Inflammation, Oxidative Stress, Metabolic Inefficiency, And Related Functional Decline of the Heart During Ex-situ Perfusion

By
Sanaz Hatami

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

Doctor of Philosophy

in

Experimental Surgery

Department of Surgery

University of Alberta

Abstract

Normothermic *ex-situ* heart perfusion (ESHP) is an emergent technique that allows for continued perfusion of the beating heart, supplying required oxygen and nutrients. Also, this technique offers a venue to assess the metabolic and functional viability of the donated heart during the period of preservation. However, myocardial function declines gradually during ