ESHP is currently limited by characteristic functional decline over the course of perfusion, which limits the post-procurement preservation period[110,111]. The exact mechanism behind this process is unknown, however our group has been on the leading edge of investigation within this area. Our studies have revealed that functional decline during normothermic ESHP in the working mode is associated with an intermingling of dysregulated metabolism, oxidative stress, and inflammation[112]. The evidence for each is discussed in the following sections.

1.5.1 **The role of leukocytes and cell death in functional decline during ESHP**

A variety of oxidative stress markers such as malondialdehyde (MDA) and global carbonylation and sulfonation of proteins are increased concomitantly with the concentration of reduced glutathione being reduced during ESHP[113]. While such oxidative stress markers could be resultant from CD68+ cell (a marker for macrophages, monocytes, and neutrophils) infiltration of the myocardium, observed during ESHP[114], perfusion with a leuko-reduced perfusate resulted in neither a reprieve from decline in functional parameters, nor exacerbations in oxidative stress markers. In fact, leuko-reduction resulted in increased TNF-α concentration by mid-perfusion, increased protein carbonylation, and worsened dP/dT minimum preservation by late perfusion[113]. Additionally, working mode (WM) perfusion (which in comparison to non-working mode (NWM) has featured improved functional preservation, reductions in

NfKB activation and inflammatory cytokine expression, and oxidative stress) had a greater total leukocyte infiltrate compared to non-working mode perfusion[114]. Taken together, these results suggest that during ESHP, oxidative stress occurs within the heart and initiates inflammation, rather than the other way around; immune cells may perhaps even play a protective role during ESHP given that their loss displayed worsened biochemical and functional parameters in some regards.

An analysis of cell death following perfusion has found that the percentage of TUNEL positive cells (a marker for cell apoptosis) increases during ESHP, however the overall percentage in all groups was less than 1.5% of cells. This is corroborated with other markers of apoptosis such as cleaved caspase-3, apoptosis inducing factor (AIF) and CCAAT-enhancer-binding protein homologous protein (CHOP) trending towards increases during ESHP, but generally not being significantly increased[110]. It is concluded that the total amount of apoptotic cell death is not great enough to justify the degree of functional decline observed. However, it should be noted that though the degree of cell death may not directly explain the loss of functional output, this does not entirely rule it out as a potential contributor to the immune milieu that pervades ESHP. The kinetics of such cell death have not been characterized during ESHP. It is possible that following IRI, the increase in necrosis or apoptosis of cells within the myocardium is combatted by resident macrophages which serve to remove these cells[115,116], of which phagocytosis can occur within a matter of hours[117]. This would dampen the number of apoptotic cells observed following the end of perfusion. It has also been reported that in some instances, the TUNEL assay has failed to detect necrotic cells, presumably due to differences in how DNA cleavage occurs between apoptosis and necrosis[118,119]. This preposition would additionally aid to explain the expansion of seemingly protective CD68+ cells within the myocardium that results following reperfusion and that their loss from the perfusate actually worsens preservation of some parameters. From this perspective, the heightened infiltrate of leukocytes during WM also associates with reduced appearance of cardiac troponin-I in perfusate during WM ESHP, suggesting that these cells could contribute to either prevention of further damage, or ‘mop up’ proteins (namely, DAMPs) that contribute to the immune milieu[114]. It is therefore possible that the actions of the myocardial resident immune cells, or even infiltrating

leukocytes from the whole blood perfusate, act to stabilize the consequences of ischemia-reperfusion injury once placed on the machine perfusion apparatus, rather than exacerbate them. This would also suggest that perhaps some of the cytokine evolution during ESHP is the result of resolving, rather than aggravating processes following IRI during machine perfusion.

In competition with this hypothesis, it should be considered that ischemia can induce membrane blebbing to shed cardiac troponin into the circulation without necrosis, with the degree of ischemia thought to increase blebbing[120,121]. However, if the release of cardiac troponin-I in this manner is only dependent on the degree of ischemia apart from necrosis, it does not explain differences in NWM and WM hearts, given that in our studies they are subject to the same degree of ischemic time before reperfusion. Therefore, it may be possible that the release of cardiac troponin-I apart from necrosis is related more tightly to the degree of cellular stress rather than ischemia. More study is required with a more detailed consideration of cell death, and how inflammatory cytosolic proteins like cardiac troponin-I may be elicited during machine perfusion apart from it. Regardless, IRI and oxidative stress experienced during ESHP appears to prime these processes whether they arise from cell death or simply cellular stress.

1.5.2 **The contribution of red blood cells to oxidative stress during ESHP**

If leukocytes do not initiate oxidative stress during ESHP, the only other cellular perfusate-based component to consider would be RBCs. Breakdown of RBCs (referred to as hemolysis) experienced during normothermic ESHP is a consequence of interaction with artificial/manufactured components of the apparatus and is also observed in extracorporeal membrane oxygenation (ECMO) and cardiopulmonary bypass (CPB) as a complication[122–125]. During ECMO, severe hemolysis has been emphasized as an independent predictor of mortality[125]. Another study showed that simply the occurrence of hemolysis was associated with increased mortality, being higher in non-survivors[124]. Given that hemoglobin (Hb) is filtered by the kidney, making it more susceptible to the toxicities described below, it has been shown to independently associate with kidney failure in pediatric ECMO[123]. ESHP is no exception as an

extracorporeal apparatus; in fact, the organ is theoretically more susceptible to such an insult given the lack of continuous support of the other organs that would be present during CPB or ECMO like the kidney and liver. Further, the burden of cytotoxicity is not spread between many organs and tissues as it would be in CPD and ECMO. In ESHP, it is solely focused within the lone organ being perfused.

Hemolysis is an incredibly oxidative and inflammatory occurrence. When RBCs break, they release free Hb. Once released, Hb, and particularly the iron stored within its heme molecules, no longer benefits from the reducing environment of the RBC, enabling tetrameric Hb to both dissociate into alpha-beta dimers and become oxidized[126]. Such dimers readily translocate across endothelial barriers to accumulate within membranes and interstitial spaces, and act as potent nitric oxide scavengers to mediate impairments in vasodilation[126,127]. Reaction of Hb with nitric oxide oxidizes Hb into methemoglobin (metHb), which further promotes the release of free heme. Heme acts as a damage signal, binding to inflammatory toll-like receptors that can prompt NfKB activation on endothelial cells[128]. Heme also intercalates into lipid membranes whereby its iron can catalyze the oxidation of the cell membrane to form lipid peroxides; this promotes membrane permeability and activates signalling to trigger inflammation[129]. Indeed, hemolysis is a feed-forward process since free heme undermines the integrity of intact RBCs, triggering them to release their own free heme. Apart from the liberation of free heme, breakdown of hemoglobin into such metabolites is a major contributor of free iron that, though mitigated by transferrin found in the plasma, reacts with hydrogen peroxide to produce potently toxic hydroxyl radicals via the classic Fenton reaction[126,129,130].

Hemolysis is typically mitigated in-vivo by the presence of acute phase proteins from the liver such as haptoglobin and hemopexin. These proteins scavenge the freed hemoglobin to prevent interim toxicity[131] before transporting it to processing systems: haptoglobin captures free hemoglobin and transports it to CD163+ macrophages of the reticuloendothelial system for processing. Hemopexin similarly complexes free hemoglobin but instead transports it to the liver for uptake via CD91[126,129]. Once these two scavenging systems are exhausted, albumin as a last stand is capable of binding

free heme and is known to be uptaken into cells via CD71 and acted on by heme-oxygenase-1[132].

Regarding the ESHP apparatus, such detoxifying mechanisms that handle the excessive hemolysis are not present, therefore the byproducts of hemolysis are free to wreak havoc on the isolated organ. Indeed, we have shown that during perfusion, the amount of plasma free hemoglobin rises steadily over the course of perfusion and is elevated by using whole blood versus a dilute whole blood perfusate; this is associated with increased nitrotyrosine (a marker of peroxynitrite formation) and protein carbonyl content within the coronary arteries of hearts perfused with whole blood versus dilute whole blood[133]. This suggests that hemolysis directly from the provision of a greater amount of red blood cells contributes to oxidative stress within the vasculature.

1.5.3 **Observed metabolic dysfunction during ESHP: linkage to oxidative stress**

The current approach to substrate provision during ESHP is to provide post-prandial (post-meal) levels of glucose and insulin. The only clinically approved TransMedics OCS Heart perfusion protocol calls for the addition of 80 IU of insulin to the perfusate[89], constituting a final concentration of >50 IU/L which is significantly greater than even physiological post-prandial serum insulin of up to 0.3 IU/L. This represents a metabolic outlay during ESHP similar to diabetic individuals and may contribute to metabolic dysregulation in a similar fashion to that observed in diabetic cardiomyopathies[134]. Results from our own model utilizing post-prandial glucose and insulin have shown that the heart experiences disturbances in glycolytic pathways which limits energetic efficiency of glucose. This has been corroborated by a study from another group[135]. During ESHP, glucose uptake increases over time, yet myocardial oxygen consumption declines[110]. Following ESHP, the activity of pyruvate kinase, a rate limiting enzyme for flux into the tricarboxylic acid (TCA) cycle, is decreased[110]. This suggests that the use of glucose for high energy oxidative phosphorylation is impaired.

Concomitantly, there is a transition from glucose oxidation towards anabolic pathways such as the pentose phosphate pathway (PPP) and hexose biosynthesis

pathways (HBP), evidenced by increased glucose-6-phosphate dehydrogenase activity and protein O-GlcNacylation (markers of flux through the PPP and HBP respectively) during ESHP[113]. These results suggest that the heart may experience an energy-deplete state as it diverts metabolism away from glucose oxidation towards combatting oxidative stress. Indeed, reduced glutathione (the heart's main anti-oxidant) is decreased during ESHP[113], perhaps as a result of perpetual oxidative stress while isolated on the ESHP platform[136]. While diversion of glucose towards the PPP can produce anti-oxidant reduced glutathione, it may also stimulate oxidative stress by providing substrate for NADPH oxidases, which generate ROS[137]. Provision of pyruvate during late perfusion resulted in a partial reversion in the loss of cardiac index during ESHP[110], perhaps through a bypass of the glycolytic and PPP/HBP pathways to feed directly into glucose oxidation. This is supported by another study comparing glucose and pyruvate supplementation during hypothermic ESHP, concluding that pyruvate more efficiently contributed to the TCA cycle[135]. Therefore, we surmise that provision of substrates that can contribute directly to oxidative phosphorylation during ESHP have great potential to limit oxidative stress and ameliorate associated functional decline.

It is well appreciated that donor organs being preserved ex-situ suffer from steady functional decline, contrasted by the observation that donor organs, once attached to the circulatory system of a recipient, last for weeks to years. The marriage of the perfusatory apparatus and the circulatory system of a live animal during cross circulation has gained much attention for its ability to recondition donor lungs, and has also been investigated in the heart[138–141]. In a landmark study by Hozain *et al.*, human lungs unsuitable for transplant were reconditioned over 24 hours of xenogenic cross-circulation, with PaO_2/FiO_2 ratios increasing a mean of 135 mmHg to a mean of roughly 320 mmHg, marking a significant increase in functional recruitment and thus transplantability[140]. They report significantly reduced inflammatory cytokines within the bronchoalveolar lavage (BAL), decreased tissue infiltrate of neutrophils, decreased apoptosis score on histology, and a significant reduction in serum P-selectin (a marker for endothelial damage). Similarly, hearts subjected to continuous xenogenic cross-circulation benefitted from increased ex-situ viability time, reported to be up to 3 days, associated with significantly decreased edema formation[139]. Despite these fascinating

results, either group reports no discrete mechanism for the observed functional improvements.

There are two hypotheses for the mediated functional benefit to the donor organ in this setting: one, cross-circulation of the plasma allows for function-deteriorating molecules to be filtered out as a consequence of the live animal's hepatorenal system; and two, molecules supplied to the plasma by the introduced body system (eg. nutrients, hormones etc.) maintain vasomotor tone as well as endothelial and parenchymal tissue integrity such that function of the donor organ is better maintained throughout perfusion[138,139]. Given that physiologic maintenance of organ function *in-vivo* is the consequence of homeostatic mechanisms facilitated by organ-organ interactions, it is a small leap to assume that each of these hypotheses likely contributes to the observed benefit during ex-situ organ perfusion.

Research is ongoing to decipher the molecules being either subtracted from or added to the perfusate. Hemofiltration shows much promise as an approach to investigate the subtractive hypothesis, as important substrates can be monitored over time within the perfusate and hemofiltrate and associated with functional benefits, without confounding additions by unknown sources, as would be the case with cross-circulation. For ESHP, Johnson *et al.* reports that in comparison to controls, hearts subjected to continuous hemofiltration during ESHP showed decreased edema, reduced histological damage scoring, and a lack of increase in coronary resistance. It may be that hemofiltration removed edema-inducing factors which decreased coronary occlusion, therefore mitigating anaerobic respiration and myocardial damage over the course of the run[142]. The prospect of hemofiltration in combatting induced edema has also been studied in the context of the lungs, where hyper concentration of the perfusate by continuous hemofiltration without fluid replacement was able to mediate up to a third reduction in gained lung fluid, though did not affect P/F ratios[143].

Another hypothesis, alluded to by Nilsson and colleagues, is that hemofiltration may be able to remove free hemoglobin released by hemolysis during perfusion[143]. They point out that free hemoglobin is able to scavenge nitric oxide, inhibiting its ability to induce vasodilation; thus, a removal of free hemoglobin by the hemofilter may explain

the lack of increase in coronary resistance observed during continuous hemofiltration of donor hearts ex-situ[144,145]. It is also known that free hemoglobin is a cellular toxin, mediating oxidative stress, endothelial cell injury, and inflammation in settings of high hemolysis[146]. This sentiment is echoed in cross-circulation of donor hearts, whereby blood based cross-circulation was reported to yield higher injury scores on histology than plasma only cross-circulation despite little other reported differences[138].

Washout of cytokines, and perhaps inflammatory damage associated molecular patterns (DAMPs), by either hemofiltration or xenogenic cross-circulation may contribute to their affects on perfused organs. This explanation seems particularly applicable in the study by Hozain *et al.*, given that the steady decrease of inflammatory cytokines as measured in bronchoalveolar lavage (BAL) along with reduced leukocyte infiltrate could be most associated with the functional benefit to the lung during cross circulation[140]. This is reinforced by other studies, whereby cytokine adsorbers utilized to sequester the inflammatory milieu were associated with improved lung function, edema formation, and reduced incidence of PGD following EVLP[147,148]. It is also known that DAMPs, also released during IRI, steadily increase over the course of EVLP, and are associated with increased TNF-α secretion as a downstream result of DAMP induced signalling[149,150]. It has also shown that M30 and high mobility group box-1 protein (HMGB-1) are significantly increased during EVLP in a group of lungs with PGD grade III[151]. These results are yet to be shown during ESHP. Perhaps the deleterious effects of these circulating inflammatory mediators can be averted by their removal during continuous hemofiltration, resulting in better control of inflammatory processes during ex-situ preservation. A more detailed biochemical characterization of the metabolic, inflammatory, and oxidative profile of cross-circulated and hemofiltered thoracic organs is warranted to identify potential mediators of these functional changes. Such identified mediators of functional deterioration during ex-situ perfusion would become obvious targets to effect longer and higher quality preservation of all donor organs, perhaps even beyond the lungs or heart.

1.6 **Conclusion**

Heart failure is an escalating threat that is best combated by transplantation, however the supply of donor organs with which we can utilize to provide gold-standard treatment are limited by logistics and pathology incurred during transport. Out of body organ perfusion apparatuses such as ESHP are an exciting venue to improve viability time with the additional prospects of facilitating suboptimal donor organ inclusivity and even reconditioning donor organs through focused treatment via the platform. The field must first overcome the mechanisms that pervade functional decline on the platform, which will require in depth study and optimization of perfusion strategy. Hypotheses regarding the effects of subtraction or addition of substrates in the perfusate in order to effect greater functional preservation during ex-situ organ perfusion suggest that there is a great deal left to study in terms of perfusate composition. What substrates must be present, in what quantities, and which must be removed or limited? Given our previous work ruling out leukocytes as a blood-based contributor to the immuno-oxidative milieu that pervades functional decline, the current thesis explores components of the blood and plasma as parts of the perfusate that could be leveraged to investigate mechanisms behind functional decline during ESHP. Through manipulating the amounts of these components in the perfusate, we provide a variety of insights that can be explored as remediators of functional decline and damage endured during ESHP, with additional relevance to other extracorporeal perfusion systems that utilize blood.

1.7 Hypotheses

In the current thesis, the hypotheses I hope to explore are as follows:

1. Functional decline cannot be ameliorated via the provision of increased oxygen delivery during normothermic ex-situ heart perfusion.

2. Increasing hematocrit during ex-situ heart perfusion will exacerbate functional decline by increasing unmitigated hemolytic burden.

3. Plasma proteins can provide beneficial effects to the preservation of the vasculature during ex-situ heart perfusion; dilution of such proteins facilitates endothelial dysfunction.

1.8 References

1. Heidenreich PA, Bozkurt B, Aguilar D, et al. 2022 AHA/ACC/HFSA Guideline for the Management of Heart Failure: A Report of the American College of Cardiology/American Heart Association Joint Committee on Clinical Practice Guidelines. *Circulation*. 2022;145(18):e895-e1032. doi:10.1161/CIR.0000000000001063

2. Savarese G, Becher PM, Lund LH, Seferovic P, Rosano GMC, Coats AJS. Global burden of heart failure: a comprehensive and updated review of epidemiology. *Cardiovasc Res*. 2023;118(17):3272-3287. doi:10.1093/cvr/cvac013

3. Tsao CW, Aday AW, Almarzooq ZI, et al. Heart Disease and Stroke Statistics-2023 Update: A Report From the American Heart Association. *Circulation*. 2023;147(8):e93-e621. doi:10.1161/CIR.0000000000001123

4. Lindsay P, Anderson K, Ducharme A, et al. THE STATE OF HEART FAILURE SERVICES IN CANADA: FINDINGS OF THE HEART & STROKE NATIONAL HEART FAILURE RESOURCES AND SERVICES INVENTORY. *Canadian Journal of Cardiology*. 2022;38(10):S188-S189. doi:10.1016/j.cjca.2022.08.153

5. Lesyuk W, Kriza C, Kolominsky-Rabas P. Cost-of-illness studies in heart failure: a systematic review 2004–2016. *BMC Cardiovascular Disorders*. 2018;18(1):74. doi:10.1186/s12872-018-0815-3

6. Shah A, Gandhi D, Srivastava S, Shah KJ, Mansukhani R. Heart Failure: A Class Review of Pharmacotherapy. *P T*. 2017;42(7):464-472.

7. Jessup M, Abraham WT, Casey DE, et al. 2009 focused update: ACCF/AHA Guidelines for the Diagnosis and Management of Heart Failure in Adults: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines: developed in collaboration with the International Society for Heart and Lung Transplantation. *Circulation*. 2009;119(14):1977-2016. doi:10.1161/CIRCULATIONAHA.109.192064

8. WRITING COMMITTEE MEMBERS, Yancy CW, Jessup M, et al. 2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology Foundation/American Heart Association Task Force on practice guidelines. *Circulation*. 2013;128(16):e240-327. doi:10.1161/CIR.0b013e31829e8776

9. Tromp J, Ouwerkerk W, Van Veldhuisen DJ, et al. A Systematic Review and Network Meta-Analysis of Pharmacological Treatment of Heart Failure With Reduced Ejection Fraction. *JACC: Heart Failure*. 2022;10(2):73-84. doi:10.1016/j.jchf.2021.09.004

10. Alraies MC, Eckman P. Adult heart transplant: indications and outcomes. *J Thorac Dis*. 2014;6(8):1120-1128. doi:10.3978/j.issn.2072-1439.2014.06.44

11. Linden PK. History of Solid Organ Transplantation and Organ Donation. *Critical Care Clinics*. 2009;25(1):165-184. doi:10.1016/j.ccc.2008.12.001

12. Stolf NAG. History of Heart Transplantation: a Hard and Glorious Journey. *Braz J Cardiovasc Surg*. 2017;32(5):423-427. doi:10.21470/1678-9741-2017-0508

13. Brink JG, Hassoulas J. The first human heart transplant and further advances in cardiac transplantation at Groote Schuur Hospital and the University of Cape Town. *Cardiovasc J Afr*. 2009;20(1):31-35.

14. Monteagudo Vela M, García Sáez D, Simon AR. Current approaches in retrieval and heart preservation. *Ann Cardiothorac Surg*. 2018;7(1):67-74. doi:10.21037/acs.2018.01.06

15. Venuta F, Van Raemdonck D. History of lung transplantation. *J Thorac Dis*. 2017;9(12):5458-5471. doi:10.21037/jtd.2017.11.84

16. Margreiter R. History of Lung and Heart-Lung Transplantation, With Special Emphasis on German-Speaking Countries. *Transplantation Proceedings*. 2016;48(8):2779-2781. doi:10.1016/j.transproceed.2016.07.015

17. Bezinover D, Saner F. Organ transplantation in the modern era. *BMC Anesthesiol*. 2019;19:32. doi:10.1186/s12871-019-0704-z

18. Panchabhai TS, Chaddha U, McCurry KR, Bremner RM, Mehta AC. Historical perspectives of lung transplantation: connecting the dots. *Journal of Thoracic Disease*. 2018;10(7). doi:10.21037/jtd.2018.07.06

19. Khush KK, Cherikh WS, Chambers DC, et al. The International Thoracic Organ Transplant Registry of the International Society for Heart and Lung Transplantation: Thirty-sixth adult heart transplantation report - 2019; focus theme: Donor and recipient size match. *J Heart Lung Transplant*. 2019;38(10):1056-1066. doi:10.1016/j.healun.2019.08.004

20. National data - OPTN. Accessed February 1, 2024. Available from: https://optn.transplant.hrsa.gov/data/view-data-reports/national-data/#

21. Hatami S, Freed DH. Machine Perfusion of Donor Heart: State of the Art. *Curr Transpl Rep*. 2019;6(3):242-250. doi:10.1007/s40472-019-00251-4

22. Wang L, MacGowan GA, Ali S, Dark JH. Ex situ heart perfusion: The past, the present, and the future. *The Journal of Heart and Lung Transplantation*. 2021;40(1):69-86. doi:10.1016/j.healun.2020.10.004

23. White CW, Messer SJ, Large SR, et al. Transplantation of Hearts Donated after Circulatory Death. *Frontiers in Cardiovascular Medicine*. 2018;5. Accessed May 7, 2022. https://www.frontiersin.org/article/10.3389/fcvm.2018.00008

24. Stehlik J, Edwards LB, Kucheryavaya AY, et al. The Registry of the International Society for Heart and Lung Transplantation: Twenty-eighth Adult Heart Transplant Report--2011. *J Heart Lung Transplant*. 2011;30(10):1078-1094. doi:10.1016/j.healun.2011.08.003

25. Jeevanandam V, Furukawa S, Prendergast TW, Todd BA, Eisen HJ, McClurken JB. Standard criteria for an acceptable donor heart are restricting heart transplantation. *Ann Thorac Surg*. 1996;62(5):1268-1275. doi:10.1016/0003-4975(96)00626-1

26. Bloomworks. The Costly Effects of an Outdated Organ Donation System. Accessed October 28, 2023. Available from: https://bloomworks.digital/organdonationreform/Introduction/

27. Bakhtiyar SS, Godfrey EL, Ahmed S, et al. Survival on the Heart Transplant Waiting List. *JAMA Cardiology*. 2020;5(11):1227-1235. doi:10.1001/jamacardio.2020.2795

28. Marsia S, Khan A, Khan M, et al. Heart transplantation after the circulatory death; The ethical dilemma. *Indian Heart J*. 2018;70(Suppl 3):S442-S445. doi:10.1016/j.ihj.2018.08.010

29. Gordon JK, McKinlay J. Physiological changes after brain stem death and management of the heart-beating donor. *Continuing Education in Anaesthesia Critical Care & Pain*. 2012;12(5):225-229. doi:10.1093/bjaceaccp/mks026

30. Collins MJ, Moainie SL, Griffith BP, Poston RS. Preserving and evaluating hearts with ex vivo machine perfusion: an avenue to improve early graft performance and expand the donor pool. *Eur J Cardiothorac Surg*. 2008;34(2):318-325. doi:10.1016/j.ejcts.2008.03.043

31. Russo MJ, Chen JM, Sorabella RA, et al. The effect of ischemic time on survival after heart transplantation varies by donor age: An analysis of the United Network for Organ Sharing database. *The Journal of Thoracic and Cardiovascular Surgery*. 2007;133(2):554-559. doi:10.1016/j.jtcvs.2006.09.019

32. Banner NR, Thomas HL, Curnow E, et al. The Importance of Cold and Warm Cardiac Ischemia for Survival After Heart Transplantation. *Transplantation*. 2008;86(4):542-547. doi:10.1097/TP.0b013e31818149b9

33. Anderson K, Rucker A, Mulvihill M, Yerokun B, Hartwig M. Impact of Ischemic Time on Primary Graft Dysfunction: A National Analysis. *The Journal of Heart and Lung Transplantation*. 2018;37(4):S459. doi:10.1016/j.healun.2018.01.1194

34. Aldam PS. The physiological changes of circulatory death with respect to organ donation [dissertation]. Cambridge (UK): University of Cambridge; 2019

35. Goila AK, Pawar M. The diagnosis of brain death. *Indian J Crit Care Med*. 2009;13(1):7-11. doi:10.4103/0972-5229.53108

36. McKeown DW, Bonser RS, Kellum JA. Management of the heartbeating brain-dead organ donor. *Br J Anaesth*. 2012;108 Suppl 1:i96-107. doi:10.1093/bja/aer351

37. Canada T. Canadian Motor Vehicle Traffic Collision Statistics: 2021. Transport Canada. April 13, 2023. Accessed February 1, 2024. https://tc.canada.ca/en/road-transportation/statistics-data/canadian-motor-vehicle-traffic-collision-statistics-2021

38. Wittwer T, Wahlers T. Marginal donor grafts in heart transplantation: lessons learned from 25 years of experience. *Transpl Int*. 2008;21(2):113-125. doi:10.1111/j.1432-2277.2007.00603.x

39. Bifulco O, Bottio T, Caraffa R, et al. Marginal versus Standard Donors in Heart Transplantation: Proper Selection Means Heart Transplant Benefit. *J Clin Med*. 2022;11(9):2665. doi:10.3390/jcm11092665

40. Yazji JH, Garg P, Wadiwala I, et al. Expanding Selection Criteria to Repairable Diseased Hearts to Meet the Demand of Shortage of Donors in Heart Transplantation. *Cureus*. 2022;14(5):e25485. doi:10.7759/cureus.25485

41. Christie IG, Chan EG, Ryan JP, et al. National Trends in Extended Criteria Donor Utilization and Outcomes for Lung Transplantation. *The Annals of Thoracic Surgery*. 2021;111(2):421-426. doi:10.1016/j.athoracsur.2020.05.087

42. Emmanuel S, Jansz P, Hayward CS, Macdonald P. Marginal Cardiac Donors and Transplant Outcomes: An 11-Year Experience. *The Journal of Heart and Lung Transplantation*. 2022;41(4):S367. doi:10.1016/j.healun.2022.01.1481

43. Messer S, Lannon J, Wong E, et al. The Potential of Transplanting Hearts From Donation After Circulatory Determined Death (DCD) Donors Within the United Kingdom. *The Journal of Heart and Lung Transplantation*. 2015;34(4):S275. doi:10.1016/j.healun.2015.01.772

44. Messer S, Page A, Berman M, et al. First to 50: Early Outcomes Following Heart Transplantation at Royal Papworth Hospital from Donation after Circulatory Determined Death (DCD) Donors. *The Journal of Heart and Lung Transplantation*. 2019;38(4):S43. doi:10.1016/j.healun.2019.01.090

45. Siddiqi HK, Trahanas J, Xu M, et al. Outcomes of Heart Transplant Donation After Circulatory Death. *J Am Coll Cardiol*. 2023;82(15):1512-1520. doi:10.1016/j.jacc.2023.08.006

46. Canadian Institute for Health Information. Summary statistics on organ transplants, wait-lists, and donors. Accessed August 12, 2024. https://www.cihi.ca/en/summary-statistics-on-organ-transplants-wait-lists-and-donors#:~:text=Key%20findings,46%25%20were%20from%20unrelated%20donors.

47. Herrera Fortin T, Calin E, Ducharme A, et al. The Potential for Heart Donation After Death Determination by Circulatory Criteria (DCD) in the Province of Quebec. *CJC Open*. Published online May 16, 2024. doi:10.1016/j.cjco.2024.05.004

48. Singh SSA, Dalzell JR, Berry C, Al-Attar N. Primary graft dysfunction after heart transplantation: a thorn amongst the roses. *Heart Fail Rev*. 2019;24(5):805-820. doi:10.1007/s10741-019-09794-1

49. Du Y, Demillard LJ, Ren J. Catecholamine-induced cardiotoxicity: A critical element in the pathophysiology of stroke-induced heart injury. *Life Sciences*. 2021;287:120106. doi:10.1016/j.lfs.2021.120106

50. Tsai YH, Lin JY, Huang YY, Wong JM. Cushing response-based warning system for intensive care of brain-injured patients. *Clin Neurophysiol*. 2018;129(12):2602-2612. doi:10.1016/j.clinph.2018.09.010

51. White CW, Lillico R, Sandha J, et al. Physiologic Changes in the Heart Following Cessation of Mechanical Ventilation in a Porcine Model of Donation After Circulatory Death: Implications for Cardiac Transplantation. *Am J Transplant*. 2016;16(3):783-793. doi:10.1111/ajt.13543

52. Pérez López S, Otero Hernández J, Vázquez Moreno N, Escudero Augusto D, Alvarez Menéndez F, Astudillo González A. Brain death effects on catecholamine levels and subsequent cardiac damage assessed in organ donors. *J Heart Lung Transplant*. 2009;28(8):815-820. doi:10.1016/j.healun.2009.04.021

53. Egan TM. Non-heart-beating donors in thoracic transplantation. *The Journal of Heart and Lung Transplantation*. 2004;23(1):3-10. doi:10.1016/S1053-2498(02)00658-7

54. Liaudet L, Calderari B, Pacher P. Pathophysiological mechanisms of catecholamine and cocaine-mediated cardiotoxicity. *Heart Fail Rev*. 2014;19(6):815-824. doi:10.1007/s10741-014-9418-y

55. Xu H xia, Cui S mei, Zhang Y mei, Ren J. Mitochondrial Ca2+ regulation in the etiology of heart failure: physiological and pathophysiological implications. *Acta Pharmacol Sin*. 2020;41(10):1301-1309. doi:10.1038/s41401-020-0476-5

56. Sanada S, Komuro I, Kitakaze M. Pathophysiology of myocardial reperfusion injury: preconditioning, postconditioning, and translational aspects of protective measures. *Am J Physiol Heart Circ Physiol*. 2011;301(5):H1723-1741. doi:10.1152/ajpheart.00553.2011

57. de Perrot M, Liu M, Waddell TK, Keshavjee S. Ischemia–Reperfusion–induced Lung Injury. *Am J Respir Crit Care Med*. 2003;167(4):490-511. doi:10.1164/rccm.200207-670SO

58. Jahania MS, Sanchez JA, Narayan P, Lasley RD, Mentzer RM. Heart preservation for transplantation: principles and strategies. *The Annals of Thoracic Surgery*. 1999;68(5):1983-1987. doi:10.1016/S0003-4975(99)01028-0

59. Kalogeris T, Baines CP, Krenz M, Korthuis RJ. Cell Biology of Ischemia/Reperfusion Injury. *Int Rev Cell Mol Biol*. 2012;298:229-317. doi:10.1016/B978-0-12-394309-5.00006-7

60. Bovo E, Mazurek SR, Zima AV. Oxidation of ryanodine receptor after ischemia-reperfusion increases propensity of Ca2+ waves during β-adrenergic receptor stimulation. *Am J Physiol Heart Circ Physiol*. 2018;315(4):H1032-H1040. doi:10.1152/ajpheart.00334.2018

61. Hurst S, Hoek J, Sheu SS. Mitochondrial Ca2+ and Regulation of the Permeability Transition Pore. *J Bioenerg Biomembr*. 2017;49(1):27-47. doi:10.1007/s10863-016-9672-x

62. Yang Y, Jiang K, Liu X, Qin M, Xiang Y. CaMKII in Regulation of Cell Death During Myocardial Reperfusion Injury. *Frontiers in Molecular Biosciences*. 2021;8. Accessed February 4, 2024. https://www.frontiersin.org/articles/10.3389/fmolb.2021.668129

63. Liu GY, Xie WL, Wang YT, et al. Calpain: the regulatory point of myocardial ischemia-reperfusion injury. *Front Cardiovasc Med*. 2023;10:1194402. doi:10.3389/fcvm.2023.1194402

64. Wu MY, Yiang GT, Liao WT, et al. Current Mechanistic Concepts in Ischemia and Reperfusion Injury. *CPB*. 2018;46(4):1650-1667. doi:10.1159/000489241

65. Lee F, Nair V, Chih S. Cardiac allograft vasculopathy: Insights on pathogenesis and therapy. *Clin Transplant*. 2020;34(3):e13794. doi:10.1111/ctr.13794

66. Angelini A, Castellani C, Fedrigo M, et al. Coronary cardiac allograft vasculopathy versus native atherosclerosis: difficulties in classification. *Virchows Arch*. 2014;464(6):627-635. doi:10.1007/s00428-014-1586-6

67. Pober JS, Chih S, Kobashigawa J, Madsen JC, Tellides G. Cardiac allograft vasculopathy: current review and future research directions. *Cardiovasc Res*. 2021;117(13):2624-2638. doi:10.1093/cvr/cvab259

68. Greenway SC, Butts R, Naftel DC, et al. Statin therapy is not associated with improved outcomes after heart transplantation in children and adolescents. *J Heart Lung Transplant*. 2016;35(4):457-465. doi:10.1016/j.healun.2015.10.040

69. Feinberg AJ, Yarber C, Vlismas PP, Farooq M, Rochlani YM, Patel SR. Impact Of Blood Pressure Control On Risk Of Cardiac Allograft Vasculopathy After Heart Transplant. *Journal of Cardiac Failure*. 2023;29(4):620-621. doi:10.1016/j.cardfail.2022.10.183

70. Goto R, Issa F, Heidt S, Taggart D, Wood KJ. Ischemia-Reperfusion Injury Accelerates Human Antibody-Mediated Transplant Vasculopathy. *Transplantation*. 2013;96(2):139. doi:10.1097/TP.0b013e318295ee32

71. Fukunaga N, Kawajiri H, Badiwala MV, et al. Protective role of Nrf2 against ischemia reperfusion injury and cardiac allograft vasculopathy. *Am J Transplant*. 2020;20(5):1262-1271. doi:10.1111/ajt.15724

72. Liu L, Fang C, Fu W, et al. Endothelial Cell-Derived Interleukin-18 Released During Ischemia Reperfusion Injury Selectively Expands T Peripheral Helper Cells to Promote Alloantibody Production. *Circulation*. 2020;141(6):464-478. doi:10.1161/CIRCULATIONAHA.119.042501

73. Qin L, Li G, Kirkiles-Smith N, et al. Complement C5 Inhibition Reduces T Cell-Mediated Allograft Vasculopathy Caused by Both Alloantibody and Ischemia Reperfusion Injury in Humanized Mice. *Am J Transplant*. 2016;16(10):2865-2876. doi:10.1111/ajt.13834

74. Fenton M, Simmonds J, Shah V, et al. Inflammatory Cytokines, Endothelial Function, and Chronic Allograft Vasculopathy in Children: An Investigation of the Donor and Recipient Vasculature After Heart Transplantation. *American Journal of Transplantation*. 2016;16(5):1559-1568. doi:10.1111/ajt.13643

75. van Suylen V, Vandendriessche K, Neyrinck A, et al. Oxygenated machine perfusion at room temperature as an alternative for static cold storage in porcine donor hearts. *Artificial Organs*. 2022;46(2):246-258. doi:10.1111/aor.14085

76. Russo MJ, Iribarne A, Hong KN, et al. Factors associated with primary graft failure after heart transplantation. *Transplantation*. 2010;90(4):444-450. doi:10.1097/TP.0b013e3181e6f1eb

77. Yeung JC, Krueger T, Yasufuku K, et al. Outcomes after transplantation of lungs preserved for more than 12 h: a retrospective study. *The Lancet Respiratory Medicine*. 2017;5(2):119-124. doi:10.1016/S2213-2600(16)30323-X

78. Lautner LJ, Freed DH, Nagendran J, Acker JP. Current techniques and the future of lung preservation. *Cryobiology*. 2020;94:1-8. doi:10.1016/j.cryobiol.2020.04.009

79. Wicomb W, Boyd ST, Cooper DK, Rose AG, Barnard CN. Ex vivo functional evaluation of pig hearts subjected to 24 hours' preservation by hypothermic perfusion. *S Afr Med J*. 1981;60(6):245-248.

80. Wicomb WN, Cooper DKC, Barnard CN. TWENTY-FOUR-HOUR PRESERVATION OF THE PIG HEART BY A PORTABLE HYPOTHERMIC PERFUSION SYSTEM. *Transplantation*. 1982;34(5):246-250.

81. Hassanein WH, Zellos L, Tyrrell TA, et al. Continuous perfusion of donor hearts in the beating state extends preservation time and improves recovery of function. *The*

Journal of Thoracic and Cardiovascular Surgery. 1998;116(5):821-830. doi:10.1016/S0022-5223(98)00452-8

82. Ozeki T, Kwon MH, Gu J, et al. Heart preservation using continuous ex vivo perfusion improves viability and functional recovery. *Circ J*. 2007;71(1):153-159. doi:10.1253/circj.71.153

83. Fitton TP, Wei C, Lin R, et al. Impact of 24 h continuous hypothermic perfusion on heart preservation by assessment of oxidative stress. *Clinical Transplantation*. 2004;18(s12):22-27. doi:10.1111/j.1399-0012.2004.00213

84. Michel SG, La Muraglia GM, Madariaga MLL, et al. Preservation of Donor Hearts Using Hypothermic Oxygenated Perfusion. *Ann Transplant*. 2014;19:409-416. doi:10.12659/AOT.890797

85. Ferrera R, Larese A, Marcsek P, et al. Comparison of different techniques of hypothermic pig heart preservation. *The Annals of Thoracic Surgery*. 1994;57(5):1233-1239. doi:10.1016/0003-4975(94)91363-3

86. Van Caenegem O, Beauloye C, Bertrand L, et al. Hypothermic continuous machine perfusion enables preservation of energy charge and functional recovery of heart grafts in an ex vivo model of donation following circulatory death. *European Journal of Cardio-Thoracic Surgery*. 2016;49(5):1348-1353. doi:10.1093/ejcts/ezv409

87. Michel SG, Ii GML, Madariaga MLL, et al. Twelve-Hour Hypothermic Machine Perfusion for Donor Heart Preservation Leads to Improved Ultrastructural Characteristics Compared to Conventional Cold Storage. *Ann Transplant*. 2015;20:461-468. doi:10.12659/AOT.893784

88. Hatami S, White CW, Ondrus M, et al. Normothermic Ex Situ Heart Perfusion in Working Mode: Assessment of Cardiac Function and Metabolism. *J Vis Exp*. 2019;(143). doi:10.3791/58430

89. Ardehali A, Esmailian F, Deng M, et al. Ex-vivo perfusion of donor hearts for human heart transplantation (PROCEED II): a prospective, open-label, multicentre, randomised non-inferiority trial. *Lancet*. 2015;385(9987):2577-2584. doi:10.1016/S0140-6736(15)60261-6

90. White C, Ambrose E, Müller A, et al. Impact of Reperfusion Calcium and pH on the Resuscitation of Hearts Donated After Circulatory Death. *Ann Thorac Surg*. 2017;103(1):122-130. doi:10.1016/j.athoracsur.2016.05.084

91. Taylor MJ. Hypothermic Blood Substitution: Special Considerations for Protection of Cells during ex vivo and in vivo Preservation. *Transfus Med Hemother*. 2007;34(4):226-244. doi:10.1159/000104250

92. White CW, Hasanally D, Mundt P, et al. A whole blood–based perfusate provides superior preservation of myocardial function during ex vivo heart perfusion. *The*

Journal of Heart and Lung Transplantation. 2015;34(1):113-121. doi:10.1016/j.healun.2014.09.021

93. White CW, Ali A, Hasanally D, et al. A cardioprotective preservation strategy employing ex vivo heart perfusion facilitates successful transplant of donor hearts after cardiocirculatory death. *The Journal of Heart and Lung Transplantation*. 2013;32(7):734-743. doi:10.1016/j.healun.2013.04.016

94. Bishawi M, Roan JN, Milano CA, et al. A normothermic ex vivo organ perfusion delivery method for cardiac transplantation gene therapy. *Sci Rep*. 2019;9(1):8029. doi:10.1038/s41598-019-43737-y

95. Miyagi N, Rao VP, Ricci D, et al. EFFICIENT AND DURABLE GENE TRANSFER TO THE TRANSPLANTED HEART USING ADENO-ASSOCIATED VIRUS 9 VECTOR. *J Heart Lung Transplant*. 2008;27(5):554-560. doi:10.1016/j.healun.2008.01.025

96. Cypel M, Liu M, Rubacha M, et al. Functional Repair of Human Donor Lungs by IL-10 Gene Therapy. *Science Translational Medicine*. 2009;1(4):4ra9-4ra9. doi:10.1126/scitranslmed.3000266

97. Yeung JC, Wagnetz D, Cypel M, et al. Ex vivo adenoviral vector gene delivery results in decreased vector-associated inflammation pre- and post-lung transplantation in the pig. *Mol Ther*. 2012;20(6):1204-1211. doi:10.1038/mt.2012.57

98. Machuca TN, Cypel M, Bonato R, et al. Safety and Efficacy of *Ex Vivo* Donor Lung Adenoviral IL-10 Gene Therapy in a Large Animal Lung Transplant Survival Model. *Human Gene Therapy*. 2017;28(9):757-765. doi:10.1089/hum.2016.070

99. Tenderich G, El-Banayosy A, Rosengard B, et al. 10: Prospective multi-center European trial to evaluate the safety and performance of the Organ Care System for heart transplants (PROTECT). *The Journal of Heart and Lung Transplantation*. 2007;26(2):S64. doi:10.1016/j.healun.2006.11.024

100. Transmedics. Transmedics Organ Care System Heart User Guide. Accessed August 12, 2024. https://www.accessdata.fda.gov/cdrh_docs/pdf18/P180051S001D.pdf

101. Hamed A, Tsui S, Huber J, Lin R, Poggio EC, Ardehali A. 19: Serum Lactate Is a Highly Sensitive and Specific Predictor of Post Cardiac Transplant Outcomes Using the Organ Care System. *The Journal of Heart and Lung Transplantation*. 2009;28(2):S71. doi:10.1016/j.healun.2008.11.025

102. Freed DH, White CW. Donor heart preservation: straight up, or on the rocks? *The Lancet*. 2015;385(9987):2552-2554. doi:10.1016/S0140-6736(15)60614-6

103. Stamp NL, Shah A, Vincent V, et al. Successful Heart Transplant after Ten Hours Out-of-body Time using the TransMedics Organ Care System. *Heart, Lung and Circulation*. 2015;24(6):611-613. doi:10.1016/j.hlc.2015.01.005

104. Schroder JN, D'Alessandro D, Esmailian F, et al. Successful Utilization of Extended Criteria Donor (ECD) Hearts for Transplantation - Results of the OCS™ Heart EXPAND Trial to Evaluate the Effectiveness and Safety of the OCS Heart System to Preserve and Assess ECD Hearts for Transplantation. *The Journal of Heart and Lung Transplantation*. 2019;38(4):S42. doi:10.1016/j.healun.2019.01.088

105. Schroder JN, Shah A, Pretorius V, et al. Expanding Heart Transplants from Donors After Circulatory Death (DCD) - Results of the First Randomized Controlled Trial Using the Organ Care System (OCS™) Heart - (OCS DCD Heart Trial). *The Journal of Heart and Lung Transplantation*. 2022;41(4):S72. doi:10.1016/j.healun.2022.01.165

106. Messer S, Page A, Axell R, et al. Outcome after heart transplantation from donation after circulatory-determined death donors. *The Journal of Heart and Lung Transplantation*. 2017;36(12):1311-1318. doi:10.1016/j.healun.2017.10.021

107. Rojas SV, Avsar M, Ius F, et al. Ex-Vivo Preservation with the Organ Care System in High Risk Heart Transplantation. *Life*. 2022;12(2):247. doi:10.3390/life12020247

108. Alomari M, Garg P, Yazji JH, et al. Is the Organ Care System (OCS) Still the First Choice With Emerging New Strategies for Donation After Circulatory Death (DCD) in Heart Transplant? *Cureus*. 14(6):e26281. doi:10.7759/cureus.26281

109. Fleck TPK, Ayala R, Kroll J, et al. Ex Vivo Allograft Perfusion for Complex Pediatric Heart Transplant Recipients. *The Annals of Thoracic Surgery*. 2021;112(4):1275-1280. doi:10.1016/j.athoracsur.2020.12.025

110. Hatami S, White CW, Shan S, et al. Myocardial Functional Decline During Prolonged Ex Situ Heart Perfusion. *The Annals of Thoracic Surgery*. 2019;108(2):499-507. doi:10.1016/j.athoracsur.2019.01.076

111. Olkowicz M, Ribeiro RVP, Yu F, et al. Dynamic Metabolic Changes During Prolonged Ex Situ Heart Perfusion Are Associated With Myocardial Functional Decline. *Frontiers in Immunology*. 2022;1:859506. doi:10.3389/fimmu.2022.859506

112. Hatami S, Qi X, White C, et al. OXIDATIVE STRESS AND RELATED RESPONSES ARE INDUCED DURING EX-SITU HEART PERFUSION AND MAY CONTRIBUTE TO THE FUNCTIONAL DECLINE OF THE EX-SITU-PERFUSED HEART. *Canadian Journal of Cardiology*. 2019;35(10):S106. doi:10.1016/j.cjca.2019.07.499

113. Hatami S, Qi X, Khan M, et al. Oxidative stress and related metabolic alterations are induced in ex situ perfusion of donated hearts regardless of the ventricular load

or leukocyte depletion. *American Journal of Transplantation*. Published online January 2023:S1600613522302174. doi:10.1016/j.ajt.2022.11.027

114. Hatami S, White CW, Qi X, et al. Immunity and Stress Responses Are Induced During Ex Situ Heart Perfusion. *Circ: Heart Failure*. 2020;13(6). doi:10.1161/CIRCHEARTFAILURE.119.006552

115. Davies LC, Jenkins SJ, Allen JE, Taylor PR. Tissue-resident macrophages. *Nat Immunol*. 2013;14(10):986-995. doi:10.1038/ni.2705

116. Peet C, Ivetic A, Bromage DI, Shah AM. Cardiac monocytes and macrophages after myocardial infarction. *Cardiovasc Res*. 2020;116(6):1101-1112. doi:10.1093/cvr/cvz336

117. Brouckaert G, Kalai M, Krysko DV, et al. Phagocytosis of Necrotic Cells by Macrophages Is Phosphatidylserine Dependent and Does Not Induce Inflammatory Cytokine Production. *Mol Biol Cell*. 2004;15(3):1089-1100. doi:10.1091/mbc.E03-09-0668

118. Kari S, Subramanian K, Altomonte IA, Murugesan A, Yli-Harja O, Kandhavelu M. Programmed cell death detection methods: a systematic review and a categorical comparison. *Apoptosis*. 2022;27(7):482-508. doi:10.1007/s10495-022-01735-y

119. Hayashi R, Ito Y, Matsumoto K, Fujino Y, Otsuki Y. Quantitative differentiation of both free 3'-OH and 5'-OH DNA ends between heat-induced apoptosis and necrosis. *J Histochem Cytochem*. 1998;46(9):1051-1059. doi:10.1177/002215549804600909

120. Hickman PE, Potter JM, Aroney C, et al. Cardiac troponin may be released by ischemia alone, without necrosis. *Clin Chim Acta*. 2010;411(5-6):318-323. doi:10.1016/j.cca.2009.12.009

121. Mair J, Lindahl B, Hammarsten O, et al. How is cardiac troponin released from injured myocardium? *European Heart Journal: Acute Cardiovascular Care*. 2018;7(6):553-560. doi:10.1177/2048872617748553

122. Bhirowo YP, Raksawardana YK, Setianto BY, et al. Hemolysis and cardiopulmonary bypass: meta-analysis and systematic review of contributing factors. *Journal of Cardiothoracic Surgery*. 2023;18(1):291. doi:10.1186/s13019-023-02406-y

123. Dalton HJ, Cashen K, Reeder RW, et al. Hemolysis During Pediatric Extracorporeal Membrane Oxygenation: Associations With Circuitry, Complications, and Mortality. *Pediatr Crit Care Med*. 2018;19(11):1067-1076. doi:10.1097/PCC.0000000000001709

124. Lehle K, Philipp A, Zeman F, et al. Technical-Induced Hemolysis in Patients with Respiratory Failure Supported with Veno-Venous ECMO - Prevalence and Risk Factors. *PLoS One*. 2015;10(11):e0143527. doi:10.1371/journal.pone.0143527

125. Omar HR, Mirsaeidi M, Socias S, et al. Plasma Free Hemoglobin Is an Independent Predictor of Mortality among Patients on Extracorporeal Membrane Oxygenation Support. *PLoS One*. 2015;10(4):e0124034. doi:10.1371/journal.pone.0124034

126. Pires IS, Berthiaume F, Palmer AF. Engineering Therapeutics to Detoxify Hemoglobin, Heme, and Iron. *Annual Review of Biomedical Engineering*. 2023;25(1):1-21. doi:10.1146/annurev-bioeng-081622-031203

127. Reiter CD, Wang X, Tanus-Santos JE, et al. Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. *Nat Med*. 2002;8(12):1383-1389. doi:10.1038/nm1202-799

128. Vallelian F, Buehler PW, Schaer DJ. Hemolysis, free hemoglobin toxicity, and scavenger protein therapeutics. *Blood*. 2022;140(17):1837-1844. doi:10.1182/blood.2022015596

129. Chiabrando D, Vinchi F, Fiorito V, Mercurio S, Tolosano E. Heme in pathophysiology: a matter of scavenging, metabolism and trafficking across cell membranes. *Front Pharmacol*. 2014;5:61. doi:10.3389/fphar.2014.00061

130. Ovalle R. A History of the Fenton Reactions (Fenton Chemistry for Beginners). In: *Reactive Oxygen Species*. IntechOpen; 2022. doi:10.5772/intechopen.99846

131. Schaer CA, Deuel JW, Schildknecht D, et al. Haptoglobin Preserves Vascular Nitric Oxide Signaling during Hemolysis. *Am J Respir Crit Care Med*. 2016;193(10):1111-1122. doi:10.1164/rccm.201510-2058OC

132. De Simone G, Varricchio R, Ruberto TF, di Masi A, Ascenzi P. Heme Scavenging and Delivery: The Role of Human Serum Albumin. *Biomolecules*. 2023;13(3):575. doi:10.3390/biom13030575

133. Qi X. Regulation of Coronary Artery Flow during Normothermic Ex-Situ Heart Perfusion [dissertation]. Edmonton (AB): University of Alberta; 2020.

134. Palomer X, Salvadó L, Barroso E, Vázquez-Carrera M. An overview of the crosstalk between inflammatory processes and metabolic dysregulation during diabetic cardiomyopathy. *Int J Cardiol*. 2013;168(4):3160-3172. doi:10.1016/j.ijcard.2013.07.150

135. Cobert ML, Peltz M, West LM, Merritt ME, Jessen ME. Glucose is an Ineffective Substrate for Preservation of Machine Perfused Donor Hearts. *Journal of Surgical Research*. 2012;173(2):198-205. doi:10.1016/j.jss.2011.05.028

136. Hatami S, Hefler J, Freed DH. Inflammation and Oxidative Stress in the Context of Extracorporeal Cardiac and Pulmonary Support. *Front Immunol*. 2022;13:831930. doi:10.3389/fimmu.2022.831930

137. Ge T, Yang J, Zhou S, Wang Y, Li Y, Tong X. The Role of the Pentose Phosphate Pathway in Diabetes and Cancer. *Frontiers in Endocrinology*. 2020;11. Accessed December 28, 2022. https://www.frontiersin.org/articles/10.3389/fendo.2020.00365

138. Church JT, Alghanem F, Deatrick KB, et al. Normothermic Ex Vivo Heart Perfusion: Effects of Live Animal Blood and Plasma Cross Circulation. *ASAIO Journal*. 2017;63(6):766-773. doi:10.1097/MAT.0000000000000583

139. McLeod JS, Poling C, Church JT, et al. Ex Vivo Heart Perfusion for 72 Hours Using Plasma Cross Circulation. *ASAIO Journal*. 2020;66(7):753-759. doi:10.1097/MAT.0000000000001061

140. Hozain AE, O'Neill JD, Pinezich MR, et al. Xenogeneic cross-circulation for extracorporeal recovery of injured human lungs. *Nat Med*. 2020;26(7):1102-1113. doi:10.1038/s41591-020-0971-8

141. Guenthart BA, O'Neill JD, Kim J, et al. Regeneration of severely damaged lungs using an interventional cross-circulation platform. *Nat Commun*. 2019;10(1):1985. doi:10.1038/s41467-019-09908-1

142. Johnson MD, Fallon BP, Langley M, et al. Prolonged (24-hour) Normothermic ex vivo Heart Perfusion Facilitated by Perfusate Hemofiltration. *ASAIO Journal*. 2022;68(10):1282-1289. doi:10.1097/MAT.0000000000001649

143. Nilsson T, Hansson C, Wallinder A, et al. Hemofiltration in ex vivo lung perfusion-a study in experimentally induced pulmonary edema. *J Thorac Cardiovasc Surg*. 2016;151(2):570-575.e1. doi:10.1016/j.jtcvs.2015.06.046

144. Deem S, Berg JT, Kerr ME, Swenson ER. Effects of the RBC membrane and increased perfusate viscosity on hypoxic pulmonary vasoconstriction. *J Appl Physiol (1985)*. 2000;88(5):1520-1528. doi:10.1152/jappl.2000.88.5.1520

145. Tchouta L, Drake D, Hoenerhoff M, et al. Twenty-four-hour normothermic perfusion of isolated ex vivo hearts using plasma exchange. *The Journal of Thoracic and Cardiovascular Surgery*. 2022;164(1):128-138. doi:10.1016/j.jtcvs.2020.11.158

146. Kumar S, Bandyopadhyay U. Free heme toxicity and its detoxification systems in human. *Toxicol Lett*. 2005;157(3):175-188. doi:10.1016/j.toxlet.2005.03.004

147. Iskender I, Cosgun T, Arni S, et al. Cytokine filtration modulates pulmonary metabolism and edema formation during ex vivo lung perfusion. *The Journal of Heart and Lung Transplantation*. 2018;37(2):283-291. doi:10.1016/j.healun.2017.05.021

148. Ghaidan H, Stenlo M, Niroomand A, et al. Reduction of primary graft dysfunction using cytokine adsorption during organ preservation and after lung transplantation. *Nat Commun*. 2022;13(1):4173. doi:10.1038/s41467-022-31811-5

149. Gloria JN, Yerxa J, Kesseli SJ, et al. Subnormothermic ex vivo lung perfusion attenuates graft inflammation in a rat transplant model. *The Journal of Thoracic and Cardiovascular Surgery*. 2022;164(2):e59-e70. doi:10.1016/j.jtcvs.2021.01.066

150. Hasenauer A, Bédat B, Parapanov R, et al. Effects of cold or warm ischemia and ex-vivo lung perfusion on the release of damage associated molecular patterns and inflammatory cytokines in experimental lung transplantation. *The Journal of Heart and Lung Transplantation*. 2021;40(9):905-916. doi:10.1016/j.healun.2021.05.015

151. Hashimoto K, Cypel M, Juvet S, et al. Higher M30 and high mobility group box 1 protein levels in ex vivo lung perfusate are associated with primary graft dysfunction after human lung transplantation. *J Heart Lung Transplant*. Published online June 21, 2017:S1053-2498(17)31870-3. doi:10.1016/j.healun.2017.06.005

Chapter 2: Elevation of perfusate hemoglobin exacerbates functional decline during ex-situ heart perfusion through hemolysis

The following chapter is currently in preparation for publication in a peer-reviewed journal.

2.1 Abstract

Background:

Ex-situ heart perfusion is a novel technology which may enable better functional preservation of hearts for transplant compared to traditional cold storage. However, hearts suffer functional deterioration during perfusion. The optimal Hb values required during ESHP are not clear: it is generally assumed that higher Hb concentration provides a benefit during normothermic ESHP. The purpose of the current study was to assess the effect of Hb concentration on functional preservation during ESHP. We hypothesized that increased levels of hemoglobin within the ESHP circuit would predispose the heart to increased hemolytic burden.

Methods:

The hearts of Yorkshire pigs weighing 45-55 kg were procured and subject to 11 hours of perfusion on an ESHP apparatus capable of working mode perfusion. Hearts were allocated to one of four groups: concentrated blood (CB, ~12 g/L, n=4), whole blood (WB, ~8 g/L, n=6), plasma (~4 g/L, n=6), and control group (~4 g/L, n=5). To achieve the desired Hb levels, the circuit was primed with donor animal WB and combined with either packed red blood cells (in CB group), donor plasma obtained via centrifugation of donor whole blood (in plasma group), or modified Krebs-Heinseleit buffer with bovine serum albumin in the control group to achieve desired hemoglobin levels. Heart weight was recorded before and after perfusion. Perfusate ion concentrations were measured via arterial blood gas analysis, with samples acquired for later biochemical analysis. Functional measurements were recorded bi-hourly (1-hour post-reperfusion (T1), T3, T5, etc.) and compared between groups. A variety of hemolytic markers such as cell-free hemoglobin, transferrin saturation, and perfusate potassium were monitored over time. One- or two-way analysis of variance with Tukey’s post-hoc test were used to determine whether statistically significant differences between groups were present.

Results

Hearts allocated to the CB group experienced increased rates of functional decline, with percent of baseline cardiac index (mean at T11, CB vs. Control: 20.6% vs. 75.3%,

$p<0.05$), stroke work (mean at T11: 17.0% vs. 58.1%, $p<0.05$), and left ventricle dP/dT maximum (mean at T11: 30.7% vs. 68.9%, $p<0.05$) and minimum (mean at T11: 26.0% vs. 80.9%, $p<0.05$) dropping significantly more compared to control by late perfusion. This was mirrored in cardiac troponin-I measurements in the perfusate over time, which were increased in the CB group (mean at T11, 99.1 vs. 28.7 ng/mL, $p<0.05$). Hearts within the CB group did have an initially increased MVO_2 (mean at T1: 8.6 vs. 3.0 mL O_2/min/g, $p<0.05$) which paired with initially increased cardiac output, however this declined over the course of perfusion. The CB group displayed significant increases in markers of hemolysis compared to control, such as perfusate potassium (mean at T11, 9.1 vs. 6.2 mmol/L, $p<0.05$), cell-free hemoglobin (at T11, 3.7 vs. 1.9 g/L, $p<0.05$), and iron binding capacity saturation (at T11, 227% vs. 115%, $p<0.05$) compared to controls.

Conclusion

While increasing perfusate hemoglobin can raise myocardial oxygen consumption and cardiac output during working mode ESHP, functional decline is exacerbated by hemolysis over the course of perfusion. Hemolysis and saturation of plasma iron binding capacity may contribute to the oxidative milieu present during ESHP. Mitigation of hemolytic byproducts may enable improved preservation during prolonged ESHP.

2.2 Introduction

Ex-situ heart perfusion (ESHP) is an advanced technology for the transportation of donor hearts, marking a significant improvement over static cold storage by facilitating greater donation distance and accommodating for extended criteria or DCD hearts[1]. Whilst static cold storage can in simple fashion preserve donor hearts for around 4-6 hours of out of body time[2–4], successful transplantation following 10-11 hours of normothermic heart perfusion have been reported[5], showing the efficacy of ESHP. In the TransMedics Organ Care System (OCS) clinical trials[6,7], it was noted that ESHP yielded a significant increase in out of body time with non-inferior post-transplant outcomes whilst also facilitating greater utilization of marginal criteria hearts. Despite these encouraging results, our group and others have documented functional decline that coincides with ESHP[8,9]. While being a concerning phenomenon for clinicians in terms of post-transplant success, decline effectively caps out of body time of the organ, representing a barrier to further widening of the transplantation bottleneck.

Identifying optimizations to various aspects of the perfusion apparatus are likely to mitigate functional decline on the platform. The perfusion solution or ‘perfusate’ is a major opportunity for improvement, given that it hosts a plethora of variables that are ripe for optimization. Currently, the TransMedics OCS uses donor whole blood to constitute its perfusate[10]. Donors can be anemic and may require hemodynamic support with fluids, leading to hemodilution[11]. Since the perfusion apparatus requires a large volume of blood (roughly 1.5 litres) to constitute the perfusate which may necessitate supplementation with buffer, this may mitigate optimal oxygen delivery. Some experimental apparatuses, including our own, have used a mixture of buffer (Krebs-Heinseleit buffer supplemented with albumin) and donor animal blood, constituting lower than physiological levels of hemoglobin[9,12]. Therefore, one may surmise that functional decline could result from inadequate oxygen delivery to the isolated organ over the course of perfusion. Addition of blood products to the circuit have been trialled, however have not been successful due to untenable ionic derangements[13,14].

Previously, we have shown that for short term perfusion (up to 5 hours of working mode), whole blood versus dilute whole blood (1:1 donor whole blood with albuminated Krebs-

Heinseleit buffer) does not appear to impact functional preservation[15]. We have also shown that whole blood in comparison to blood substitutes provides the most optimal preservation of ultrastructure on histology[16]. However, whether elevation of hemoglobin and therefore oxygen delivery can ameliorate functional decline, especially over longer periods, is unclear.

In the use of a blood based perfusate, oxygen delivery must be balanced with the potential for hemolysis to occur over the course of perfusion. Therefore, we hypothesized that elevation of hematocrit would boost oxygen delivery, however would also lead to worsened accumulation of hemolytic byproducts during perfusion. We show in the following work that concentration of the blood based perfusate via the addition of packed RBCs from a cell saver surprisingly leads to worsened functional decline, which associates with elevations in cell-free hemoglobin in the perfusate.

2.3 Methods

2.3.1 Procurement Surgery

Following approval from the University of Alberta Animal Care and Use Committee (AUP00000943), Yorkshire pigs weighing 45-55kg (16-18 weeks of age) were subject to heart procurement surgery, as previously described[12]. Following sedation using 20 mg/kg of ketamine and 0.6 mg/kg of atropine and intubation with mechanical ventilation using 1-2% sevoflurane, a 6F fluids catheter was placed into the internal jugular vein. A minimal volume (<500mL) of PlasmaLyte A was delivered during the course of surgery prior to procurement to avoid hemodilution.

Following placement of the fluids line, 1000 U/kg of heparin was delivered intravenously. A midline sternotomy was performed, and a two-stage venous cannula was placed into the right atrium. Blood was collected as outlined in the experimental groups below to formulate the perfusate of the circuit. A cardioplegia needle was then placed in the ascending aorta; ~100mL of blood was collected to complete the Del-Nido cardioplegia solution. The animal was then euthanized by exsanguination and cardioplegia was delivered, then the heart was procured. The heart was weighed following excision to get the pre-perfusion weight. The time between exsanguination and reperfusion on the perfusion apparatus (in minutes) was recorded as the ischemic time.

2.3.2 Experimental Groups

Four groups differing in perfusate formulation during ESHP were established, with hearts being allocated to each experimental group. The ‘control’ group (designated the control as the historically used perfusate condition[12]) had a perfusate consisting of 800mL of collected donor whole blood mixed with 800mL of modified Krebs Heinseleit buffer with 8% albumin, as used previously[12,17] (ingredients in Supplementary Table 1). The ‘whole blood’ (WB) group had a perfusate consisting entirely of collected donor whole blood (1600mL). To establish the ‘concentrated blood’ (CB) group, the circuit was primed with 800mL of donor whole blood. The rest of the animal’s blood was then run through a cell saver (LivaNova Xtra, LivaNova Cardiopulmonary, United Kingdom) to obtain a red cell concentrate that was added to the whole blood prime to achieve a

hemoglobin of ~12-13 g/dL. To establish the ‘plasma’ group, the circuit was again first primed with 800mL of donor whole blood. The rest of the donor animal’s blood was collected and centrifuged at 1500g for 30 minutes in 50mL falcon tubes, with ~800mL of plasma added back to the whole blood prime.

2.3.3 Perfusion Apparatus & Procedure

A custom ESHP apparatus was constructed as previously described[12]. In brief, a membrane oxygenator (Capiox FX25, Terumo Cardiovascular) and heat exchanger, reservoir (custom), two centrifugal pumps (Medtronic), and an arterial line filter (Medtronic AF100) connected with tubing are used as components for the ESHP circuit. The heart rests on a custom-made silicone pad with an embedded magnet that attracts a magnet cannula sewn directly into the left atrium following heart procurement. The aortic arch and pulmonary artery are secured to cannulas integrated within the silicone pad with silk ties. Pump RPM of both the aortic (Ao) and left atrial (LA) centrifugal pumps supplying the respective cannulas are controlled by computer software, enabling both non-working mode and working mode perfusion modes to be achieved. Flow of both the LA pump and coronary return line (from the pulmonary artery cannula) are measured using flow sensors (Transonic Systems Inc.), with pressure from various parts of the perfusion apparatus being measured by pressure transducers. All data is fed into a custom software which displays pressures and flow rates in real time, enabling the calculation/measurement of key cardiac functional parameters.

Physiologic working mode is established by computerized control of LA pump rounds per minute (RPM), which adjusts according to the instantaneously sensed left atrial pressure (LAP) to maintain a desired LAP set by the user (6 mmHg in this study). Measurements of perfusate oximetry as well as ionic and metabolite concentrations (eg. lactate, glucose) are measured regularly using arterial blood gas analysis (Radiometer ABL, Denmark). During perfusion, glucose (25% w/v) is infused to target concentrations of 6-10 mmol/L. Insulin (2U/hr) and dobutamine (5 mcg/min) are also infused for hormonal support.

Following heart procurement, the magnet cannula was sewn into the LA on the backtable and the heart is then fastened to the silicone pad via this magnet; the aorta

and pulmonary arteries are attached to the appropriate cannulas and reperfusion is commenced at a mean aortic pressure of 30 mmHg and LAP of ~0 mmHg to achieve non-working mode perfusion (constituting 'time 0', T0). Non-working mode perfusion is held for the first hour. A heat exchanger is used to gradually rewarm the perfusate and heart to 38.5°C (porcine body temperature) during this hour. Following 1 hour into perfusion (denoted T1), hearts are transitioned into working mode perfusion by gradually increasing the desired LAP on the computer software (up to 6 mmHg). Afterwards a pigtail catheter is introduced into the left ventricle via a sheath cannula placed in the subclavian artery following procurement to enable LV pressure sensing. Baseline functional measurements including heart rate, systolic, diastolic, and mean pressure, LV dP/dT maximum and minimum, left atrial flow (representing cardiac output during working mode), stroke work, and coronary flow are recorded at T1, then every two hours afterward until T11. At each functional measurement (T1, T3, T5, etc.), arterial and venous blood gas measurements are taken as well as a sample of the perfusate for downstream analyses. Following T11, the heart is dismounted, weighed, and right and left ventricle samples of the heart are excised and flash frozen in liquid nitrogen before being stored at -80°C.

2.3.4 Data Analysis

Formulae

The formulae used for calculation of cardiac functional parameters are as follows:

$$Weight\ Gain\ (\%) = \frac{Heart\ Weight\ Final - Heart\ Weight\ Initial}{Heart\ Weight\ Initial} * 100\%$$

$$Cardiac\ Index\ (mL/\ min/g) = \frac{Left\ Atrial\ Flow\ (mL/min)}{Initial\ Heart\ Weight\ (g)}$$

$$Indexed\ Coronary\ Flow\ (mL/\ min/g) = \frac{Coronary\ Blood\ Flow\ (mL/min)}{Initial\ Heart\ Weight\ (g)}$$

$$Oxygen\ Extraction\ (\%) = \left(1 - \frac{CvO_2}{CaO_2}\right) * 100\%$$

$$MVO_2\left(\frac{mL\ O_2}{min * g}\right) = \frac{\left((CaO_2 - CvO_2) * Coronary\ Blood\ Flow\ (mL/min)\right)}{Initial\ Heart\ Weight\ (g)}$$

$$CaO_2\ or\ CvO_2 = \left(1.34\ \left(\frac{mL\ O_2}{g\ Hb}\right) * Hb\ \left(\frac{g}{dL}\right) * \frac{O_2\ sat\ (\%)}{100\%}\right) + 0.00289\ \left(\frac{mL\ O_2}{100\ mL * mmHg\ O_2}\right) * O_2\ (mmHg)$$

Where CaO_2 uses ABG data from an arterial sample, and CvO_2 uses a venous sample.

2.3.5 Experimental Assays

Assessment of biochemical parameters in the perfusate were accomplished via commercial kits: Total NO_x (Abcam, ab65328), Total Iron Binding Capacity (TIBC) and serum iron (Abcam ab239715), and cardiac troponin-I (Life Diagnostics Inc. CTNI-9-HS) were measured via the referenced kits according to manufacturer instructions. Assessment of supernatant (cell-free) hemoglobin was accomplished via colorimetric determination in plasma using Drabkin's reagent.

2.3.6 Comparisons & Statistics

Data is displayed in figures and tables as mean ± standard error of the mean (SEM) error bars. Intergroup differences were compared using one- or two-way repeated measures analysis of variance (ANOVA) with multiple comparisons accounted for by Tukey's multiple comparison test. An adjusted p-value <0.05 was considered a statistically significant difference.

2.4 Results

2.4.1 Heart Characteristics

Intergroup differences (Table 1) in ischemic time, post-procurement weight, and weight difference (%) were negligible ($p>0.05$). While non-significant in comparison to the control group, there was a trend towards greater weight gain in the CB group and lower weight gain in the plasma group ($p<0.05$ between CB group and plasma group). Achieved hemoglobin was expectably greater in the whole blood and concentrated blood group, with means of run averages of 9.7 and 13.2 g/dL, respectively ($p<0.05$ each in comparison to control).

2.4.2 Myocardial Metabolism

Myocardial oxygen consumption (MVO_2, Figure 2.2A) was significantly elevated in comparison to control in the CB group compared to control during early perfusion (T1, T3, T5, $p<0.05$). WB perfusion also elevated MVO_2, however this was not statistically significant compared to control group until mid-late perfusion (T5, T7, T9, $p<0.05$). Plasma group showed a similarly elevated MVO_2, however this was only statistically significant at T5 (mean 4.4 vs. 2.1 mL/min/100g, $p<0.05$). Expressed as a percentage of baseline (Figure 2.2B), no groups were statistically significant in comparison to the control group however WB group trended towards greater preservation of MVO_2 during late perfusion.

Glucose was kept within post-prandial ranges during the course of perfusion, being kept between 5-10 mmol/L (Figure 2.5). Lactate appeared to initially decrease within the plasma, WB, and CB groups at T3 in comparison to the control group, which elevated from its baseline value at T1 (mean lactate at T3: 4.9 vs. 2.1, 1.2, 1.1 for PL, WB, and CB groups respectively, $p<0.05$ each). Lactate trends appeared to increase thereafter, with plasma group but not control group having significantly greater lactate levels than the WB or CB group by T11 ($p<0.05$).

2.4.3 Myocardial Function

Cardiac indices (Figure 2.1A) were initially significantly elevated in WB, CB, and to a lesser extent in PL perfusion groups in comparison to control, which remained significant for various periods across perfusion: PL group was significantly elevated until T3, CB group until T5, and WB group until T9 ($p<0.05$). Taken as a percentage of the baseline value at T1 (Figure 2.1B), the CB group had a significantly decreased percent of baseline value compared to control by T9 and T11 (mean at T9: 82.0% vs. 27.7%, T11: 75.3% vs. 20.6%, $p<0.05$ each). Stroke work (Figure 2.1C) trended towards a similar pattern as cardiac index however was not statistically significant between the groups at any point; CB group vs. control group approached significance by T9 and T11 ($p=0.085$ and $p=0.053$ respectively). When expressed as a percentage of baseline (Figure 2.1D), stroke work preservation in percent was significantly decreased within the CB group compared to control group at T7, T9, and T11 ($p<0.05$ each). Similarly, LV dP/dT max and min preservation (Figures 2.1E, 2.1F) was significantly decreased in the CB group compared to control at T9 and T11 ($p<0.05$ each).

2.4.4 Vascular Outcomes

In each group, indexed coronary flow (iCF) trends (Figure 2.3A) appeared to elevate from its T1 value before gradually trending downwards; this was most apparent in the CB group. iCF was significantly elevated compared to control in the CB group at T1, remaining elevated until T7; WB group iCF was elevated at T3 and remained elevated until T11 ($p<0.05$). PL group trended towards an elevated iCF compared to control however this was not statistically significant at any time point. Oxygen extraction (Figure 3B) generally decreased over time, and appeared to be decreased in the WB and CB groups compared to the control group, being statistically significant from the control group at most time points. Levels of nitrite and nitrates (NO_x, Figure 2.3C) steadily increased over the course of perfusion. In the CB group, levels of nitrates/nitrites were significantly higher at T5 and T11 in comparison to the control group ($p<0.05$).

2.4.5 Hemolytic Markers

Perfusate potassium (Figure 2.4A) elevated over the course of perfusion in all groups, however perfusate potassium was significantly greater in the CB group compared to the control at T3 onwards ($p<0.05$); the WB group was significantly greater than the control group at T9 onwards ($p<0.05$). PL group trended towards being elevated in comparison to the control group however was not statistically significant at any time point. Supernatant (cell-free) hemoglobin (Figure 2.4B) was significantly raised within the CB group at T5 and T11 compared to control ($p<0.05$), ending at a mean value of 3.7 g/L. No other group was statistically different compared to control. When cell-free hemoglobin was taken as a percentage of total hemoglobin (percent hemolysis, Figure 4C), by T11 the WB group had the lowest percent hemolysis (1.48%) followed by CB group (2.85%), both significantly lower than the control group (4.29%, $p<0.05$ each).

Perfusate total iron binding capacity (TIBC) and serum iron was assessed at T11 in all groups. TIBC was significantly increased in the WB group and trended towards increases in the CB and plasma groups (Figure 2.6A). Serum iron was significantly elevated compared to control in the WB group and the CB group, with CB group having the highest mean serum iron (264.6 µmol/L). Assessing the TIBC saturation found that all groups hovered at approximately 100% TIBC saturation except for the CB group, which had a mean saturation of 227% and was significantly increased from each of the other groups ($p<0.05$).

2.4.6 Cardiac Troponin-I

Cardiac Troponin-I (cTn-I) in perfusate increased over time in all groups, becoming significant compared to the control in the CB group, WB group, and plasma group by T5 and remaining elevated by T11 (Figure 2.7). The CB group had the greatest cTn-I concentration at both T5 and T11 (66.4 ng/mL and 99.5 ng/mL respectively).

2.5 Discussion

We hypothesized that increasing hematocrit within the ESHP circuit would lead to increased hemolytic byproducts being developed over the course of perfusion. Indeed, we saw that within the CB group that function declined most rapidly over the course of perfusion: preservation of baseline cardiac index, stroke work, and LV dP/dT min and max were significantly reduced by late perfusion. MVO_2, while initializing at increased levels, dropped precipitously in the CB group. Greater decline of function in this group coincided with the development of cell-free hemoglobin that was significantly elevated by T5 in the CB group in comparison to the control group. Perfusate potassium levels grew at an increased pace in the CB group, followed by the WB group, indicating that potassium-rich red blood cells were breaking down. These results suggest that hemolysis is an important phenomenon to mitigate during ESHP and more generally, ex-situ organ perfusion utilizing blood based perfusate. Without mechanisms to handle the breakdown of blood-based perfusates, an 'upper limit' to organ preservation is set as continuous hemolysis steadily deposits toxic byproducts into the perfusate that become a barrier to efficacious preservation.

One such byproduct of hemolysis, apart from free heme, is free iron. We show here that by late perfusion, all groups were roughly 100% saturated in iron binding capacity with the CB group especially suffering from iron overload. Free iron is readily uptaken by L-type calcium channels and contributes to the cytoplasmic labile iron pool[18]. While longitudinal iron exposure typically leads to dilated cardiomyopathy in the intact organism, acute exposures during ESHP likely contribute to the oxidative stress endured (particularly lipid peroxidation) through Fenton-type reactions[19]. This implies that bolstering iron sequestration capacity during ex-vivo organ perfusion with blood based perfusates can be a valuable strategy to lessen oxidative burden over longer term perfusion. This could also be accomplished via a hemofiltration-based strategy. A variety of studies from the Bartlett laboratory[20–22] have reported the ability to extend ESHP for up to 24 hours using plasma exchange, whereby hemofiltered volume is replaced by fresh frozen plasma (FFP). While a resource intensive method (using 1 mL per hour per gram of heart tissue[21] which for 24 hours of perfusion in a 280 gram heart

would be nearly 6.7 liters of FFP), the removal of hemolytic byproducts and serum iron may explain the method's ability to effectively prolong perfusion time.

The presence of free iron within the perfusate during late perfusion and presumably increased labile iron within the cytoplasm of ex-situ perfused hearts has implications for cell death, via ferroptosis. Lipid peroxide formation and depletion of reduced glutathione within the cell, which we have detected evidence of both in ex-situ perfused hearts[17], have been suggested to precipitate ferroptosis[23]. As opposed to traditional apoptosis, ferroptosis is not characterized by the cleavage of DNA or chromatin condensation, but rather permeabilization of the cell membrane and dissipation of mitochondrial membrane potential[24]. Previous attempts to characterize the degree of cell death during ESHP via TUNEL assay may therefore fail to capture the true scope of cell death, explaining why a low degree of apoptotic cell death was observed within ex-situ hearts perfused for prolonged periods[8]. Ferroptosis also yields the release of arachidonic acid mediators, which have also been detected via metabolomics studies in ex-situ perfused hearts[9]. Further investigation into the contribution of iron overload and ferroptosis to functional decline in the context of ex-situ heart perfusion is warranted, and may prove to be another targetable phenomenon to effect improved myocardial preservation.

Note that for the CB group, a cell salvage device was used to concentrate the perfusate with packed red blood cells. It is known that mechanical cell salvage devices reduce RBC deformability and 2,3-DPG content[25], indicative of damage sustained, likely due to intense mechanical stress experienced during suction and high-speed centrifugation in the machine[26]. While this process itself does not lyse the RBCs, it appears to predispose them to lysis as they are circulated through the ESHP apparatus. While one might expect given these cellular lesions that the CB group would have the highest proportion of RBCs lysed, we found that the plasma and control groups appeared to have a greater percentage of hemolysis. This may suggest that increased perfusate viscosity can be protective against hemolysis on the platform, but nonetheless the most important endpoint is cell-free hemoglobin, which was highest in the CB group.

This has implications for the use of blood products during ESHP. Given that stored RBC units are susceptible to the storage lesion that presents similarly to the mechanical

damage endured during cell-salvage, this would suggest that products stored for longer periods of time may be unsuitable for application to this context. In studies purporting supplementation of ex-situ heart perfusion with blood products, the main hurdles have been the immediate hyperkalemia and hypocalcemia that result[13,27]. Likewise, we found that hyperkalemia manifested within the CB group.

While elevation of hematocrit would certainly boost oxygen delivery, we did see that MVO_2 appeared to be increased by increasing hemoglobin concentrations in the system: within the CB group, by T1, MVO_2 was significantly increased and trended towards increases in the WB group. Simultaneously, cardiac index in both the WB and CB groups were significantly elevated, with indexed coronary flow also elevating in each in comparison to control. This suggests that increasing oxygen delivery by way of elevating hematocrit can boost functional performance during ESHP following the transition to working mode (note that hearts had an hour to acclimate to perfusate conditions during rewarming), indicating that hemodilution with buffer may cut oxygen delivery needed for better performance. However, this increased output during ESHP did not necessarily lead to better functional preservation, as the control group still trended towards the highest preservation of baseline function by the end of perfusion despite having the lowest actual cardiac index values. This may suggest that oxygenation provided by increased hematocrit in the CB group was feeding oxidative processes rather than being allocated to cardiac metabolism.

Interestingly, the plasma group also seemed to have similar elevation in function akin to the WB and CB groups, albeit to a lesser degree, despite having similar achieved hemoglobin levels. This suggests that other substrates that are a part of the plasma, but not Krebs-Heinseleit buffer, contribute to influencing this change. A likely candidate is free fatty acids: the processed bovine serum albumin with which the KHB is composed are devoid of fatty acids, however the albumin derived from the plasma is likely to have conjugated free fatty acids bound. We have noted previously that during ESHP in conditions similar to the control group in the current study, free fatty acids are readily consumed before T5[8]. Reconstituting the circuit with a greater amount of free fatty acids, this may have elevated early perfusion output via increased substrate provision.

Whether increased performance during ESHP correlates with better post-transplantation outcomes is yet to be studied, given that clinical ESHP platforms do not currently have the capability to directly measure myocardial function during perfusion[28]. While we have previously shown that functional parameters such as stroke work better correlate with myocardial performance over metabolic ones such as lactate and MVO_2[29], these are not yet correlated with post-transplant success. The variation in initial cardiac indices in our study as a result of differing perfusate compositions at least suggests that substrate availability influences performance on the apparatus. This means that the context (ie. the perfusate conditions) in which the heart is placed needs to be accounted for should performance markers like cardiac index be associated with post-transplant success.

One could reason that an elevated metabolic state without the proper support during ESHP is likely to fuel maladaptive processes (eg. oxidative stress, as we have associated with functional decline previously[17]) that lead to worsened tissue preservation and presumably worsened outcomes following transplantation. We have shown previously that initial elevations in myocardial work during ESHP due to a hanging versus supported orientation of the heart led to swifter decline in function: perfusion of hearts oriented in a hanging/suspended fashion started off with greater cardiac index, but also declined significantly more than supported hearts[30].

The notion of substrate availability as a context for myocardial performance during ESHP is supported by the lactate levels we observed between the groups: at T3, we saw that in the control group, there was a significant elevation in lactate that did not appear to be tied to glucose levels. This suggests that an elevated metabolic state, as denoted by the trends towards greater MVO_2 and cardiac output in groups other than the control group, influences lactate levels. Lactate has been shown in clinical settings to be a predictor of transplant success[31], however this has been under contention[32]. Presuming that substrates such as free fatty acids are in shorter supply within the control group as compared to the blood groups which run out before mid-perfusion, this may suggest that lactate evolution in the perfusate is subject to free fatty acid availability, rather than pathologic anaerobic metabolism as postulated by the canonical understanding of lactate biology. Given these hearts operated at a higher output during

early perfusion, this would presumably accelerate glycolytic flux which would create demand for pyruvate, leading to comparatively increased lactate consumption in the blood and plasma groups.

Nonetheless, the lack of association with functional decline is consistent with our previous studies which have shown that lactate correlates poorly with myocardial performance[29], and clinical studies that show that even a low lactate can fail to predict transplant success[5]. If this were the case, we should have observed within the CB group that lactate evolution would dominate given that this group declined the most functionally. However, we saw that its lactate levels were identical to that of the WB group. This would suggest lactate is heavily influenced by metabolism rather than the result of processes that mediate permanent damage to the heart.

A much more correlable perfusate marker to myocardial functional decline would be cardiac troponin-I (cTn-I), which we found here to be especially elevated in the CB group. Interestingly, in the plasma group cTn-I appeared to be significantly elevated as well. This may be related to the reperfusion procedure: whilst the rest of the animal's blood was being centrifuged, the hearts in the plasma group were being reconstituted on an apparatus which had very low priming volume, requiring reperfusion to commence at lower pressures. This may have mediated an extended warm ischemic time which contributed to elevated cTn-I. It is also possible that increased metabolism in these groups also mediated elevations in cTn-I, given that all of the groups with elevated cTn-I also had higher cardiac index at early perfusion. This would suggest that maladaptive metabolic processes contribute to a state of myocardial stress; this requires further study.

In our study, we showed that as hemoglobin elevates, coronary flow also increased. In all groups, by T3 there was an elevation in coronary flow which may suggest loss of tone, presumably due to ischemia-reperfusion injury. Nonetheless, one would expect that as hemoglobin within the system increases, coronary flow would decrease since oxygen demands of the heart can be satisfied with equivalent flow. This is provided that performance stays constant, but performance of the heart initially was increased in all groups compared to control, denoted by elevated cardiac index and trends towards

increased MVO_2 which were significant at various points across the perfusion. As alluded to above, this suggests that myocardial performance is limited in the control group by the availability of metabolic substrate in the perfusate. In the CB group, MVO_2 was greater than the WB group however did not have significantly greater coronary flow or performance, or lower oxygen extraction, suggesting that at physiologic hemoglobin levels, demand for oxygen is satisfied; however, it begs the question what the oxygen being consumed was going towards. This may have been over-oxygenation which could contribute to the formation of oxidative stress which may have contributed to the functional decline observed.

We did see that in the CB group that indexed coronary flow initially increased similarly to the WB group, but after peaking steadily decreased, unlike the WB group. This may be suggestive of the severe hemolysis occurring that appeared by T5, as cell-free hemoglobin is known to be a scavenger of nitric oxide. We also measured that there was increased levels of nitrates and nitrites within the perfusate in the CB group which may suggest that an especially pro-oxidative environment is abrogating nitric oxide signalling and therefore vasodilation. However, there is no significant difference between the WB group and the CB group in terms of NO_x. It may be that a greater degree of NO oxidation occurred within the tissue rather than the perfusate, given that peroxynitrite is highly reactive agent. Therefore, nitrosylation of proteins could be assessed to see whether this occurred and may have contributed to dysfunction in vasodilation in the CB group versus the WB group.

2.5.1 Limitations

Despite hemolysis as a consequence to functional preservation being an important conclusion we were able to draw from elevating hematocrit, excessive hemolysis in the CB group prevented us from assessing the hypothesis that elevated oxygen delivery could mitigate functional decline over the course of perfusion. We did see that in the WB group, which was less influenced by hemolytic outcomes, that MVO_2 preservation trended towards increases during later perfusion, however this did not coincide with increased preservation of cardiac index or stroke work. This suggests that hemolysis

contributes to functional decline, and can predominate if severe, but other contributing processes are at play.

Another limitation of the current study was that differences in perfusate viscosity due to altered hematocrit cannot be controlled for. However, this is unavoidable given that viscosity changes in tandem with blood hematocrit[33].

Another limitation is that our model utilizes hearts from pre-pubescent female pigs, precluding analysis of sex mediated responses to the experimental conditions. Finally, it should be noted that our model is ‘minimally damaging’, given that hearts are not subject to the catecholamine storm that would be endured by hearts donated within the clinical setting in DBD or DCD scenarios[34]. Therefore, the preservation times in this study are somewhat idealized, but this can also be seen to lessen the potential for our experimental variable, hemoglobin concentration, to be confounded.

2.6 Tables & Figures

Table 2.1. Procurement and perfusion parameters, presented as mean (SEM).

Experimental Group	**Control (n=5)**	**Whole Blood (n=6)**	**Concentrated Blood (n=4)**	**Plasma Group (n=6)**
Ischemic Time (min)	21.5 (1.1)	21.2 (1.5)	22.7 (0.9)	20.8 (0.7)
Post-procurement heart weight (g)	240.8 (12.5)	246.7 (6.9)	236.8 (9.0)	225.9 (4.8)
Post-perfusion heart weight (g)	307.8 (17.3)	313.3 (7.5)	319.5 (16.4)	277.5 (13.6)
Weight gain (% increase)	27.9 (3.4)	27.3 (3.3)	34.9 (3.8)	22.5 (3.7)
Achieved Hb (g/dL)	4.7 (0.2)	9.7 (0.3)*	13.2 (0.3)*	4.8 (0.3)

***=**$p<0.05$ vs. control via ANOVA with Tukey post-hoc test.

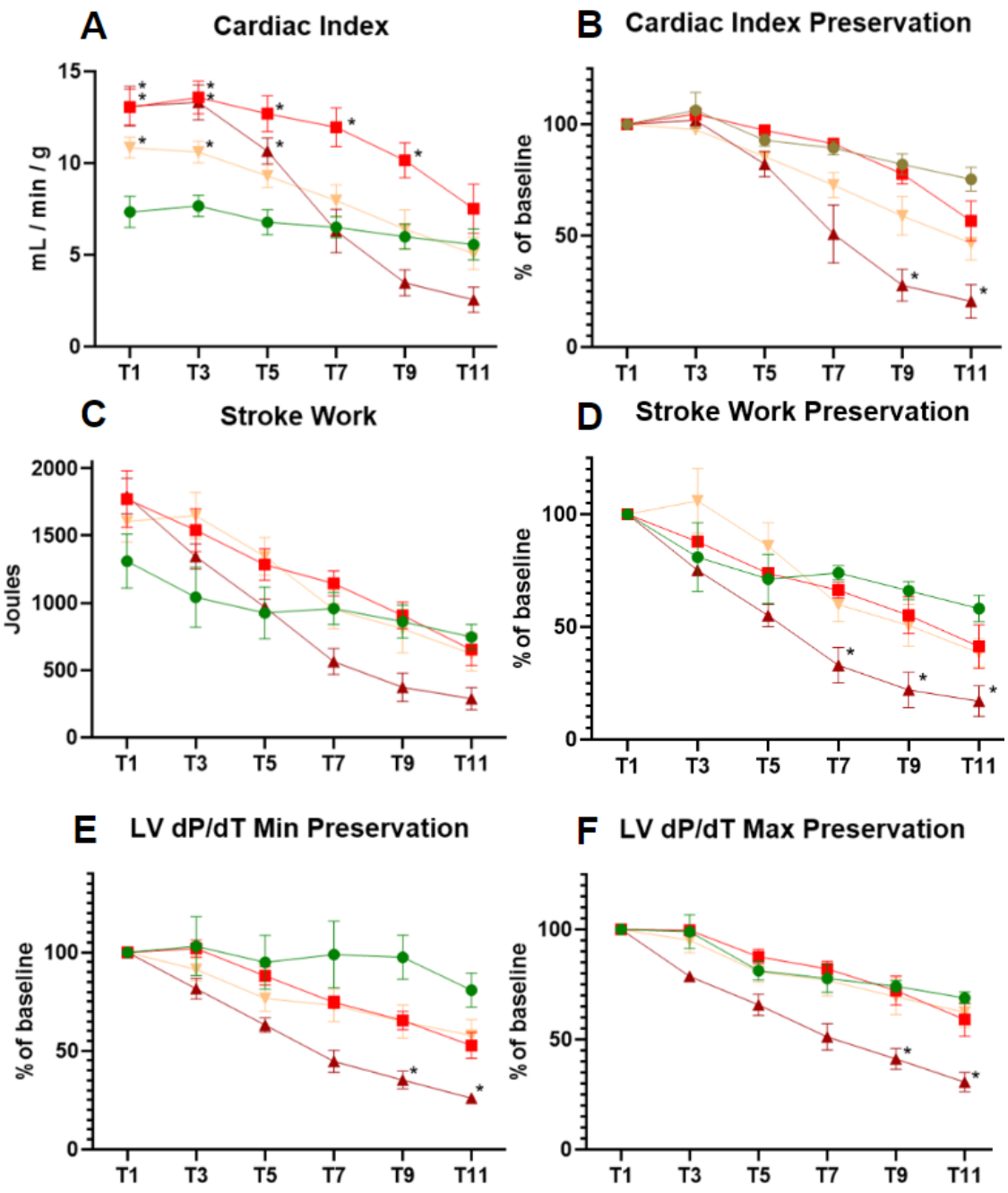

Figure 2.1. Myocardial functional parameters over the course of perfusion, from 1 hour post-reperfusion (T1) to 11 hours post-reperfusion (T11). **A)** Cardiac Index, **B)** Cardiac Index Preservation (percentage of T1 over time), **C)** Stroke work, **D)** Stroke Work Preservation, **E)** Left Ventricular dP/dT Minimum, **F)** Left Ventricular dP/dT Maximum. Displaying mean ± SEM error bars, *=$p<0.05$ compared to Control.

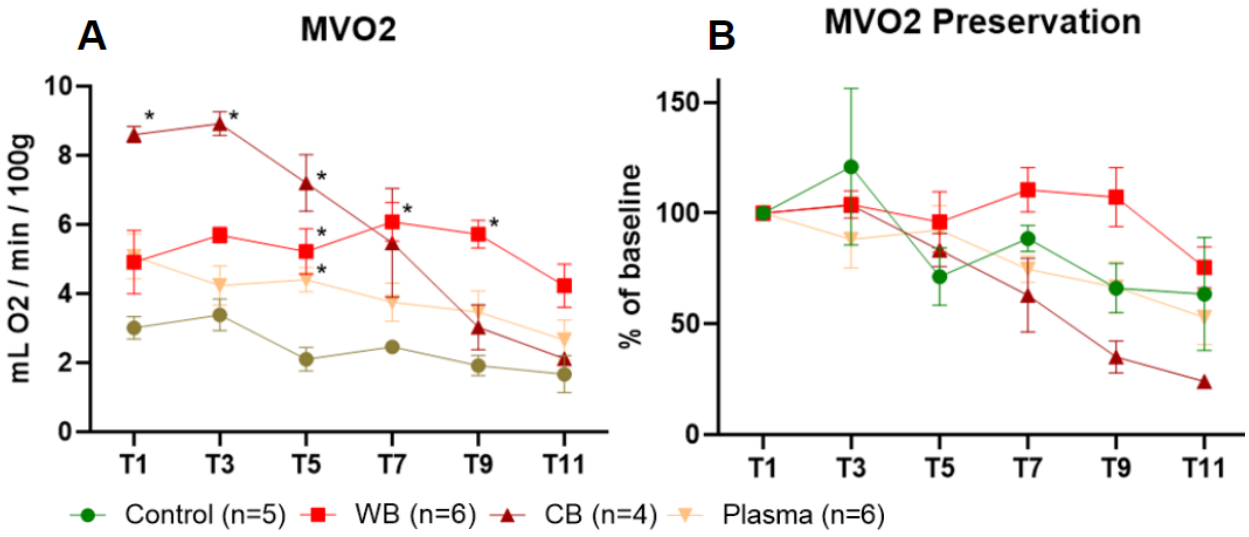

Figure 2.2. A) Myocardial oxygen consumption (MVO_2), **B)** MVO_2 Preservation (percentage of T1 value). Displaying mean ± SEM error bars, *=p<0.05 compared to Control.

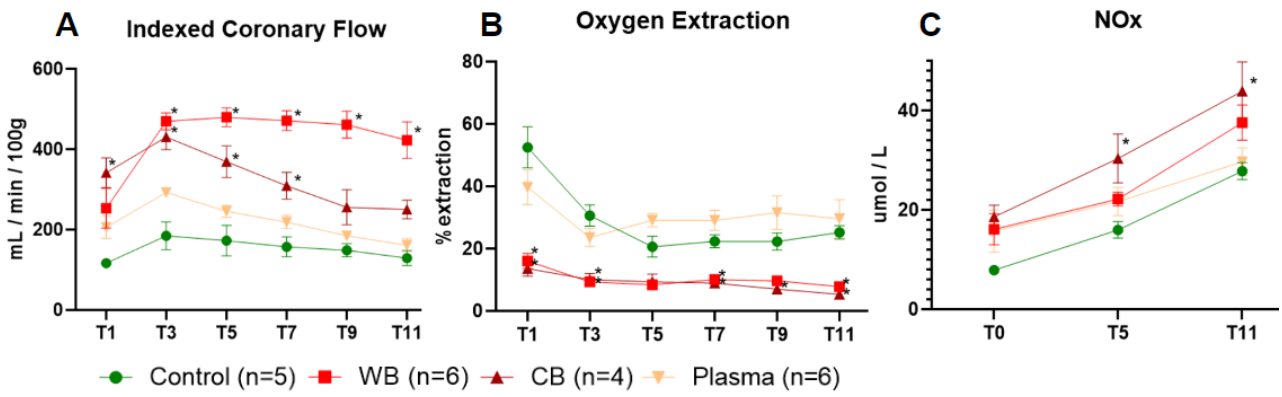

Figure 2.3. A) Indexed Coronary Flow, **B)** Oxygen Extraction, **C)** Perfusate Nitrites and Nitrates (NO_x). Displaying mean ± SEM error bars, *=$p<0.05$ compared to Control.

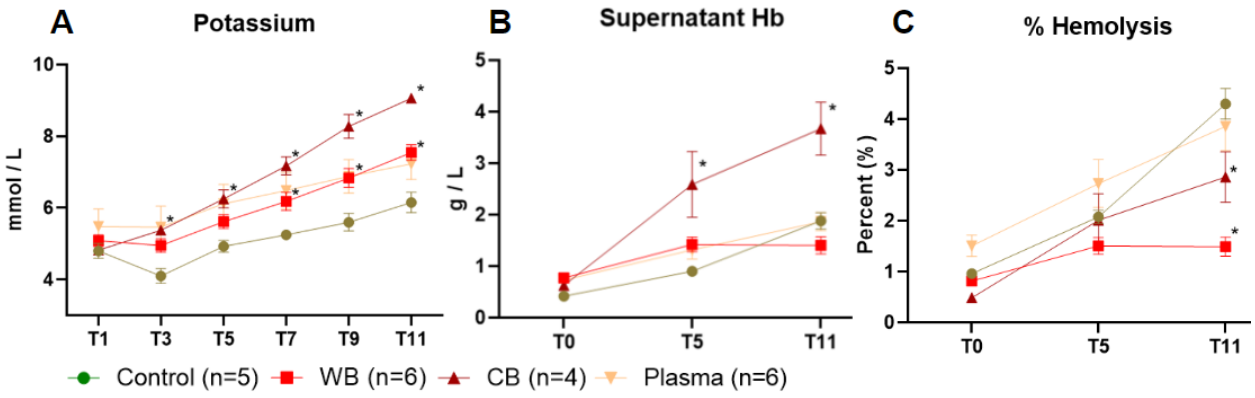

Figure 2.4. Hemolytic markers from perfusate. **A)** Perfusate potassium, **B)** Supernatant (cell-free) hemoglobin (Hb), **C)** Percent hemolysis (percentage total Hb which is supernatant Hb). Displaying mean ± SEM error bars, *=p<0.05 compared to Control.

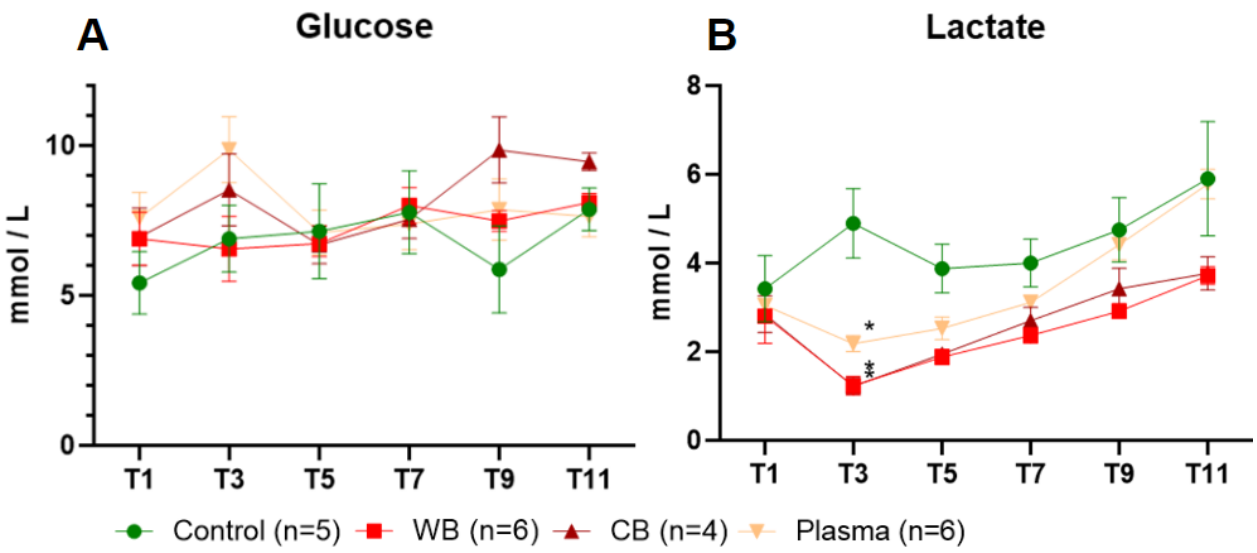

Figure 2.5. Perfusate glucose and lactate measured by ABG. **A)** Glucose, **B)** Lactate. Displaying mean ± SEM error bars, *=p<0.05 compared to Control.

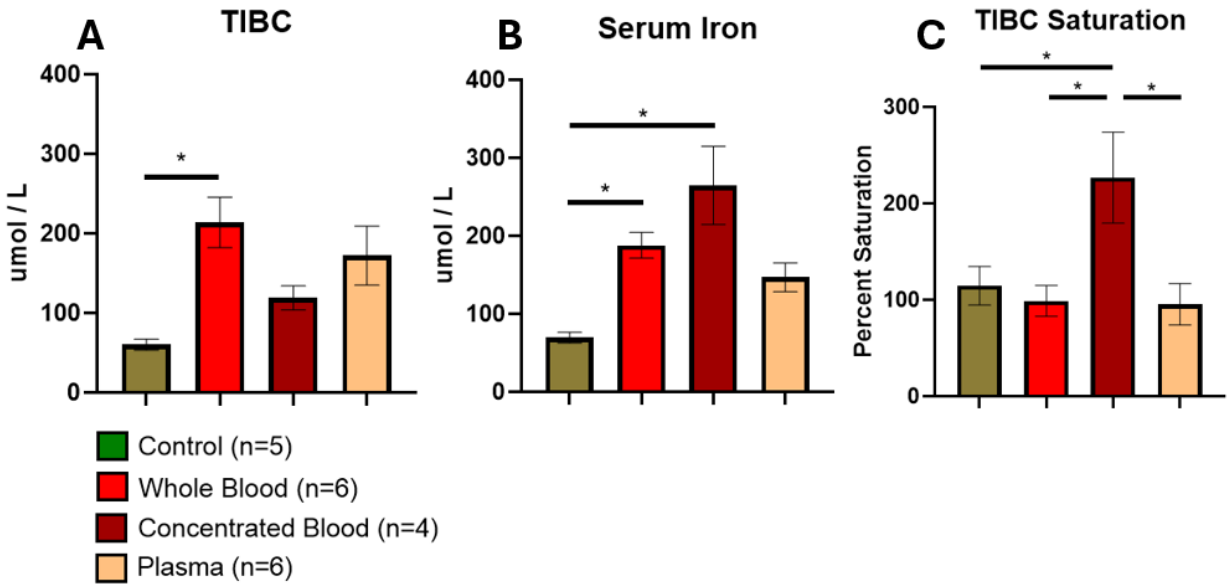

Figure 2.6. **A)** Total iron binding capacity (TIBC), **B)** serum iron, and **C)** transferrin saturation of perfusate at T11. Displaying mean ± SEM error bars, *=$p<0.05$ compared to Control.

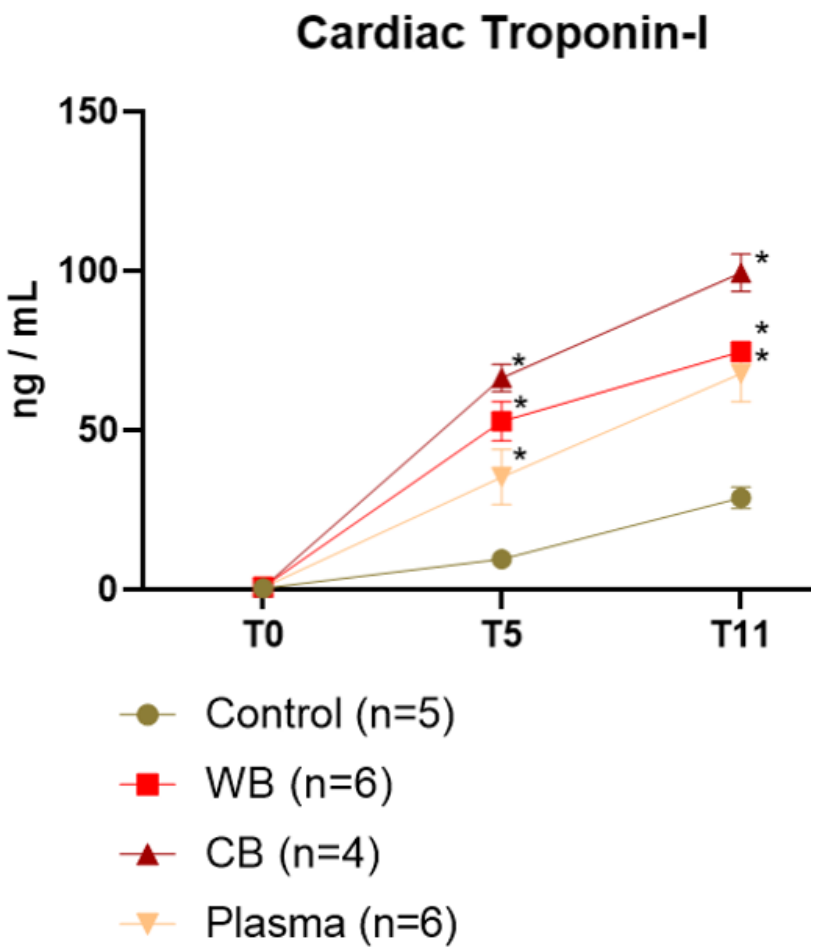

Figure 2.7. Cardiac troponin-I in perfusate. Displaying mean ± SEM error bars, *=p<0.05 compared to Control.

Supplementary Tables

Supplementary Table 2.1: Ingredients for modified Krebs-Heinseleit Buffer.

Reagent	Final Concentration (mmol/L)
Glucose	10
Sodium Chloride (NaCl)	85
Potassium Chloride (KCl)	4.6
Sodium Bicarbonate ($NaHCO_3$)	25
Sodium Phosphate, ($NaHPO_4$)	1.2
Calcium Chloride ($CaCl_2$)	1.25
Magnesium Chloride ($MgCl_2$)	1.2
Sodium Pyruvate	5
Sodium Acetate	10
Bovine Serum Albumin (BSA)	8% w/v

2.7 References

1. Wagner MJ, Hatami S, Freed DH. Thoracic organ machine perfusion: A review of concepts with a focus on reconditioning therapies. *Frontiers in Transplantation*. 2023;2. Accessed August 13, 2023. https://www.frontiersin.org/articles/10.3389/frtra.2023.1060992

2. Russo MJ, Iribarne A, Hong KN, et al. Factors associated with primary graft failure after heart transplantation. *Transplantation*. 2010;90(4):444-450. doi:10.1097/TP.0b013e3181e6f1eb

3. Russo MJ, Chen JM, Sorabella RA, et al. The effect of ischemic time on survival after heart transplantation varies by donor age: An analysis of the United Network for Organ Sharing database. *The Journal of Thoracic and Cardiovascular Surgery*. 2007;133(2):554-559. doi:10.1016/j.jtcvs.2006.09.019

4. Banner NR, Thomas HL, Curnow E, et al. The Importance of Cold and Warm Cardiac Ischemia for Survival After Heart Transplantation. *Transplantation*. 2008;86(4):542-547. doi:10.1097/TP.0b013e31818149b9

5. Stamp NL, Shah A, Vincent V, et al. Successful Heart Transplant after Ten Hours Out-of-body Time using the TransMedics Organ Care System. *Heart, Lung and Circulation*. 2015;24(6):611-613. doi:10.1016/j.hlc.2015.01.005

6. Ardehali A, Esmailian F, Deng M, et al. Ex-vivo perfusion of donor hearts for human heart transplantation (PROCEED II): a prospective, open-label, multicentre, randomised non-inferiority trial. *Lancet*. 2015;385(9987):2577-2584. doi:10.1016/S0140-6736(15)60261-6

7. Bakhtiyar SS, Sakowitz S, Verma A, Chervu NL, Benharash P. Expanded Criteria Donor Heart Allograft Utilization: National Trends and Outcomes. *The Annals of Thoracic Surgery*. 2023;116(6):1250-1258. doi:10.1016/j.athoracsur.2023.09.013

8. Hatami S, White CW, Shan S, et al. Myocardial Functional Decline During Prolonged Ex Situ Heart Perfusion. *The Annals of Thoracic Surgery*. 2019;108(2):499-507. doi:10.1016/j.athoracsur.2019.01.076

9. Olkowicz M, Ribeiro RVP, Yu F, et al. Dynamic Metabolic Changes During Prolonged Ex Situ Heart Perfusion Are Associated With Myocardial Functional Decline. *Frontiers in Immunology*. 2022;1:859506. doi:10.3389/fimmu.2022.859506

10. Transmedics Inc. TransMedics Organ Care System (TM) OCS Heart User Guide. Published online 2021. https://www.accessdata.fda.gov/cdrh_docs/pdf18/P180051S001D.pdf

11. Gunst J, Souter MJ. Management of the brain-dead donor in the intensive care unit. *Intensive Care Med*. Published online April 10, 2024. doi:10.1007/s00134-024-07409-4

12. Hatami S, White CW, Ondrus M, et al. Normothermic Ex Situ Heart Perfusion in Working Mode: Assessment of Cardiac Function and Metabolism. *J Vis Exp*. 2019;(143). doi:10.3791/58430

13. Chew HC, Scheuer S, Dhital K, Macdonald P. Banked blood for normothermic machine perfusion of the donor heart: A clinical perspective. *The Journal of Heart and Lung Transplantation*. 2019;38(12):1322. doi:10.1016/j.healun.2019.09.001

14. Chew H, Cheong C, Fulton M, et al. (96) - Outcome After Warm Machine Perfusion (WMP) Recovery of Marginal Brain Dead (MBD) and Donation After Circulatory Death (DCD) Heart Transplantation. *The Journal of Heart and Lung Transplantation*. 2017;36(4, Supplement):S45-S46. doi:10.1016/j.healun.2017.01.107

15. Qi X. Regulation of Coronary Artery Flow during Normothermic Ex-Situ Heart Perfusion [dissertation]. Edmonton (AB): University of Alberta; 2020.

16. White CW, Hasanally D, Mundt P, et al. A whole blood–based perfusate provides superior preservation of myocardial function during ex vivo heart perfusion. *The Journal of Heart and Lung Transplantation*. 2015;34(1):113-121. doi:10.1016/j.healun.2014.09.021

17. Hatami S, Qi X, Khan M, et al. Oxidative stress and related metabolic alterations are induced in ex situ perfusion of donated hearts regardless of the ventricular load or leukocyte depletion. *American Journal of Transplantation*. Published online January 2023:S1600613522302174. doi:10.1016/j.ajt.2022.11.027

18. Oudit GY, Trivieri MG, Khaper N, Liu PP, Backx PH. Role of L-type Ca2+ channels in iron transport and iron-overload cardiomyopathy. *J Mol Med (Berl)*. 2006;84(5):349-364. doi:10.1007/s00109-005-0029-x

19. Murphy CJ, Oudit GY. Iron-Overload Cardiomyopathy: Pathophysiology, Diagnosis, and Treatment. *Journal of Cardiac Failure*. 2010;16(11):888-900. doi:10.1016/j.cardfail.2010.05.009

20. Spencer BL, Wilhelm SK, Stephan C, et al. Extending heart preservation to 24 h with normothermic perfusion. *Front Cardiovasc Med*. 2024;11. doi:10.3389/fcvm.2024.1325169

21. Spencer BL, Wilhelm SK, Urrea KA, et al. Twenty-Four Hour Normothermic Ex Vivo Heart Perfusion With Hemofiltration In an Adult Porcine Model. *Transplant Proc*. 2023;55(9):2241-2246. doi:10.1016/j.transproceed.2023.08.014

22. Johnson MD, Fallon BP, Langley M, et al. Prolonged (24-hour) Normothermic ex vivo Heart Perfusion Facilitated by Perfusate Hemofiltration. *ASAIO Journal*. 2022;68(10):1282-1289. doi:10.1097/MAT.0000000000001649

23. Dixon SJ, Olzmann JA. The cell biology of ferroptosis. *Nat Rev Mol Cell Biol*. 2024;25(6):424-442. doi:10.1038/s41580-024-00703-5

24. Xie Y, Hou W, Song X, et al. Ferroptosis: process and function. *Cell Death Differ*. 2016;23(3):369-379. doi:10.1038/cdd.2015.158

25. Gu YJ, Vermeijden WJ, Vries AJ de, Hagenaars JAM, Graaff R, Oeveren W van. Influence of Mechanical Cell Salvage on Red Blood Cell Aggregation, Deformability, and 2,3-Diphosphoglycerate in Patients Undergoing Cardiac Surgery With Cardiopulmonary Bypass. *The Annals of Thoracic Surgery*. 2008;86(5):1570-1575. doi:10.1016/j.athoracsur.2008.07.052

26. Carroll C, Young F. Intraoperative cell salvage. *BJA Education*. 2021;21(3):95-101. doi:10.1016/j.bjae.2020.11.007

27. Chew HC, Gao L, Villaneuva J, et al. Ex-Vivo Perfusion of Donor Hearts: The Feasibility of Banked Blood for Normothermic Machine Perfusion. *OBM Transplantation*. 2019;3(2):1-24. doi:10.21926/obm.transplant.1902067

28. White CW, Messer SJ, Large SR, et al. Transplantation of Hearts Donated after Circulatory Death. *Front Cardiovasc Med*. 2018;5:8. doi:10.3389/fcvm.2018.00008

29. White CW, Ambrose E, Müller A, et al. Assessment of donor heart viability during ex vivo heart perfusion. *Can J Physiol Pharmacol*. 2015;93(10):893-901. doi:10.1139/cjpp-2014-0474

30. Hatami S, Qi X, White CW, et al. The Position of the Heart During Normothermic Ex Situ Heart Perfusion is an Important Factor in Preservation and Recovery of Myocardial Function. *ASAIO Journal*. 2021;67(11):1222-1231. doi:10.1097/MAT.0000000000001386

31. Hamed A, Tsui S, Huber J, Lin R, Poggio EC, Ardehali A. 19: Serum Lactate Is a Highly Sensitive and Specific Predictor of Post Cardiac Transplant Outcomes Using the Organ Care System. *The Journal of Heart and Lung Transplantation*. 2009;28(2):S71. doi:10.1016/j.healun.2008.11.025

32. Freed DH, White CW. Donor heart preservation: straight up, or on the rocks? *The Lancet*. 2015;385(9987):2552-2554. doi:10.1016/S0140-6736(15)60614-6

33. Trejo-Soto C, Hernández-Machado A. Normalization of Blood Viscosity According to the Hematocrit and the Shear Rate. *Micromachines (Basel)*. 2022;13(3):357. doi:10.3390/mi13030357

34. Aldam PS. The physiological changes of circulatory death with respect to organ donation [dissertation]. Cambridge (UK): University of Cambridge; 2019

Chapter 3: The impacts of fluids provision & plasma components on coronary functionality during ex-situ heart perfusion

The following chapter is currently in preparation for publication in a peer-reviewed journal.

3.1 Abstract

Background:

Ex-situ heart perfusion (ESHP) enables improved preservation of donor hearts during transit compared to cold storage. However, alongside a loss of heart function during ESHP, a loss of coronary vascular resistance (CVR) is also observed, a marker of endothelial dysfunction. We have noted previously that perturbations of the endothelial glycocalyx, a structure implicated in regulation of vasomotor tone, occur during ESHP, especially when whole blood perfusate is diluted with manufactured buffer. During the studies featured in the previous chapter, we observed that control runs provided a low amount of crystalloid fluid during procurement appeared to be saved from development of supraphysiologic coronary flow. In the current chapter we analyze coronary flow rates from historical ESHP runs wherein a high degree of crystalloid fluid was given, comparing them to newer ESHP runs in which either low amounts of fluids have been provided during procurement or plasma proteins have been supplemented within the perfusate during ESHP.

Methods

Hearts were procured from yorkshire pigs weighing 45-55 kg and perfused for 11 hours on an ESHP apparatus capable of working mode perfusion. Hearts were perfused with either a 1:1 mixture of donor animal whole blood (WB) and plasma (Plasma group, n=6), 1:1 mixture of WB and Krebs-Heiseleit buffer (KHB) with 16% bovine serum albumin (hi-ALB group, n=5), or 1:1 mixture of WB with KHB with 8% bovine serum (Low Fluids). Each of these groups were provided <500mL of crystalloid fluid during procurement. These were compared to historical ESHP runs (denoted High Fluids group, n=6) in which it was noted that ~2.5 litres of crystalloid fluid was provided during procurement. Functional measurements and arterial blood gas analysis were carried out bi-hourly and compared between groups. Markers of endothelial glycocalyx breakdown such as syndecan-1 and hyaluronan were assessed using enzyme-linked immunosorbent assay (ELISA).

Results

There were few differences in functional preservation between the groups. The Plasma group started off at higher mean cardiac index values compared to Low Fluids group (10.8 vs. 7.3 mL/min/g, $p<0.05$), and trended towards higher initial stroke work. Indexed coronary flow was significantly raised from all groups within the High Fluids group from T3 onwards, continuing to rise throughout perfusion. Perfusate hyaluronan at T0 was significantly raised in the High Fluids group compared to Low Fluids group. Hyaluronan raised in the Low Fluids group by T5 but remained low in the hiALB and Plasma groups (in ng/mL, hiALB 72.9 vs. plasma 81.6, low fluids 442.1 & 297.3 for low fluids and high fluids respectively; $p<0.05$ for each of hiALB and Plasma vs. low fluids). Syndecan-1 trended towards higher values in both the Low Fluids and High Fluids groups compared to Plasma and hiALB at perfusion start (in ng/mL: 17.4, 17.8, 4.3, 2.5 respectively), but was not statistically significant.

Conclusion

Early disturbances marked by deposition of certain endothelial glycocalyx components within the perfusate during ESHP perhaps due to fluid provision during procurement are associated with supraphysiologic coronary flow during ESHP. Provision of plasma proteins, either by direct provision of donor plasma or supplementation of buffer with albumin, can ameliorate glycocalyx damage during early perfusion (up to 5 hours). This suggests the importance of plasma components in preservation of vascular integrity during ESHP.

3.2 Introduction

Vascular preservation is an important consideration during ex-situ heart perfusion (ESHP). As the interface between the perfusate and the tissue, the endothelium plays a significant role in regulating flow and acting as a barrier to the formation of edema, which is of concern in machine perfusion settings, especially hypothermic ESHP[1]. Beyond acute effects observed immediately following machine perfusion, cardiac allograft vasculopathy (CAV) is a severely limiting factor in regard to graft longevity following transplantation, with almost 50% being diagnosed with this currently incurable condition by year 10 post-transplant[2]. The exact mechanism behind CAV is unknown, but it is thought to be a disease of the endothelium lensed by both immunologic and non-immunologic risk factors, including acute conditions experienced during the transplant process[3]. Modulation of the interface between the circulating perfusate and the donated organ's vasculature during machine perfusion could therefore be an important strategy to better preserve vascular health, and mitigate the development of post-transplant pathology such as acute rejection and CAV. The ESHP platform is an excellent opportunity to apply therapeutic regimes and strategies to bolster graft function post-transplant[1].

Indeed, markers of endothelium damage are present during ESHP. In previous studies we have noted that coronary vascular resistance appears to be progressively lost during ESHP, leading to supraphysiologic coronary flow that is uncoupled from myocardial performance[4]. In a physiological setting, one would expect that coronary flow adapts to meet the metabolic demands of the myocardium[5], however in the setting of ESHP, coronary flow has been observed to increase whilst myocardial function declines[6]. This inability to appropriately regulate flow suggests that damage is sustained to the coronary endothelium, presumably by the immuno-oxidative milieu that pervades machine perfusion settings and the onset of ischemia-reperfusion injury[7,8].

Linking together these observations is an endothelial structure called the endothelial glycocalyx (EGX). The EGX is as a protruding mesh of both plasma- and endothelium-derived proteins, glycoproteins, and proteoglycans found between the lumen and endothelial cells. The structure protrudes into the vessel lumen, with many

key functions relevant to observations made during ESHP: 1) it has been shown to act as a flow mechanotransducer, enabling endothelial sensing of flow to trigger vasoactivity through regulating nitric oxide production[9,10], 2) it forms a size-exclusionary and charge selective barrier that contributes to endothelial permeability, and 3) it houses vasculo-protective anticoagulant and antioxidative enzymes that dock within the structure[11]. Disturbance of the EGX leads to the release of its components such as syndecan-1, hyaluronan, heparan sulfate, etc. into the circulation, which is the primary way that EGX damage is detected. Indeed, the EGX is known to be sensitive to inflammation & oxidative stress (such as that produced by ischemia-reperfusion injury)[12], and therefore liable to the conditions endured during ESHP[8].

In this chapter, as a type of secondary analysis I compared ESHP which were given a large amount of crystalloid fluid during procurement to runs in which minimal fluids were provided, or whereby the perfusate is supplemented with plasma proteins from either donor plasma supplementation or albumin supplementation via the perfusate. I show preliminarily that coronary resistance is impaired between runs with high or low fluids during procurement. Supplementation of plasma proteins either by donor plasma or albumin via the perfusate during ESHP appeared to ameliorate glycocalyx component deposition at early time perfusion periods. This lends support to the hypothesis that impaired coronary vascular resistance during ESHP may be the result of glycocalyx breakdown, and can be influenced by fluids during procurement preceding the immuno-oxidative milieu present during ESHP[8].

3.3 Methods

The relevant methodology for ESHP and data analysis is described in Chapter 2. Under ethics approval AUP00000943, animal usage, heart procurement and machine perfusion procedure and apparatus are all identical for the experiment described in this chapter. The only difference in methods is the establishment of groups, outlined below.

3.3.1 Experimental Groups

The ‘high fluids’ group (n=6) describes runs from 2018-2019 in which a high degree of fluids (2.5-3L of lactated ringers saline) were provided during the procurement

process. The ‘low fluids’ group (n=5) was fluids restricted during procurement, using at most 500mL of PlasmaLyte A. The Plasma (n=6) and high-albumin (hiALB, n=5) groups were also fluids restricted, again using at most 500mL of PlasmaLyte A. In the plasma group, a whole blood prime (800mL) was used to reperfuse the heart whilst blood was centrifuged at 1500g for 30 minutes in 50mL falcon tubes, with ~800mL of plasma being added back to the whole blood prime. The high albumin group is similar to the ‘low fluids’ group however instead of using modified KHB with 8% w/v BSA, 16% w/v BSA was used instead (see Supplementary Table 1 for modified KHB ingredients). All groups were perfused for nearly 12 hours on the custom ESHP apparatus outlined in Chapter 2, with measurement of functional variables and sampling procedures remaining the same.

3.3.2 Experimental Assays

Perfusate biochemical markers were measured using kits. Cardiac troponin-I was evaluated using a kit from Life Diagnostics Inc. (CTNI-9-HS); hyaluronan using a kit from Echelon Biosciences (K-4800); syndecan-1 using a kit from Abcam (ab46506). Manufacturer instructions were followed for each kit.

3.3.3 Data Analysis & Comparisons

Variables such as weight gain, cardiac index, indexed coronary flow, oxygen extraction, and MVO_2 are calculated as described in Chapter 2 methods. The ‘low fluids’ group was designated as the control group for which all groups were compared to.

Data is displayed in figures and tables as mean ± standard error of the mean (SEM) error bars. Intergroup differences were compared using one- or two-way analysis of variance (ANOVA) with multiple comparisons accounted for by Tukey’s multiple comparison test. An adjusted p-value <0.05 was considered a statistically significant difference.

3.4 Results

3.4.1 Heart Characteristics & Weight Gain

Initial heart characteristics and weight gain are presented in Table 3.1 and Figure 2.1. There were no significant differences in weight gain between any of the groups ($p>0.05$, Figure 3.1), however weight gain in the Plasma and hiALB groups trended towards lower values (Mean: Plasma, 22.5%; hiALB, 19.7%; Low Fluids, 27.9%).

3.4.2 Functional Results

Cardiac index within the Plasma group was significantly greater at early time periods (T1, T3) in comparison to the Low Fluids group ($p<0.05$). Cardiac index preservation (%) was significantly decreased ($p<0.05$) compared to Low Fluids group at T11 in the Plasma group (Figure 3.2B). While not significant, stroke work (Figure 3.2C) similarly trended towards greater values at early perfusion (T1, T3, T5). Otherwise, markers of cardiac function did not significantly differ between groups.

3.4.3 Myocardial Metabolism Markers

As previously noted, myocardial oxygen consumption (MVO_2) trended towards higher values in the plasma group at all time points, and was statistically significant ($p<0.05$) in comparison to the Low Fluids group at T5 (Figure 3.3A). All groups' MVO_2 appeared to decrease over the course of perfusion. Mean lactate in the high fluids group appeared to remain stable between 3-4 mmol/L. Lactate was only significantly different from the low fluids group in the plasma group at T3 ($p<0.05$, Figure 3.3B). Glucose was kept within 5-10 mmol/L and was relatively stable within this range, with no statistically significant differences present at any timepoint (Figure 3.3C).

3.4.4 Coronary Flow and Oxygen Extraction

In all groups but the high fluids group, indexed coronary flow appeared to peak at T3 before decreasing back to levels near baseline. Indexed coronary flow was significantly increased in the high fluids group compared to the low fluids group at all time points (Figure 3.4A). This associated with a trend towards decreased oxygen

extraction in the high fluids group, which was significant in comparison to the low fluids group at T3, T7, and T9 (Figure 3.4B).

3.4.5 Perfusate Biomarkers

Levels of hyaluronan, syndecan-1, and cardiac troponin-I were measured within the perfusate at T0 (baseline), T5 (mid-perfusion), and T11 (late perfusion). Hyaluronan was significantly increased within the high fluids group even at baseline in comparison to the low fluids group (Figure 3.5A). In the hiALB and Plasma groups, hyaluronan levels were significantly decreased ($p<0.05$) in perfusate at T5 in comparison to the low fluids group. Hyaluronan in all groups elevated by T11, however there was no significant intergroup differences at this timepoint.

For syndecan-1, there were no significant differences at any timepoint however syndecan-1 trended towards elevations at baseline in both the low and high fluids groups in comparison to the hiALB and Plasma groups (Figure 3.5B). The low fluids group appeared to have a decreasing syndecan-1 content over time.

Cardiac troponin-I elevated over time in all groups, significantly moreso ($p<0.05$) in the Plasma and High Fluids groups in comparison to Low Fluids and hiALB groups (Figure 3.5C).

3.5 Discussion

We observed that between the High Fluids (>2.5L) and Low Fluids (<500mL) groups coronary flow was significantly increased at all points across the perfusion period. Similarly, the Plasma and hiALB groups, which also had low fluids (<500mL) provision during procurement, presented similar coronary flow patterns across an extended period of ESHP. While cardiac functional output was unchanged between any of the groups except for plasma, which as previously discussed may be the result of increased free fatty acid provision, this profound difference in coronary flow suggests that the degree of fluids provision during procurement can influence coronary flow once on the ESHP apparatus.

Since the EGX is known to participate in vessel tone regulation, we hypothesized that disturbance of the EGX may mediate the striking differences in coronary flow observed here. As a dynamic structure which forms a Le-Chetalier-like equilibrium with components within the plasma[13], it is also sensitive to hemodilution. It has been shown that plasma proteins such as albumin interact at the lumen-endothelial barrier and actually forms part of the EGX mesh, contributing to its negative charge and stabilizing the other proteins that compose its structure[11,13]; addition of fluids in surgical scenarios has been shown to increase the levels of circulating EGX markers in serum[14]. Indeed, we saw that in the group provided a higher amount of fluids during procurement that hyaluronan was significantly elevated within serum at the very beginning of perfusion (T0); syndecan-1 trended towards higher values initially in the low and high fluids groups, being lower in plasma and hiALB groups.

We have observed previously in a comparison between dilute whole blood and whole blood perfusate during short perfusion times (<6 hours), which would also differ in the degree of EGX stabilizing plasma proteins present, that coronary blood flow ended up increased in the dilute blood group, with higher levels of hyaluronan deposition and increased levels of cTn-I during perfusion[6]. An unpairing between coronary flow and left ventricular stroke work was observed within dilute blood group perfusion, signalling an inability to regulate coronary vascular tone[6]. Outside of our research group's results, hearts that during ESHP underwent plasma exchange featured a greater coronary

vascular resistance over the course of perfusion[15]. The preliminary results shown here reinforce that replenishment or disturbance of plasma proteins during ESHP impacts coronary tone regulation.

Interestingly, loss of the glycocalyx has thus far been associated with vasoconstriction given that the glycocalyx is thought to control flow-mediated tone via activation of eNOS and subsequent NO production[16]. In this manner, loss of the glycocalyx is anticipated to result in vasoconstriction. However, studies which have identified the EGX to regulate tone through NO production have been conducted in-vitro, typically using cultured cells[16–18]. Evidence in the context of ESHP has thus far shown that conditions which disturb the glycocalyx seem to be associated with the effect of mediating increased coronary blood flow, with replenishment of plasma proteins having the opposite effect. It should be noted that the glycocalyx does takes up considerable space within resistance mediating arteries, stretching up to micrometeres into the vessel that can be observed on histology as a zone of RBC exclusion from the lumen-endothelial interface[11]. Via Poiseuille's equation, which states that at a constant pressure difference across a vascular bed (the ESHP runs featured here are at a constant perfusion pressure of 40 mmHg), even small increases in vessel radius (that could be yielded by glycocalyx loss) can mediate large increases in flow. Further, studies that have focused on EGX mediated vasodilation via NO production are isolated from the inflammatory and altered metabolic conditions that pervade ESHP, therefore it is likely that many interacting effects on the coronary vasculature are at play to produce the results observed here.

Nonetheless, the fact that provision of increased amounts of plasma proteins via plasma or albumin supplementation in the perfusate could reduce hyaluronan deposition suggests an approach to protect the EGX (and presumably, the endothelium) during ESHP. This may be a modality with which vascular outcomes and rejection processes following transplantation can be mitigated, as the EGX can influence the vascular permeability of allo-antigens through the endothelium, recruitment of leukocytes, and hosts a variety of vasculo-protective enzymes[11]. In the field currently, there are few efforts to provide vasculo-protection during ESHP, in favour of

preservation of myocardial performance for which vasculoprotection and myocardial performance are anticipated to go hand in hand. Provided that the low fluids condition in comparison to the high fluids condition was able to reduce cTnI levels independently of myocardial performance during ESHP suggests these two areas do not necessarily go hand in hand during ESHP. More study is required to better characterize how fluid conditions act upon coronary vascular resistance and overall vascular protection during ESHP.

3.5.1 Limitations

As these are preliminary results for consideration that require further study and characterization, there are some limitations that should be noted. Firstly, the High Fluids group and Low Fluids group ESHP runs were accomplished by different personnel at different periods in our research group's experience with heart procurement and management during ESHP. While care has been taken to ensure that discrepancies in methods are minimized, this could factor into the differences observed here. Different fluids were used between the High Fluids and Low Fluids groups (Lactated Ringers vs. PlasmaLyte A). This should not affect the interpretation regarding the relationship between EGX disturbance and plasma protein availability, as these are both crystalloid fluids lacking any plasma components. Nonetheless, it is a notable difference in treatment between these groups.

3.5.2 Future Directions

While it follows that lower fluids provision during heart and blood procurement before ESHP would lead to less dilution of circulating plasma proteins in the perfusate, since plasma protein supplementation was only experienced by some hearts after reconstitution on the ESHP apparatus, this did not allow us to assess whether plasma protein supplementation during ESHP could ameliorate potential vascular disturbances from high fluids provision during procurement. In the future, another experimental group whereby high amounts of crystalloid fluid are provided during procurement followed by reconstitution of the heart in a high plasma protein perfusate (such as that in the hiALB

group) during ESHP could show us whether the elevated coronary flow phenotype is reversible during ESHP. Alternatively, albumin could be removed from the KHB to see if hemodilution once on the ESHP apparatus can influence coronary flow. If coronary flow is influenced in either of these scenarios, would lend greater credence to the proposition that ESHP could be used as a platform for focused vascular protection/reconditioning via plasma protein supplementation.

There are a variety of assays that could be carried out to better elucidate the relationship between EGX disturbance and the striking difference in coronary flow between the High Fluids and Low Fluids groups. Assay of the nitric oxides produced in the perfusate would provide insight into whether NO production is impaired through glycocalyx disturbance in this setting. Characterization of vasodilatory factors related to high fluids provision (for example, atrial natriuretic peptide[19]) would also be insightful.

3.5.3 Conclusion

Provision of a high amount of fluids during heart procurement seems to produce robust differences in coronary vascular resistance during ESHP. Concomitantly, high fluids appeared to mediate disturbance in hyaluronan, indicating that high fluids provision could mediate damage to the vasculature to predispose these differences. More study is required, though the results here suggest the importance of plasma proteins during ESHP.

3.6 Tables & Figures

Table 2.1. Procurement and perfusion parameters, mean (SEM).

Experimental Group	Low Fluids (Control, n=5)	High Fluids (n=6)	hiALB (n=5)	Plasma (n=6)
Ischemic Time (min)	21.5 (1.1)	21.5 (1.2)	21.0 (1.2)	20.8 (0.7)
Post-procurement heart weight (g)	240.8 (12.5)	250.2 (10.6)	251.1 (5.6)	225.9 (4.8)
Post-perfusion heart weight (g)	307.8 (17.3)	315.5 (9.8)	300.4 (7.9)	277.5 (13.6)
Achieved Hb (g/dL)	4.7 (0.2)	4.7 (0.1)	4.6 (0.2)	4.8 (0.3)

***=**$p<0.05$ vs. low fluids via ANOVA with Tukey post-hoc test.

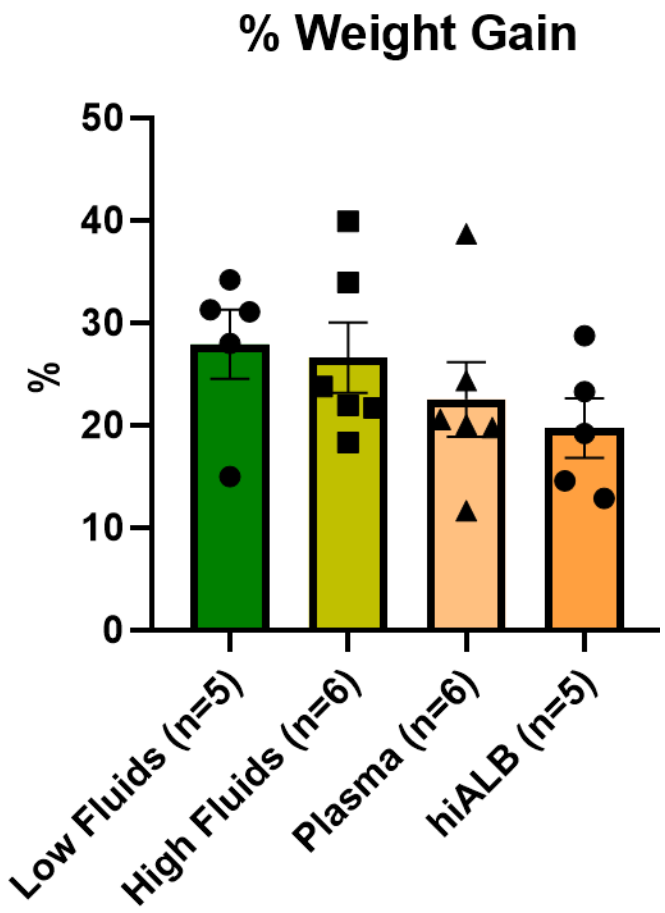

Figure 3.1. Percentage weight gain of hearts subject to ESHP. Displaying mean ± SEM error bars.

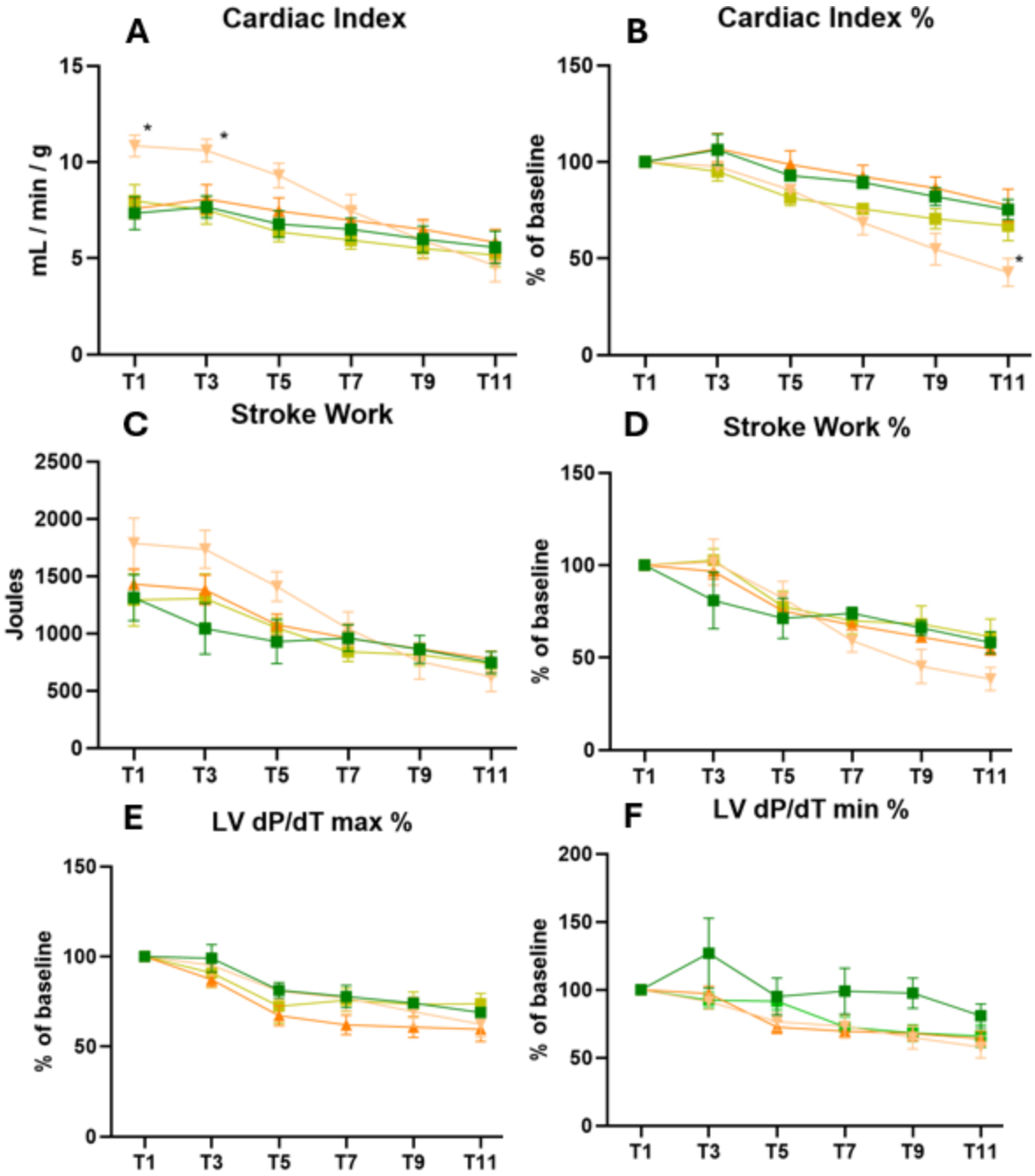

Figure 3.2. Myocardial functional parameters over the course of perfusion, from 1 hour post-reperfusion (T1) to 11 hours post-reperfusion (T11). **A)** Cardiac Index, **B)** Cardiac Index Preservation (percentage of T1 over time), **C)** Stroke work, **D)** Stroke Work Preservation, **E)** Left Ventricular dP/dT Minimum, **F)** Left Ventricular dP/dT Maximum. Displaying mean ± SEM error bars, *=p<0.05 compared to Low Fluids.

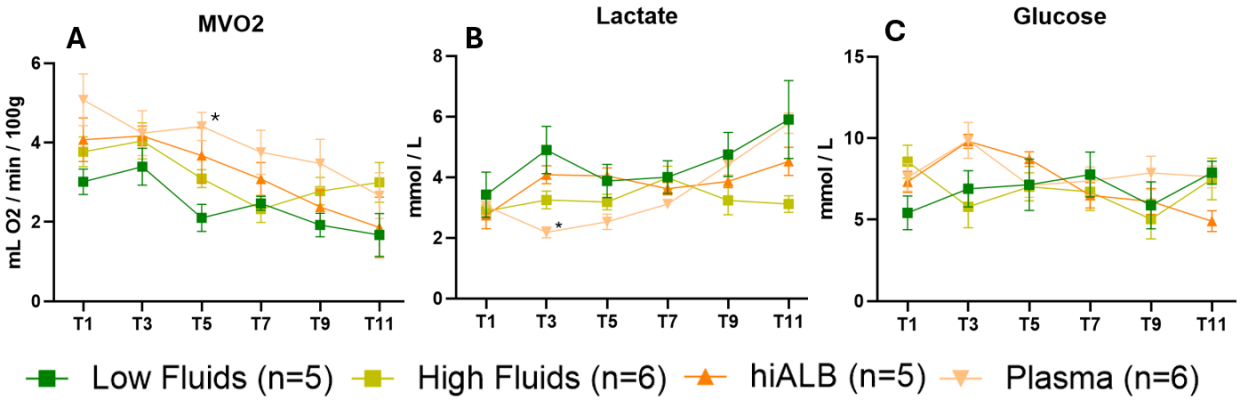

Figure 3.3. Myocardial metabolic markers. **A)** Myocardial oxygen consumption (MVO_2), **B)** perfusate lactate concentration, **C)** perfusate glucose concentration. Displaying mean ± SEM error bars, *=$p<0.05$ compared to Low Fluids.

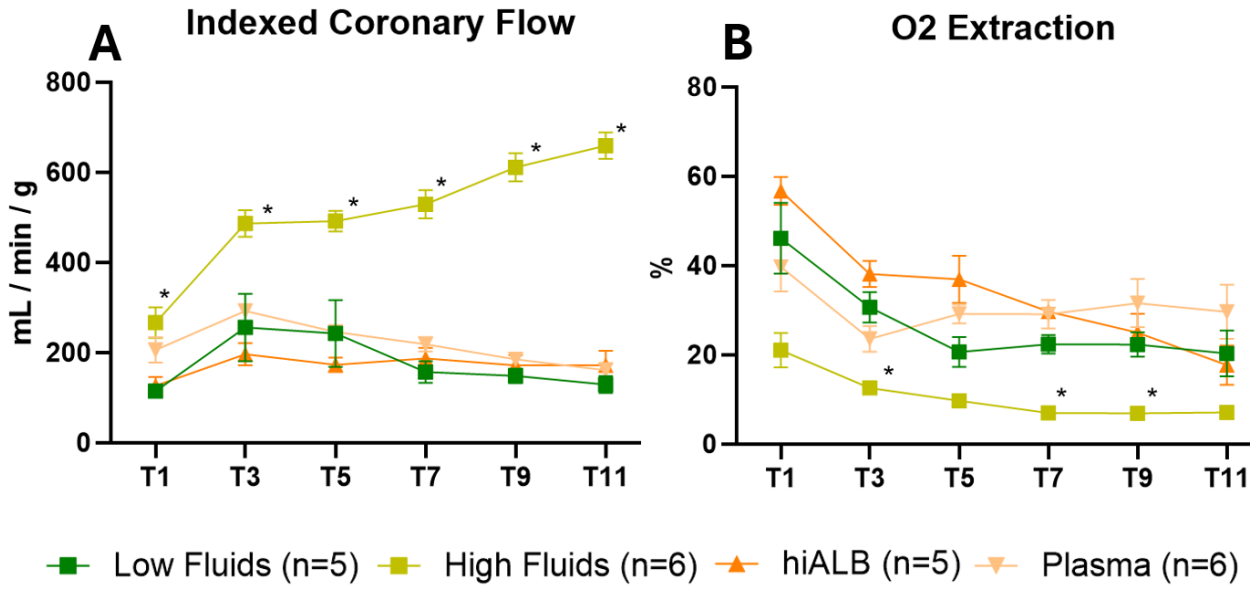

Figure 3.4. A) Indexed Coronary Flow, **B)** Oxygen Extraction. Displaying mean ± SEM error bars, *=p<0.05 compared to Low Fluids.

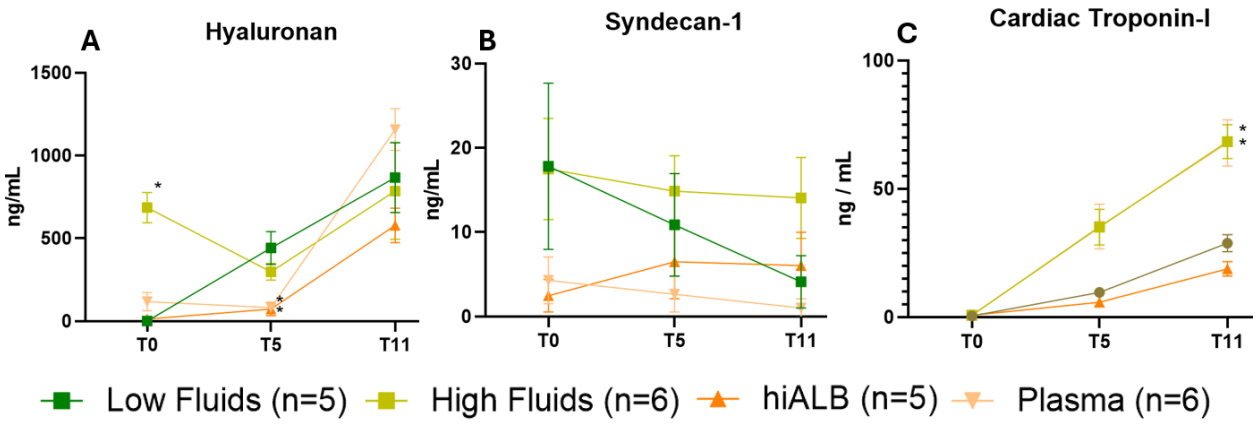

Figure 3.5. Endothelial glycocalyx breakdown markers and cardiac troponin-I concentrations in perfusate. **A)** Hyaluronan concentration, **B)** Syndecan-1 concentration, **C)** Cardiac troponin-I concentration. Displaying mean ± SEM error bars, *=p<0.05 compared to Low Fluids.

3.7 References

1. Wagner MJ, Hatami S, Freed DH. Thoracic organ machine perfusion: A review of concepts with a focus on reconditioning therapies.

2. Khush KK, Cherikh WS, Chambers DC, et al. The International Thoracic Organ Transplant Registry of the International Society for Heart and Lung Transplantation: Thirty-sixth adult heart transplantation report - 2019; focus theme: Donor and recipient size match. *J Heart Lung Transplant*. 2019;38(10):1056-1066. doi:10.1016/j.healun.2019.08.004

3. Pober JS, Chih S, Kobashigawa J, Madsen JC, Tellides G. Cardiac allograft vasculopathy: current review and future research directions. *Cardiovasc Res*. 2021;117(13):2624-2638. doi:10.1093/cvr/cvab259

4. Qi X, Hatami S, Bozso S, et al. The evaluation of constant coronary artery flow versus constant coronary perfusion pressure during normothermic ex situ heart perfusion. *The Journal of Heart and Lung Transplantation*. 2022;41(12):1738-1750. doi:10.1016/j.healun.2022.08.009

5. Goodwill AG, Dick GM, Kiel AM, Tune JD. Regulation of Coronary Blood Flow. *Compr Physiol*. 2017;7(2):321-382. doi:10.1002/cphy.c160016

6. Qi X. Regulation of Coronary Artery Flow during Normothermic Ex-Situ Heart Perfusion [dissertation]. Edmonton (AB): University of Alberta; 2020.

7. Hatami S, Hefler J, Freed DH. Inflammation and Oxidative Stress in the Context of Extracorporeal Cardiac and Pulmonary Support. *Front Immunol*. 2022;13:831930. doi:10.3389/fimmu.2022.831930

8. Hatami S, Qi X, Khan M, et al. Oxidative stress and related metabolic alterations are induced in ex situ perfusion of donated hearts regardless of the ventricular load or leukocyte depletion. *American Journal of Transplantation*. Published online January 2023:S1600613522302174. doi:10.1016/j.ajt.2022.11.027

9. Florian JA, Kosky JR, Ainslie K, Pang Z, Dull RO, Tarbell JM. Heparan Sulfate Proteoglycan Is a Mechanosensor on Endothelial Cells. *Circulation Research*. 2003;93(10):e136-e142. doi:10.1161/01.RES.0000101744.47866.D5

10. Tarbell JM, Weinbaum S, Kamm RD. Cellular fluid mechanics and mechanotransduction. *Ann Biomed Eng*. 2005;33(12):1719-1723. doi:10.1007/s10439-005-8775-z

11. Reitsma S, Slaaf DW, Vink H, van Zandvoort MAMJ, oude Egbrink MGA. The endothelial glycocalyx: composition, functions, and visualization. *Pflugers Arch*. 2007;454(3):345-359. doi:10.1007/s00424-007-0212-8

12. Duni A, Liakopoulos V, Koutlas V, Pappas C, Mitsis M, Dounousi E. The Endothelial Glycocalyx as a Target of Ischemia and Reperfusion Injury in Kidney Transplantation—Where Have We Gone So Far? *Int J Mol Sci*. 2021;22(4):2157. doi:10.3390/ijms22042157

13. Aldecoa C, Llau JV, Nuvials X, Artigas A. Role of albumin in the preservation of endothelial glycocalyx integrity and the microcirculation: a review. *Ann Intensive Care*. 2020;10(1):85. doi:10.1186/s13613-020-00697-1

14. Torres Filho IP, Torres LN, Salgado C, Dubick MA. Plasma syndecan-1 and heparan sulfate correlate with microvascular glycocalyx degradation in hemorrhaged rats after different resuscitation fluids. *Am J Physiol Heart Circ Physiol*. 2016;310(11):H1468-1478. doi:10.1152/ajpheart.00006.2016

15. Tchouta L, Drake D, Hoenerhoff M, et al. Twenty-four-hour normothermic perfusion of isolated ex vivo hearts using plasma exchange. *The Journal of Thoracic and Cardiovascular Surgery*. 2022;164(1):128-138. doi:10.1016/j.jtcvs.2020.11.158

16. Foote CA, Soares RN, Ramirez-Perez FI, et al. Endothelial Glycocalyx. *Compr Physiol*. 2022;12(4):3781-3811. doi:10.1002/cphy.c210029

17. Bartosch AMW, Mathews R, Tarbell JM. Endothelial Glycocalyx-Mediated Nitric Oxide Production in Response to Selective AFM Pulling. *Biophys J*. 2017;113(1):101-108. doi:10.1016/j.bpj.2017.05.033

18. Dragovich MA, Chester D, Fu BM, et al. Mechanotransduction of the endothelial glycocalyx mediates nitric oxide production through activation of TRP channels. *American Journal of Physiology-Cell Physiology*. 2016;311(6):C846-C853. doi:10.1152/ajpcell.00288.2015

19. Song W, Wang H, Wu Q. Atrial Natriuretic Peptide in Cardiovascular Biology and Disease (NPPA). *Gene*. 2015;569(1):1-6. doi:10.1016/j.gene.2015.06.029

Chapter 4: Summary and Future Directions

4.1 Summary, Future Directions, and Discussion

Through the work presented in this thesis, I emphasize here the importance of perfusate optimization, specifically with regard to blood components such as red blood cells and plasma proteins, as an important frontier for improvement within ex-situ heart perfusion and more broadly, machine perfusion of donor organs in general.

In chapter two, I show that by increasing the hemoglobin content within the perfusate via clinically relevant means that this predisposes the ex-situ heart to an increased level of hemolytic burden, which associates with quickened functional decline. Hemolysis is an unavoidable phenomenon in machine perfusion settings utilizing blood-based perfusates. As a toxicity that accumulates within the perfusate over time, if undealt with, it undoubtedly sets an upper limit on preservation time. Hemofiltration of the perfusate to continuously remove released free hemoglobin from hemolysis seems like a valuable approach to mitigate this process, as has been done recently[1,2], however the imposition of continuous hemofiltration requires an ideal replacement fluid to be infused concomitantly that is reminiscent of what is being lost via the hemofilter. The most ideal fluid to infuse would be fresh frozen plasma isolated from the donor. Though, this is resource intensive towards the blood bank, with costliness rising with the length of preservation required. On the other hand, use of an artificially produced replacement fluid perpetuates the limitations imposed by manufactured buffers, utilized in the context of machine perfusion to supplement volume. These buffers do not perfectly recapitulate donor plasma, leading to increasing washout of potentially key plasma proteins. Further study is needed to define the most optimal manufactured buffer that can be utilized for ESHP such that hemofiltration approaches are subtracting unwanted substrates without

removing those necessary for efficacious preservation. This is much easier said than done given the multiplicity of variables at play and the effects of perfusate ingredients across the tissue, from the myocardium to the coronary vasculature.

We surmise that the increased rate of functional decline in hearts subject to high hemolytic burden is due to the exacerbation of oxidative stress, with the induction of oxidative stress during ESHP seeming to be a central molecular paradigm for functional decline on the platform[3]. It is possible that aside from hemolysis, an increased provision of oxygen carriers in the perfusate through concentration of RBC content led to over-oxygenation of the tissue, perhaps contributing to oxidative stress. The difference in functional preservation in this setting provides us the opportunity to investigate molecular differences that can be associated with functional decline, such as oxidative stress markers. As a future direction, it should be confirmed that in hearts experiencing increased rates of functional decline that these hearts are also experiencing heightened amounts of oxidative stress induction. This would reinforce that interventions that mitigate contributors to oxidative stress are likely to make preservation on the ESHP platform not only lengthier, but also more efficacious.

There are implications here for the importance of red blood cell quality during ESHP as well. By utilizing the cell saver, the mechanical manipulation of the blood presumably reduced its quality leading to its lysis over time[4]. In this regard, should prolonged perfusion to the period of days to weeks be accomplished, it seems pertinent that the red blood cells within the blood based perfusate will also require tending to or replacement. Given that only so much blood volume can be taken from the donor (an especially limiting factor if you consider that there are platforms for the other

transplantable solid organs as well), making the perfusate itself modular and interchangeable, like an ‘oil change’ would be an interesting future direction from this work. Any interventions to the perfusate that focus on providing resistance to the red blood cells to deterioration during ESHP would also be a welcome improvement.

The importance of plasma proteins within the perfusate during ESHP is implied through the analyses displayed in chapter three. It appears that when an increased amount of fluids are provided that would dilute plasma proteins that this predisposes hearts to development of concerning supraphysiologic coronary flow rates during ESHP. Such would be the case with hemofiltration, as alluded to above. In chapter three I suggest that the endothelial glycocalyx may be an important player in this phenomenon given its sensitivity to fluid loading conditions and functions its structure mediates when intact. I show preliminarily that disturbance to the endothelial glycocalyx (that is increased by fluids provision) can be prevented through artificial supplementation of the perfusate buffer with increased concentrations of plasma proteins. Given that this was a comparison to historical runs that were conducted within a different era of our research group’s experience with ESHP, a study whereby it is the primary aim to vary plasma protein concentrations within the perfusate and determine the corresponding effect on coronary flow rates and endothelial glycocalyx disturbance is needed.

However, given that plasma proteins act to preserve the endothelial glycocalyx, a structure with many functions that would be therapeutically relevant for allografts[5], supplementation of these proteins during ESHP could be a modality to effect improved preservation, especially to the vasculature itself. Such interventions specific to the vasculature could have a great deal of impact given that rejection processes and

development of cardiac allograft vasculopathy begin as perturbations to the coronary endothelium[6]. It follows that in comparison to the intact organism whose vasculature is privy to undiluted whole blood that hosts vasculo-protective factors, dilution of such factors with manufactured buffers or crystalloid fluids that do not include these factors would constitute an insult to the vasculature. This has wide implications for the plethora of crystalloid fluids provided perioperatively, via cardioplegia, machine perfusion perfusates, et cetera. By investigating the impact of fluids on the coronary endothelium during ESHP, this can inform procurement practices. Concentration of vasculoprotective factors (perhaps endothelial glycocalyx upholding factors), which are disturbed via crystalloid fluids administration, within the ESHP perfusate could be an important way to mitigate post-transplant maladies to the vasculature in the future.

4.2 References

1. Johnson MD, Fallon BP, Langley M, et al. Prolonged (24-hour) Normothermic ex vivo Heart Perfusion Facilitated by Perfusate Hemofiltration. *ASAIO Journal*. 2022;68(10):1282-1289. doi:10.1097/MAT.0000000000001649

2. McLeod JS, Poling C, Church JT, et al. Ex Vivo Heart Perfusion for 72 Hours Using Plasma Cross Circulation. *ASAIO Journal*. 2020;66(7):753-759. doi:10.1097/MAT.0000000000001061

3. Hatami S. Inflammation, Oxidative Stress, Metabolic Inefficiency, And Related Functional Decline of the Heart During Ex-situ Perfusion [dissertation]. Edmonton (CA): University of Alberta, 2021.

4. Vercaemst L. Hemolysis in Cardiac Surgery Patients Undergoing Cardiopulmonary Bypass: A Review in Search of a Treatment Algorithm. *J Extra Corpor Technol*. 2008;40(4):257-267.

5. Foote CA, Soares RN, Ramirez-Perez FI, et al. Endothelial Glycocalyx. *Compr Physiol*. 2022;12(4):3781-3811. doi:10.1002/cphy.c210029

6. Pober JS, Chih S, Kobashigawa J, Madsen JC, Tellides G. Cardiac allograft vasculopathy: current review and future research directions. *Cardiovasc Res*. 2021;117(13):2624-2638. doi:10.1093/cvr/cvab259

Bibliography

Heidenreich PA, Bozkurt B, Aguilar D, et al. 2022 AHA/ACC/HFSA Guideline for the Management of Heart Failure: A Report of the American College of Cardiology/American Heart Association Joint Committee on Clinical Practice Guidelines. *Circulation*. 2022;145(18):e895-e1032. doi:10.1161/CIR.0000000000001063

Savarese G, Becher PM, Lund LH, Seferovic P, Rosano GMC, Coats AJS. Global burden of heart failure: a comprehensive and updated review of epidemiology. *Cardiovasc Res*. 2023;118(17):3272-3287. doi:10.1093/cvr/cvac013

Tsao CW, Aday AW, Almarzooq ZI, et al. Heart Disease and Stroke Statistics-2023 Update: A Report From the American Heart Association. *Circulation*. 2023;147(8):e93-e621. doi:10.1161/CIR.0000000000001123

Lindsay P, Anderson K, Ducharme A, et al. THE STATE OF HEART FAILURE SERVICES IN CANADA: FINDINGS OF THE HEART & STROKE NATIONAL HEART FAILURE RESOURCES AND SERVICES INVENTORY. *Canadian Journal of Cardiology*. 2022;38(10):S188-S189. doi:10.1016/j.cjca.2022.08.153

Lesyuk W, Kriza C, Kolominsky-Rabas P. Cost-of-illness studies in heart failure: a systematic review 2004–2016. *BMC Cardiovascular Disorders*. 2018;18(1):74. doi:10.1186/s12872-018-0815-3

Shah A, Gandhi D, Srivastava S, Shah KJ, Mansukhani R. Heart Failure: A Class Review of Pharmacotherapy. *P T*. 2017;42(7):464-472.

Jessup M, Abraham WT, Casey DE, et al. 2009 focused update: ACCF/AHA Guidelines for the Diagnosis and Management of Heart Failure in Adults: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines: developed in collaboration with the International Society for Heart and Lung Transplantation. *Circulation*. 2009;119(14):1977-2016. doi:10.1161/CIRCULATIONAHA.109.192064

WRITING COMMITTEE MEMBERS, Yancy CW, Jessup M, et al. 2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology Foundation/American Heart Association Task Force on practice guidelines. *Circulation*. 2013;128(16):e240-327. doi:10.1161/CIR.0b013e31829e8776

Tromp J, Ouwerkerk W, Van Veldhuisen DJ, et al. A Systematic Review and Network Meta-Analysis of Pharmacological Treatment of Heart Failure With Reduced Ejection Fraction. *JACC: Heart Failure*. 2022;10(2):73-84. doi:10.1016/j.jchf.2021.09.004

Alraies MC, Eckman P. Adult heart transplant: indications and outcomes. *J Thorac Dis*. 2014;6(8):1120-1128. doi:10.3978/j.issn.2072-1439.2014.06.44

Linden PK. History of Solid Organ Transplantation and Organ Donation. *Critical Care Clinics*. 2009;25(1):165-184. doi:10.1016/j.ccc.2008.12.001

Stolf NAG. History of Heart Transplantation: a Hard and Glorious Journey. *Braz J Cardiovasc Surg*. 2017;32(5):423-427. doi:10.21470/1678-9741-2017-0508

Brink JG, Hassoulas J. The first human heart transplant and further advances in cardiac transplantation at Groote Schuur Hospital and the University of Cape Town. *Cardiovasc J Afr*. 2009;20(1):31-35.

Monteagudo Vela M, García Sáez D, Simon AR. Current approaches in retrieval and heart preservation. *Ann Cardiothorac Surg*. 2018;7(1):67-74. doi:10.21037/acs.2018.01.06

Venuta F, Van Raemdonck D. History of lung transplantation. *J Thorac Dis*. 2017;9(12):5458-5471. doi:10.21037/jtd.2017.11.84

Margreiter R. History of Lung and Heart-Lung Transplantation, With Special Emphasis on German-Speaking Countries. *Transplantation Proceedings*. 2016;48(8):2779-2781. doi:10.1016/j.transproceed.2016.07.015

Bezinover D, Saner F. Organ transplantation in the modern era. *BMC Anesthesiol*. 2019;19:32. doi:10.1186/s12871-019-0704-z

Panchabhai TS, Chaddha U, McCurry KR, Bremner RM, Mehta AC. Historical perspectives of lung transplantation: connecting the dots. *Journal of Thoracic Disease*. 2018;10(7). doi:10.21037/jtd.2018.07.06

Khush KK, Cherikh WS, Chambers DC, et al. The International Thoracic Organ Transplant Registry of the International Society for Heart and Lung Transplantation: Thirty-sixth adult heart transplantation report - 2019; focus theme: Donor and recipient size match. *J Heart Lung Transplant*. 2019;38(10):1056-1066. doi:10.1016/j.healun.2019.08.004

Organ Procurement and Transplantation Network. National data. Accessed February 1, 2024. Available from: https://optn.transplant.hrsa.gov/data/view-data-reports/national-data/#

Hatami S, Freed DH. Machine Perfusion of Donor Heart: State of the Art. *Curr Transpl Rep*. 2019;6(3):242-250. doi:10.1007/s40472-019-00251-4

Wang L, MacGowan GA, Ali S, Dark JH. Ex situ heart perfusion: The past, the present, and the future. *The Journal of Heart and Lung Transplantation*. 2021;40(1):69-86. doi:10.1016/j.healun.2020.10.004

White CW, Messer SJ, Large SR, et al. Transplantation of Hearts Donated after Circulatory Death. *Frontiers in Cardiovascular Medicine*. 2018;5. Accessed May 7, 2022. https://www.frontiersin.org/article/10.3389/fcvm.2018.00008

Stehlik J, Edwards LB, Kucheryavaya AY, et al. The Registry of the International Society for Heart and Lung Transplantation: Twenty-eighth Adult Heart Transplant Report--2011. *J Heart Lung Transplant*. 2011;30(10):1078-1094. doi:10.1016/j.healun.2011.08.003

Jeevanandam V, Furukawa S, Prendergast TW, Todd BA, Eisen HJ, McClurken JB. Standard criteria for an acceptable donor heart are restricting heart transplantation. *Ann Thorac Surg*. 1996;62(5):1268-1275. doi:10.1016/0003-4975(96)00626-1

Bloomworks. The Costly Effects of an Outdated Organ Donation System. Accessed October 28, 2023. Available from: https://bloomworks.digital/organdonationreform/Introduction/

Bakhtiyar SS, Godfrey EL, Ahmed S, et al. Survival on the Heart Transplant Waiting List. *JAMA Cardiology*. 2020;5(11):1227-1235. doi:10.1001/jamacardio.2020.2795

Marsia S, Khan A, Khan M, et al. Heart transplantation after the circulatory death; The ethical dilemma. *Indian Heart J*. 2018;70(Suppl 3):S442-S445. doi:10.1016/j.ihj.2018.08.010

Gordon JK, McKinlay J. Physiological changes after brain stem death and management of the heart-beating donor. *Continuing Education in Anaesthesia Critical Care & Pain*. 2012;12(5):225-229. doi:10.1093/bjaceaccp/mks026

Collins MJ, Moainie SL, Griffith BP, Poston RS. Preserving and evaluating hearts with ex vivo machine perfusion: an avenue to improve early graft performance and expand the donor pool. *Eur J Cardiothorac Surg*. 2008;34(2):318-325. doi:10.1016/j.ejcts.2008.03.043

Russo MJ, Chen JM, Sorabella RA, et al. The effect of ischemic time on survival after heart transplantation varies by donor age: An analysis of the United Network for Organ Sharing database. *The Journal of Thoracic and Cardiovascular Surgery*. 2007;133(2):554-559. doi:10.1016/j.jtcvs.2006.09.019

Banner NR, Thomas HL, Curnow E, et al. The Importance of Cold and Warm Cardiac Ischemia for Survival After Heart Transplantation. *Transplantation*. 2008;86(4):542-547. doi:10.1097/TP.0b013e31818149b9

Anderson K, Rucker A, Mulvihill M, Yerokun B, Hartwig M. Impact of Ischemic Time on Primary Graft Dysfunction: A National Analysis. *The Journal of Heart and Lung Transplantation*. 2018;37(4):S459. doi:10.1016/j.healun.2018.01.1194

Aldam PS. The physiological changes of circulatory death with respect to organ donation [dissertation]. Cambridge (UK): University of Cambridge; 2019

Goila AK, Pawar M. The diagnosis of brain death. *Indian J Crit Care Med*. 2009;13(1):7-11. doi:10.4103/0972-5229.53108

McKeown DW, Bonser RS, Kellum JA. Management of the heartbeating brain-dead organ donor. *Br J Anaesth*. 2012;108 Suppl 1:i96-107. doi:10.1093/bja/aer351

Canada T. Canadian Motor Vehicle Traffic Collision Statistics: 2021. Transport Canada. April 13, 2023. Accessed February 1, 2024. https://tc.canada.ca/en/road-transportation/statistics-data/canadian-motor-vehicle-traffic-collision-statistics-2021

Wittwer T, Wahlers T. Marginal donor grafts in heart transplantation: lessons learned from 25 years of experience. *Transpl Int*. 2008;21(2):113-125. doi:10.1111/j.1432-2277.2007.00603.x

Bifulco O, Bottio T, Caraffa R, et al. Marginal versus Standard Donors in Heart Transplantation: Proper Selection Means Heart Transplant Benefit. *J Clin Med*. 2022;11(9):2665. doi:10.3390/jcm11092665

Yazji JH, Garg P, Wadiwala I, et al. Expanding Selection Criteria to Repairable Diseased Hearts to Meet the Demand of Shortage of Donors in Heart Transplantation. *Cureus*. 2022;14(5):e25485. doi:10.7759/cureus.25485

Christie IG, Chan EG, Ryan JP, et al. National Trends in Extended Criteria Donor Utilization and Outcomes for Lung Transplantation. *The Annals of Thoracic Surgery*. 2021;111(2):421-426. doi:10.1016/j.athoracsur.2020.05.087

Emmanuel S, Jansz P, Hayward CS, Macdonald P. Marginal Cardiac Donors and Transplant Outcomes: An 11-Year Experience. *The Journal of Heart and Lung Transplantation*. 2022;41(4):S367. doi:10.1016/j.healun.2022.01.1481

Messer S, Lannon J, Wong E, et al. The Potential of Transplanting Hearts From Donation After Circulatory Determined Death (DCD) Donors Within the United Kingdom. *The Journal of Heart and Lung Transplantation*. 2015;34(4):S275. doi:10.1016/j.healun.2015.01.772

Messer S, Page A, Berman M, et al. First to 50: Early Outcomes Following Heart Transplantation at Royal Papworth Hospital from Donation after Circulatory Determined Death (DCD) Donors. *The Journal of Heart and Lung Transplantation*. 2019;38(4):S43. doi:10.1016/j.healun.2019.01.090

Siddiqi HK, Trahanas J, Xu M, et al. Outcomes of Heart Transplant Donation After Circulatory Death. *J Am Coll Cardiol*. 2023;82(15):1512-1520. doi:10.1016/j.jacc.2023.08.006

Canadian Institute for Health Information. Summary statistics on organ transplants, wait-lists, and donors. Accessed August 12, 2024. https://www.cihi.ca/en/summary-statistics-on-organ-transplants-wait-lists-and-

donors#:~:text=Key%20findings,46%25%20were%20from%20unrelated%20donors.

Herrera Fortin T, Calin E, Ducharme A, et al. The Potential for Heart Donation After Death Determination by Circulatory Criteria (DCD) in the Province of Quebec. *CJC Open*. Published online May 16, 2024. doi:10.1016/j.cjco.2024.05.004

Singh SSA, Dalzell JR, Berry C, Al-Attar N. Primary graft dysfunction after heart transplantation: a thorn amongst the roses. *Heart Fail Rev*. 2019;24(5):805-820. doi:10.1007/s10741-019-09794-1

Du Y, Demillard LJ, Ren J. Catecholamine-induced cardiotoxicity: A critical element in the pathophysiology of stroke-induced heart injury. *Life Sciences*. 2021;287:120106. doi:10.1016/j.lfs.2021.120106

Tsai YH, Lin JY, Huang YY, Wong JM. Cushing response-based warning system for intensive care of brain-injured patients. *Clin Neurophysiol*. 2018;129(12):2602-2612. doi:10.1016/j.clinph.2018.09.010

White CW, Lillico R, Sandha J, et al. Physiologic Changes in the Heart Following Cessation of Mechanical Ventilation in a Porcine Model of Donation After Circulatory Death: Implications for Cardiac Transplantation. *Am J Transplant*. 2016;16(3):783-793. doi:10.1111/ajt.13543

Pérez López S, Otero Hernández J, Vázquez Moreno N, Escudero Augusto D, Alvarez Menéndez F, Astudillo González A. Brain death effects on catecholamine levels and subsequent cardiac damage assessed in organ donors. *J Heart Lung Transplant*. 2009;28(8):815-820. doi:10.1016/j.healun.2009.04.021

Egan TM. Non-heart-beating donors in thoracic transplantation. *The Journal of Heart and Lung Transplantation*. 2004;23(1):3-10. doi:10.1016/S1053-2498(02)00658-7

Liaudet L, Calderari B, Pacher P. Pathophysiological mechanisms of catecholamine and cocaine-mediated cardiotoxicity. *Heart Fail Rev*. 2014;19(6):815-824. doi:10.1007/s10741-014-9418-y

Xu H xia, Cui S mei, Zhang Y mei, Ren J. Mitochondrial Ca2+ regulation in the etiology of heart failure: physiological and pathophysiological implications. *Acta Pharmacol Sin*. 2020;41(10):1301-1309. doi:10.1038/s41401-020-0476-5

Sanada S, Komuro I, Kitakaze M. Pathophysiology of myocardial reperfusion injury: preconditioning, postconditioning, and translational aspects of protective measures. *Am J Physiol Heart Circ Physiol*. 2011;301(5):H1723-1741. doi:10.1152/ajpheart.00553.2011

de Perrot M, Liu M, Waddell TK, Keshavjee S. Ischemia–Reperfusion–induced Lung Injury. *Am J Respir Crit Care Med*. 2003;167(4):490-511. doi:10.1164/rccm.200207-670SO

Jahania MS, Sanchez JA, Narayan P, Lasley RD, Mentzer RM. Heart preservation for transplantation: principles and strategies. *The Annals of Thoracic Surgery*. 1999;68(5):1983-1987. doi:10.1016/S0003-4975(99)01028-0

Kalogeris T, Baines CP, Krenz M, Korthuis RJ. Cell Biology of Ischemia/Reperfusion Injury. *Int Rev Cell Mol Biol*. 2012;298:229-317. doi:10.1016/B978-0-12-394309-5.00006-7

Bovo E, Mazurek SR, Zima AV. Oxidation of ryanodine receptor after ischemia-reperfusion increases propensity of Ca2+ waves during β-adrenergic receptor stimulation. *Am J Physiol Heart Circ Physiol*. 2018;315(4):H1032-H1040. doi:10.1152/ajpheart.00334.2018

Hurst S, Hoek J, Sheu SS. Mitochondrial Ca2+ and Regulation of the Permeability Transition Pore. *J Bioenerg Biomembr*. 2017;49(1):27-47. doi:10.1007/s10863-016-9672-x

Yang Y, Jiang K, Liu X, Qin M, Xiang Y. CaMKII in Regulation of Cell Death During Myocardial Reperfusion Injury. *Frontiers in Molecular Biosciences*. 2021;8. Accessed February 4, 2024. https://www.frontiersin.org/articles/10.3389/fmolb.2021.668129

Liu GY, Xie WL, Wang YT, et al. Calpain: the regulatory point of myocardial ischemia-reperfusion injury. *Front Cardiovasc Med*. 2023;10:1194402. doi:10.3389/fcvm.2023.1194402

Wu MY, Yiang GT, Liao WT, et al. Current Mechanistic Concepts in Ischemia and Reperfusion Injury. *CPB*. 2018;46(4):1650-1667. doi:10.1159/000489241

Lee F, Nair V, Chih S. Cardiac allograft vasculopathy: Insights on pathogenesis and therapy. *Clin Transplant*. 2020;34(3):e13794. doi:10.1111/ctr.13794

Angelini A, Castellani C, Fedrigo M, et al. Coronary cardiac allograft vasculopathy versus native atherosclerosis: difficulties in classification. *Virchows Arch*. 2014;464(6):627-635. doi:10.1007/s00428-014-1586-6

Pober JS, Chih S, Kobashigawa J, Madsen JC, Tellides G. Cardiac allograft vasculopathy: current review and future research directions. *Cardiovasc Res*. 2021;117(13):2624-2638. doi:10.1093/cvr/cvab259

Greenway SC, Butts R, Naftel DC, et al. Statin therapy is not associated with improved outcomes after heart transplantation in children and adolescents. *J Heart Lung Transplant*. 2016;35(4):457-465. doi:10.1016/j.healun.2015.10.040

Feinberg AJ, Yarber C, Vlismas PP, Farooq M, Rochlani YM, Patel SR. Impact Of Blood Pressure Control On Risk Of Cardiac Allograft Vasculopathy After Heart Transplant. *Journal of Cardiac Failure*. 2023;29(4):620-621. doi:10.1016/j.cardfail.2022.10.183

Goto R, Issa F, Heidt S, Taggart D, Wood KJ. Ischemia-Reperfusion Injury Accelerates Human Antibody-Mediated Transplant Vasculopathy. *Transplantation*. 2013;96(2):139. doi:10.1097/TP.0b013e318295ee32

Fukunaga N, Kawajiri H, Badiwala MV, et al. Protective role of Nrf2 against ischemia reperfusion injury and cardiac allograft vasculopathy. *Am J Transplant*. 2020;20(5):1262-1271. doi:10.1111/ajt.15724

Liu L, Fang C, Fu W, et al. Endothelial Cell-Derived Interleukin-18 Released During Ischemia Reperfusion Injury Selectively Expands T Peripheral Helper Cells to Promote Alloantibody Production. *Circulation*. 2020;141(6):464-478. doi:10.1161/CIRCULATIONAHA.119.042501

Qin L, Li G, Kirkiles-Smith N, et al. Complement C5 Inhibition Reduces T Cell-Mediated Allograft Vasculopathy Caused by Both Alloantibody and Ischemia Reperfusion Injury in Humanized Mice. *Am J Transplant*. 2016;16(10):2865-2876. doi:10.1111/ajt.13834

Fenton M, Simmonds J, Shah V, et al. Inflammatory Cytokines, Endothelial Function, and Chronic Allograft Vasculopathy in Children: An Investigation of the Donor and Recipient Vasculature After Heart Transplantation. *American Journal of Transplantation*. 2016;16(5):1559-1568. doi:10.1111/ajt.13643

van Suylen V, Vandendriessche K, Neyrinck A, et al. Oxygenated machine perfusion at room temperature as an alternative for static cold storage in porcine donor hearts. *Artificial Organs*. 2022;46(2):246-258. doi:10.1111/aor.14085

Russo MJ, Iribarne A, Hong KN, et al. Factors associated with primary graft failure after heart transplantation. *Transplantation*. 2010;90(4):444-450. doi:10.1097/TP.0b013e3181e6f1eb

Yeung JC, Krueger T, Yasufuku K, et al. Outcomes after transplantation of lungs preserved for more than 12 h: a retrospective study. *The Lancet Respiratory Medicine*. 2017;5(2):119-124. doi:10.1016/S2213-2600(16)30323-X

Lautner LJ, Freed DH, Nagendran J, Acker JP. Current techniques and the future of lung preservation. *Cryobiology*. 2020;94:1-8. doi:10.1016/j.cryobiol.2020.04.009

Wicomb W, Boyd ST, Cooper DK, Rose AG, Barnard CN. Ex vivo functional evaluation of pig hearts subjected to 24 hours' preservation by hypothermic perfusion. *S Afr Med J*. 1981;60(6):245-248.

Wicomb WN, Cooper DKC, Barnard CN. TWENTY-FOUR-HOUR PRESERVATION OF THE PIG HEART BY A PORTABLE HYPOTHERMIC PERFUSION SYSTEM. *Transplantation*. 1982;34(5):246-250.

Hassanein WH, Zellos L, Tyrrell TA, et al. Continuous perfusion of donor hearts in the beating state extends preservation time and improves recovery of function. *The*

Journal of Thoracic and Cardiovascular Surgery. 1998;116(5):821-830. doi:10.1016/S0022-5223(98)00452-8

Ozeki T, Kwon MH, Gu J, et al. Heart preservation using continuous ex vivo perfusion improves viability and functional recovery. *Circ J*. 2007;71(1):153-159. doi:10.1253/circj.71.153

Fitton TP, Wei C, Lin R, et al. Impact of 24 h continuous hypothermic perfusion on heart preservation by assessment of oxidative stress. *Clinical Transplantation*. 2004;18(s12):22-27. doi:10.1111/j.1399-0012.2004.00213

Michel SG, La Muraglia GM, Madariaga MLL, et al. Preservation of Donor Hearts Using Hypothermic Oxygenated Perfusion. *Ann Transplant*. 2014;19:409-416. doi:10.12659/AOT.890797

Ferrera R, Larese A, Marcsek P, et al. Comparison of different techniques of hypothermic pig heart preservation. *The Annals of Thoracic Surgery*. 1994;57(5):1233-1239. doi:10.1016/0003-4975(94)91363-3

Van Caenegem O, Beauloye C, Bertrand L, et al. Hypothermic continuous machine perfusion enables preservation of energy charge and functional recovery of heart grafts in an ex vivo model of donation following circulatory death. *European Journal of Cardio-Thoracic Surgery*. 2016;49(5):1348-1353. doi:10.1093/ejcts/ezv409

Michel SG, Ii GML, Madariaga MLL, et al. Twelve-Hour Hypothermic Machine Perfusion for Donor Heart Preservation Leads to Improved Ultrastructural Characteristics Compared to Conventional Cold Storage. *Ann Transplant*. 2015;20:461-468. doi:10.12659/AOT.893784

Hatami S, White CW, Ondrus M, et al. Normothermic Ex Situ Heart Perfusion in Working Mode: Assessment of Cardiac Function and Metabolism. *J Vis Exp*. 2019;(143). doi:10.3791/58430

Ardehali A, Esmailian F, Deng M, et al. Ex-vivo perfusion of donor hearts for human heart transplantation (PROCEED II): a prospective, open-label, multicentre, randomised non-inferiority trial. *Lancet*. 2015;385(9987):2577-2584. doi:10.1016/S0140-6736(15)60261-6

White C, Ambrose E, Müller A, et al. Impact of Reperfusion Calcium and pH on the Resuscitation of Hearts Donated After Circulatory Death. *Ann Thorac Surg*. 2017;103(1):122-130. doi:10.1016/j.athoracsur.2016.05.084

Taylor MJ. Hypothermic Blood Substitution: Special Considerations for Protection of Cells during ex vivo and in vivo Preservation. *Transfus Med Hemother*. 2007;34(4):226-244. doi:10.1159/000104250

White CW, Hasanally D, Mundt P, et al. A whole blood–based perfusate provides superior preservation of myocardial function during ex vivo heart perfusion. *The*

Journal of Heart and Lung Transplantation. 2015;34(1):113-121. doi:10.1016/j.healun.2014.09.021

White CW, Ali A, Hasanally D, et al. A cardioprotective preservation strategy employing ex vivo heart perfusion facilitates successful transplant of donor hearts after cardiocirculatory death. *The Journal of Heart and Lung Transplantation*. 2013;32(7):734-743. doi:10.1016/j.healun.2013.04.016

Bishawi M, Roan JN, Milano CA, et al. A normothermic ex vivo organ perfusion delivery method for cardiac transplantation gene therapy. *Sci Rep*. 2019;9(1):8029. doi:10.1038/s41598-019-43737-y

Miyagi N, Rao VP, Ricci D, et al. EFFICIENT AND DURABLE GENE TRANSFER TO THE TRANSPLANTED HEART USING ADENO-ASSOCIATED VIRUS 9 VECTOR. *J Heart Lung Transplant*. 2008;27(5):554-560. doi:10.1016/j.healun.2008.01.025

Cypel M, Liu M, Rubacha M, et al. Functional Repair of Human Donor Lungs by IL-10 Gene Therapy. *Science Translational Medicine*. 2009;1(4):4ra9-4ra9. doi:10.1126/scitranslmed.3000266

Yeung JC, Wagnetz D, Cypel M, et al. Ex vivo adenoviral vector gene delivery results in decreased vector-associated inflammation pre- and post-lung transplantation in the pig. *Mol Ther*. 2012;20(6):1204-1211. doi:10.1038/mt.2012.57

Machuca TN, Cypel M, Bonato R, et al. Safety and Efficacy of *Ex Vivo* Donor Lung Adenoviral IL-10 Gene Therapy in a Large Animal Lung Transplant Survival Model. *Human Gene Therapy*. 2017;28(9):757-765. doi:10.1089/hum.2016.070

Tenderich G, El-Banayosy A, Rosengard B, et al. 10: Prospective multi-center European trial to evaluate the safety and performance of the Organ Care System for heart transplants (PROTECT). *The Journal of Heart and Lung Transplantation*. 2007;26(2):S64. doi:10.1016/j.healun.2006.11.024

Transmedics. Transmedics Organ Care System Heart User Guide. Accessed August 12, 2024. https://www.accessdata.fda.gov/cdrh_docs/pdf18/P180051S001D.pdf

Hamed A, Tsui S, Huber J, Lin R, Poggio EC, Ardehali A. 19: Serum Lactate Is a Highly Sensitive and Specific Predictor of Post Cardiac Transplant Outcomes Using the Organ Care System. *The Journal of Heart and Lung Transplantation*. 2009;28(2):S71. doi:10.1016/j.healun.2008.11.025

Freed DH, White CW. Donor heart preservation: straight up, or on the rocks? *The Lancet*. 2015;385(9987):2552-2554. doi:10.1016/S0140-6736(15)60614-6

Stamp NL, Shah A, Vincent V, et al. Successful Heart Transplant after Ten Hours Out-of-body Time using the TransMedics Organ Care System. *Heart, Lung and Circulation*. 2015;24(6):611-613. doi:10.1016/j.hlc.2015.01.005

Schroder JN, D'Alessandro D, Esmailian F, et al. Successful Utilization of Extended Criteria Donor (ECD) Hearts for Transplantation - Results of the OCS™ Heart EXPAND Trial to Evaluate the Effectiveness and Safety of the OCS Heart System to Preserve and Assess ECD Hearts for Transplantation. *The Journal of Heart and Lung Transplantation*. 2019;38(4):S42. doi:10.1016/j.healun.2019.01.088

Schroder JN, Shah A, Pretorius V, et al. Expanding Heart Transplants from Donors After Circulatory Death (DCD) - Results of the First Randomized Controlled Trial Using the Organ Care System (OCS™) Heart - (OCS DCD Heart Trial). *The Journal of Heart and Lung Transplantation*. 2022;41(4):S72. doi:10.1016/j.healun.2022.01.165

Messer S, Page A, Axell R, et al. Outcome after heart transplantation from donation after circulatory-determined death donors. *The Journal of Heart and Lung Transplantation*. 2017;36(12):1311-1318. doi:10.1016/j.healun.2017.10.021

Rojas SV, Avsar M, Ius F, et al. Ex-Vivo Preservation with the Organ Care System in High Risk Heart Transplantation. *Life*. 2022;12(2):247. doi:10.3390/life12020247

Alomari M, Garg P, Yazji JH, et al. Is the Organ Care System (OCS) Still the First Choice With Emerging New Strategies for Donation After Circulatory Death (DCD) in Heart Transplant? *Cureus*. 14(6):e26281. doi:10.7759/cureus.26281

Fleck TPK, Ayala R, Kroll J, et al. Ex Vivo Allograft Perfusion for Complex Pediatric Heart Transplant Recipients. *The Annals of Thoracic Surgery*. 2021;112(4):1275-1280. doi:10.1016/j.athoracsur.2020.12.025

Hatami S, White CW, Shan S, et al. Myocardial Functional Decline During Prolonged Ex Situ Heart Perfusion. *The Annals of Thoracic Surgery*. 2019;108(2):499-507. doi:10.1016/j.athoracsur.2019.01.076

Olkowicz M, Ribeiro RVP, Yu F, et al. Dynamic Metabolic Changes During Prolonged Ex Situ Heart Perfusion Are Associated With Myocardial Functional Decline. *Frontiers in Immunology*. 2022;1:859506. doi:10.3389/fimmu.2022.859506

Hatami S, Qi X, White C, et al. OXIDATIVE STRESS AND RELATED RESPONSES ARE INDUCED DURING EX-SITU HEART PERFUSION AND MAY CONTRIBUTE TO THE FUNCTIONAL DECLINE OF THE EX-SITU-PERFUSED HEART. *Canadian Journal of Cardiology*. 2019;35(10):S106. doi:10.1016/j.cjca.2019.07.499

Hatami S, Qi X, Khan M, et al. Oxidative stress and related metabolic alterations are induced in ex situ perfusion of donated hearts regardless of the ventricular load or leukocyte depletion. *American Journal of Transplantation*. Published online January 2023:S1600613522302174. doi:10.1016/j.ajt.2022.11.027

Hatami S, White CW, Qi X, et al. Immunity and Stress Responses Are Induced During Ex Situ Heart Perfusion. *Circ: Heart Failure*. 2020;13(6). doi:10.1161/CIRCHEARTFAILURE.119.006552

Davies LC, Jenkins SJ, Allen JE, Taylor PR. Tissue-resident macrophages. *Nat Immunol*. 2013;14(10):986-995. doi:10.1038/ni.2705

Peet C, Ivetic A, Bromage DI, Shah AM. Cardiac monocytes and macrophages after myocardial infarction. *Cardiovasc Res*. 2020;116(6):1101-1112. doi:10.1093/cvr/cvz336

117. Brouckaert G, Kalai M, Krysko DV, et al. Phagocytosis of Necrotic Cells by Macrophages Is Phosphatidylserine Dependent and Does Not Induce Inflammatory Cytokine Production. *Mol Biol Cell*. 2004;15(3):1089-1100. doi:10.1091/mbc.E03-09-0668

Kari S, Subramanian K, Altomonte IA, Murugesan A, Yli-Harja O, Kandhavelu M. Programmed cell death detection methods: a systematic review and a categorical comparison. *Apoptosis*. 2022;27(7):482-508. doi:10.1007/s10495-022-01735-y

Hayashi R, Ito Y, Matsumoto K, Fujino Y, Otsuki Y. Quantitative differentiation of both free 3'-OH and 5'-OH DNA ends between heat-induced apoptosis and necrosis. *J Histochem Cytochem*. 1998;46(9):1051-1059. doi:10.1177/002215549804600909

Hickman PE, Potter JM, Aroney C, et al. Cardiac troponin may be released by ischemia alone, without necrosis. *Clin Chim Acta*. 2010;411(5-6):318-323. doi:10.1016/j.cca.2009.12.009

Mair J, Lindahl B, Hammarsten O, et al. How is cardiac troponin released from injured myocardium? *European Heart Journal: Acute Cardiovascular Care*. 2018;7(6):553-560. doi:10.1177/2048872617748553

Bhirowo YP, Raksawardana YK, Setianto BY, et al. Hemolysis and cardiopulmonary bypass: meta-analysis and systematic review of contributing factors. *Journal of Cardiothoracic Surgery*. 2023;18(1):291. doi:10.1186/s13019-023-02406-y

Dalton HJ, Cashen K, Reeder RW, et al. Hemolysis During Pediatric Extracorporeal Membrane Oxygenation: Associations With Circuitry, Complications, and Mortality. *Pediatr Crit Care Med*. 2018;19(11):1067-1076. doi:10.1097/PCC.0000000000001709

Lehle K, Philipp A, Zeman F, et al. Technical-Induced Hemolysis in Patients with Respiratory Failure Supported with Veno-Venous ECMO - Prevalence and Risk Factors. *PLoS One*. 2015;10(11):e0143527. doi:10.1371/journal.pone.0143527

Omar HR, Mirsaeidi M, Socias S, et al. Plasma Free Hemoglobin Is an Independent Predictor of Mortality among Patients on Extracorporeal Membrane Oxygenation Support. *PLoS One*. 2015;10(4):e0124034. doi:10.1371/journal.pone.0124034

Pires IS, Berthiaume F, Palmer AF. Engineering Therapeutics to Detoxify Hemoglobin, Heme, and Iron. *Annual Review of Biomedical Engineering*. 2023;25(1):1-21. doi:10.1146/annurev-bioeng-081622-031203

Reiter CD, Wang X, Tanus-Santos JE, et al. Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. *Nat Med*. 2002;8(12):1383-1389. doi:10.1038/nm1202-799

Vallelian F, Buehler PW, Schaer DJ. Hemolysis, free hemoglobin toxicity, and scavenger protein therapeutics. *Blood*. 2022;140(17):1837-1844. doi:10.1182/blood.2022015596

Chiabrando D, Vinchi F, Fiorito V, Mercurio S, Tolosano E. Heme in pathophysiology: a matter of scavenging, metabolism and trafficking across cell membranes. *Front Pharmacol*. 2014;5:61. doi:10.3389/fphar.2014.00061

Ovalle R. A History of the Fenton Reactions (Fenton Chemistry for Beginners). In: *Reactive Oxygen Species*. IntechOpen; 2022. doi:10.5772/intechopen.99846

Schaer CA, Deuel JW, Schildknecht D, et al. Haptoglobin Preserves Vascular Nitric Oxide Signaling during Hemolysis. *Am J Respir Crit Care Med*. 2016;193(10):1111-1122. doi:10.1164/rccm.201510-2058OC

De Simone G, Varricchio R, Ruberto TF, di Masi A, Ascenzi P. Heme Scavenging and Delivery: The Role of Human Serum Albumin. *Biomolecules*. 2023;13(3):575. doi:10.3390/biom13030575

Qi X. Regulation of Coronary Artery Flow during Normothermic Ex-Situ Heart Perfusion [dissertation]. Edmonton (AB): University of Alberta; 2020.

Palomer X, Salvadó L, Barroso E, Vázquez-Carrera M. An overview of the crosstalk between inflammatory processes and metabolic dysregulation during diabetic cardiomyopathy. *Int J Cardiol*. 2013;168(4):3160-3172. doi:10.1016/j.ijcard.2013.07.150

Cobert ML, Peltz M, West LM, Merritt ME, Jessen ME. Glucose is an Ineffective Substrate for Preservation of Machine Perfused Donor Hearts. *Journal of Surgical Research*. 2012;173(2):198-205. doi:10.1016/j.jss.2011.05.028

Hatami S, Hefler J, Freed DH. Inflammation and Oxidative Stress in the Context of Extracorporeal Cardiac and Pulmonary Support. *Front Immunol*. 2022;13:831930. doi:10.3389/fimmu.2022.831930

Ge T, Yang J, Zhou S, Wang Y, Li Y, Tong X. The Role of the Pentose Phosphate Pathway in Diabetes and Cancer. *Frontiers in Endocrinology*. 2020;11. Accessed December 28, 2022. https://www.frontiersin.org/articles/10.3389/fendo.2020.00365

Church JT, Alghanem F, Deatrick KB, et al. Normothermic Ex Vivo Heart Perfusion: Effects of Live Animal Blood and Plasma Cross Circulation. *ASAIO Journal*. 2017;63(6):766-773. doi:10.1097/MAT.0000000000000583

McLeod JS, Poling C, Church JT, et al. Ex Vivo Heart Perfusion for 72 Hours Using Plasma Cross Circulation. *ASAIO Journal*. 2020;66(7):753-759. doi:10.1097/MAT.0000000000001061

Hozain AE, O'Neill JD, Pinezich MR, et al. Xenogeneic cross-circulation for extracorporeal recovery of injured human lungs. *Nat Med*. 2020;26(7):1102-1113. doi:10.1038/s41591-020-0971-8

Guenthart BA, O'Neill JD, Kim J, et al. Regeneration of severely damaged lungs using an interventional cross-circulation platform. *Nat Commun*. 2019;10(1):1985. doi:10.1038/s41467-019-09908-1

Johnson MD, Fallon BP, Langley M, et al. Prolonged (24-hour) Normothermic ex vivo Heart Perfusion Facilitated by Perfusate Hemofiltration. *ASAIO Journal*. 2022;68(10):1282-1289. doi:10.1097/MAT.0000000000001649

Nilsson T, Hansson C, Wallinder A, et al. Hemofiltration in ex vivo lung perfusion-a study in experimentally induced pulmonary edema. *J Thorac Cardiovasc Surg*. 2016;151(2):570-575.e1. doi:10.1016/j.jtcvs.2015.06.046

Deem S, Berg JT, Kerr ME, Swenson ER. Effects of the RBC membrane and increased perfusate viscosity on hypoxic pulmonary vasoconstriction. *J Appl Physiol (1985)*. 2000;88(5):1520-1528. doi:10.1152/jappl.2000.88.5.1520

Tchouta L, Drake D, Hoenerhoff M, et al. Twenty-four-hour normothermic perfusion of isolated ex vivo hearts using plasma exchange. *The Journal of Thoracic and Cardiovascular Surgery*. 2022;164(1):128-138. doi:10.1016/j.jtcvs.2020.11.158

Kumar S, Bandyopadhyay U. Free heme toxicity and its detoxification systems in human. *Toxicol Lett*. 2005;157(3):175-188. doi:10.1016/j.toxlet.2005.03.004

Iskender I, Cosgun T, Arni S, et al. Cytokine filtration modulates pulmonary metabolism and edema formation during ex vivo lung perfusion. *The Journal of Heart and Lung Transplantation*. 2018;37(2):283-291. doi:10.1016/j.healun.2017.05.021

Ghaidan H, Stenlo M, Niroomand A, et al. Reduction of primary graft dysfunction using cytokine adsorption during organ preservation and after lung transplantation. *Nat Commun*. 2022;13(1):4173. doi:10.1038/s41467-022-31811-5

Gloria JN, Yerxa J, Kesseli SJ, et al. Subnormothermic ex vivo lung perfusion attenuates graft inflammation in a rat transplant model. *The Journal of Thoracic and Cardiovascular Surgery*. 2022;164(2):e59-e70. doi:10.1016/j.jtcvs.2021.01.066

Hasenauer A, Bédat B, Parapanov R, et al. Effects of cold or warm ischemia and ex-vivo lung perfusion on the release of damage associated molecular patterns and inflammatory cytokines in experimental lung transplantation. *The Journal of Heart and Lung Transplantation*. 2021;40(9):905-916. doi:10.1016/j.healun.2021.05.015

Hashimoto K, Cypel M, Juvet S, et al. Higher M30 and high mobility group box 1 protein levels in ex vivo lung perfusate are associated with primary graft dysfunction after human lung transplantation. *J Heart Lung Transplant*. Published online June 21, 2017:S1053-2498(17)31870-3. doi:10.1016/j.healun.2017.06.005

Wagner MJ, Hatami S, Freed DH. Thoracic organ machine perfusion: A review of concepts with a focus on reconditioning therapies. *Frontiers in Transplantation*. 2023;2. Accessed August 13, 2023. https://www.frontiersin.org/articles/10.3389/frtra.2023.1060992

Russo MJ, Iribarne A, Hong KN, et al. Factors associated with primary graft failure after heart transplantation. *Transplantation*. 2010;90(4):444-450. doi:10.1097/TP.0b013e3181e6f1eb

Russo MJ, Chen JM, Sorabella RA, et al. The effect of ischemic time on survival after heart transplantation varies by donor age: An analysis of the United Network for Organ Sharing database. *The Journal of Thoracic and Cardiovascular Surgery*. 2007;133(2):554-559. doi:10.1016/j.jtcvs.2006.09.019

Banner NR, Thomas HL, Curnow E, et al. The Importance of Cold and Warm Cardiac Ischemia for Survival After Heart Transplantation. *Transplantation*. 2008;86(4):542-547. doi:10.1097/TP.0b013e31818149b9

Stamp NL, Shah A, Vincent V, et al. Successful Heart Transplant after Ten Hours Out-of-body Time using the TransMedics Organ Care System. *Heart, Lung and Circulation*. 2015;24(6):611-613. doi:10.1016/j.hlc.2015.01.005

Ardehali A, Esmailian F, Deng M, et al. Ex-vivo perfusion of donor hearts for human heart transplantation (PROCEED II): a prospective, open-label, multicentre, randomised non-inferiority trial. *Lancet*. 2015;385(9987):2577-2584. doi:10.1016/S0140-6736(15)60261-6

Bakhtiyar SS, Sakowitz S, Verma A, Chervu NL, Benharash P. Expanded Criteria Donor Heart Allograft Utilization: National Trends and Outcomes. *The Annals of Thoracic Surgery*. 2023;116(6):1250-1258. doi:10.1016/j.athoracsur.2023.09.013

Hatami S, White CW, Shan S, et al. Myocardial Functional Decline During Prolonged Ex Situ Heart Perfusion. *The Annals of Thoracic Surgery*. 2019;108(2):499-507. doi:10.1016/j.athoracsur.2019.01.076

Olkowicz M, Ribeiro RVP, Yu F, et al. Dynamic Metabolic Changes During Prolonged Ex Situ Heart Perfusion Are Associated With Myocardial Functional Decline. *Frontiers in Immunology*. 2022;1:859506. doi:10.3389/fimmu.2022.859506

Transmedics Inc. TransMedics Organ Care System (TM) OCS Heart User Guide. Published online 2021. https://www.accessdata.fda.gov/cdrh_docs/pdf18/P180051S001D.pdf

Gunst J, Souter MJ. Management of the brain-dead donor in the intensive care unit. *Intensive Care Med*. Published online April 10, 2024. doi:10.1007/s00134-024-07409-4

Hatami S, White CW, Ondrus M, et al. Normothermic Ex Situ Heart Perfusion in Working Mode: Assessment of Cardiac Function and Metabolism. *J Vis Exp*. 2019;(143). doi:10.3791/58430

Chew HC, Scheuer S, Dhital K, Macdonald P. Banked blood for normothermic machine perfusion of the donor heart: A clinical perspective. *The Journal of Heart and Lung Transplantation*. 2019;38(12):1322. doi:10.1016/j.healun.2019.09.001

Chew H, Cheong C, Fulton M, et al. (96) - Outcome After Warm Machine Perfusion (WMP) Recovery of Marginal Brain Dead (MBD) and Donation After Circulatory Death (DCD) Heart Transplantation. *The Journal of Heart and Lung Transplantation*. 2017;36(4, Supplement):S45-S46. doi:10.1016/j.healun.2017.01.107

Qi X. Regulation of Coronary Artery Flow during Normothermic Ex-Situ Heart Perfusion [dissertation]. Edmonton (AB): University of Alberta; 2020.

White CW, Hasanally D, Mundt P, et al. A whole blood–based perfusate provides superior preservation of myocardial function during ex vivo heart perfusion. *The Journal of Heart and Lung Transplantation*. 2015;34(1):113-121. doi:10.1016/j.healun.2014.09.021

Hatami S, Qi X, Khan M, et al. Oxidative stress and related metabolic alterations are induced in ex situ perfusion of donated hearts regardless of the ventricular load or leukocyte depletion. *American Journal of Transplantation*. Published online January 2023:S1600613522302174. doi:10.1016/j.ajt.2022.11.027

Oudit GY, Trivieri MG, Khaper N, Liu PP, Backx PH. Role of L-type Ca2+ channels in iron transport and iron-overload cardiomyopathy. *J Mol Med (Berl)*. 2006;84(5):349-364. doi:10.1007/s00109-005-0029-x

Murphy CJ, Oudit GY. Iron-Overload Cardiomyopathy: Pathophysiology, Diagnosis, and Treatment. *Journal of Cardiac Failure*. 2010;16(11):888-900. doi:10.1016/j.cardfail.2010.05.009

Spencer BL, Wilhelm SK, Stephan C, et al. Extending heart preservation to 24 h with normothermic perfusion. *Front Cardiovasc Med*. 2024;11. doi:10.3389/fcvm.2024.1325169

Spencer BL, Wilhelm SK, Urrea KA, et al. Twenty-Four Hour Normothermic Ex Vivo Heart Perfusion With Hemofiltration In an Adult Porcine Model. *Transplant Proc*. 2023;55(9):2241-2246. doi:10.1016/j.transproceed.2023.08.014

Johnson MD, Fallon BP, Langley M, et al. Prolonged (24-hour) Normothermic ex vivo Heart Perfusion Facilitated by Perfusate Hemofiltration. *ASAIO Journal*. 2022;68(10):1282-1289. doi:10.1097/MAT.0000000000001649

Dixon SJ, Olzmann JA. The cell biology of ferroptosis. *Nat Rev Mol Cell Biol*. 2024;25(6):424-442. doi:10.1038/s41580-024-00703-5

Xie Y, Hou W, Song X, et al. Ferroptosis: process and function. *Cell Death Differ*. 2016;23(3):369-379. doi:10.1038/cdd.2015.158

Gu YJ, Vermeijden WJ, Vries AJ de, Hagenaars JAM, Graaff R, Oeveren W van. Influence of Mechanical Cell Salvage on Red Blood Cell Aggregation, Deformability, and 2,3-Diphosphoglycerate in Patients Undergoing Cardiac Surgery With Cardiopulmonary Bypass. *The Annals of Thoracic Surgery*. 2008;86(5):1570-1575. doi:10.1016/j.athoracsur.2008.07.052

Carroll C, Young F. Intraoperative cell salvage. *BJA Education*. 2021;21(3):95-101. doi:10.1016/j.bjae.2020.11.007

Chew HC, Gao L, Villaneuva J, et al. Ex-Vivo Perfusion of Donor Hearts: The Feasibility of Banked Blood for Normothermic Machine Perfusion. *OBM Transplantation*. 2019;3(2):1-24. doi:10.21926/obm.transplant.1902067

White CW, Messer SJ, Large SR, et al. Transplantation of Hearts Donated after Circulatory Death. *Front Cardiovasc Med*. 2018;5:8. doi:10.3389/fcvm.2018.00008

White CW, Ambrose E, Müller A, et al. Assessment of donor heart viability during ex vivo heart perfusion. *Can J Physiol Pharmacol*. 2015;93(10):893-901. doi:10.1139/cjpp-2014-0474

Hatami S, Qi X, White CW, et al. The Position of the Heart During Normothermic Ex Situ Heart Perfusion is an Important Factor in Preservation and Recovery of Myocardial Function. *ASAIO Journal*. 2021;67(11):1222-1231. doi:10.1097/MAT.0000000000001386

Hamed A, Tsui S, Huber J, Lin R, Poggio EC, Ardehali A. 19: Serum Lactate Is a Highly Sensitive and Specific Predictor of Post Cardiac Transplant Outcomes Using the Organ Care System. *The Journal of Heart and Lung Transplantation*. 2009;28(2):S71. doi:10.1016/j.healun.2008.11.025

Freed DH, White CW. Donor heart preservation: straight up, or on the rocks? *The Lancet*. 2015;385(9987):2552-2554. doi:10.1016/S0140-6736(15)60614-6

Trejo-Soto C, Hernández-Machado A. Normalization of Blood Viscosity According to the Hematocrit and the Shear Rate. *Micromachines (Basel)*. 2022;13(3):357. doi:10.3390/mi13030357

Aldam PS. The physiological changes of circulatory death with respect to organ donation [dissertation]. Cambridge (UK): University of Cambridge; 2019

Wagner MJ, Hatami S, Freed DH. Thoracic organ machine perfusion: A review of concepts with a focus on reconditioning therapies.

Khush KK, Cherikh WS, Chambers DC, et al. The International Thoracic Organ Transplant Registry of the International Society for Heart and Lung Transplantation: Thirty-sixth adult heart transplantation report - 2019; focus theme: Donor and recipient size match. *J Heart Lung Transplant*. 2019;38(10):1056-1066. doi:10.1016/j.healun.2019.08.004

Pober JS, Chih S, Kobashigawa J, Madsen JC, Tellides G. Cardiac allograft vasculopathy: current review and future research directions. *Cardiovasc Res*. 2021;117(13):2624-2638. doi:10.1093/cvr/cvab259

Qi X, Hatami S, Bozso S, et al. The evaluation of constant coronary artery flow versus constant coronary perfusion pressure during normothermic ex situ heart perfusion. *The Journal of Heart and Lung Transplantation*. 2022;41(12):1738-1750. doi:10.1016/j.healun.2022.08.009

Goodwill AG, Dick GM, Kiel AM, Tune JD. Regulation of Coronary Blood Flow. *Compr Physiol*. 2017;7(2):321-382. doi:10.1002/cphy.c160016

Qi X. Regulation of Coronary Artery Flow during Normothermic Ex-Situ Heart Perfusion [dissertation]. Edmonton (AB): University of Alberta; 2020.

Hatami S, Hefler J, Freed DH. Inflammation and Oxidative Stress in the Context of Extracorporeal Cardiac and Pulmonary Support. *Front Immunol*. 2022;13:831930. doi:10.3389/fimmu.2022.831930

Hatami S, Qi X, Khan M, et al. Oxidative stress and related metabolic alterations are induced in ex situ perfusion of donated hearts regardless of the ventricular load or leukocyte depletion. *American Journal of Transplantation*. Published online January 2023:S1600613522302174. doi:10.1016/j.ajt.2022.11.027

Florian JA, Kosky JR, Ainslie K, Pang Z, Dull RO, Tarbell JM. Heparan Sulfate Proteoglycan Is a Mechanosensor on Endothelial Cells. *Circulation Research*. 2003;93(10):e136-e142. doi:10.1161/01.RES.0000101744.47866.D5

Tarbell JM, Weinbaum S, Kamm RD. Cellular fluid mechanics and mechanotransduction. *Ann Biomed Eng*. 2005;33(12):1719-1723. doi:10.1007/s10439-005-8775-z

Reitsma S, Slaaf DW, Vink H, van Zandvoort MAMJ, oude Egbrink MGA. The endothelial glycocalyx: composition, functions, and visualization. *Pflugers Arch*. 2007;454(3):345-359. doi:10.1007/s00424-007-0212-8

Duni A, Liakopoulos V, Koutlas V, Pappas C, Mitsis M, Dounousi E. The Endothelial Glycocalyx as a Target of Ischemia and Reperfusion Injury in Kidney Transplantation—Where Have We Gone So Far? *Int J Mol Sci*. 2021;22(4):2157. doi:10.3390/ijms22042157

Aldecoa C, Llau JV, Nuvials X, Artigas A. Role of albumin in the preservation of endothelial glycocalyx integrity and the microcirculation: a review. *Ann Intensive Care*. 2020;10(1):85. doi:10.1186/s13613-020-00697-1

Torres Filho IP, Torres LN, Salgado C, Dubick MA. Plasma syndecan-1 and heparan sulfate correlate with microvascular glycocalyx degradation in hemorrhaged rats after different resuscitation fluids. *Am J Physiol Heart Circ Physiol*. 2016;310(11):H1468-1478. doi:10.1152/ajpheart.00006.2016

Tchouta L, Drake D, Hoenerhoff M, et al. Twenty-four-hour normothermic perfusion of isolated ex vivo hearts using plasma exchange. *The Journal of Thoracic and Cardiovascular Surgery*. 2022;164(1):128-138. doi:10.1016/j.jtcvs.2020.11.158

Foote CA, Soares RN, Ramirez-Perez FI, et al. Endothelial Glycocalyx. *Compr Physiol*. 2022;12(4):3781-3811. doi:10.1002/cphy.c210029

Bartosch AMW, Mathews R, Tarbell JM. Endothelial Glycocalyx-Mediated Nitric Oxide Production in Response to Selective AFM Pulling. *Biophys J*. 2017;113(1):101-108. doi:10.1016/j.bpj.2017.05.033

Dragovich MA, Chester D, Fu BM, et al. Mechanotransduction of the endothelial glycocalyx mediates nitric oxide production through activation of TRP channels. *American Journal of Physiology-Cell Physiology*. 2016;311(6):C846-C853. doi:10.1152/ajpcell.00288.2015

Song W, Wang H, Wu Q. Atrial Natriuretic Peptide in Cardiovascular Biology and Disease (NPPA). *Gene*. 2015;569(1):1-6. doi:10.1016/j.gene.2015.06.029

Johnson MD, Fallon BP, Langley M, et al. Prolonged (24-hour) Normothermic ex vivo Heart Perfusion Facilitated by Perfusate Hemofiltration. *ASAIO Journal*. 2022;68(10):1282-1289. doi:10.1097/MAT.0000000000001649

McLeod JS, Poling C, Church JT, et al. Ex Vivo Heart Perfusion for 72 Hours Using Plasma Cross Circulation. *ASAIO Journal*. 2020;66(7):753-759. doi:10.1097/MAT.0000000000001061

Hatami S. Inflammation, Oxidative Stress, Metabolic Inefficiency, And Related Functional Decline of the Heart During Ex-situ Perfusion [dissertation]. Edmonton (CA): University of Alberta, 2021.

Vercaemst L. Hemolysis in Cardiac Surgery Patients Undergoing Cardiopulmonary Bypass: A Review in Search of a Treatment Algorithm. *J Extra Corpor Technol*. 2008;40(4):257-267.

Foote CA, Soares RN, Ramirez-Perez FI, et al. Endothelial Glycocalyx. *Compr Physiol*. 2022;12(4):3781-3811. doi:10.1002/cphy.c210029

Pober JS, Chih S, Kobashigawa J, Madsen JC, Tellides G. Cardiac allograft vasculopathy: current review and future research directions. *Cardiovasc Res*. 2021;117(13):2624-2638. doi:10.1093/cvr/cvab259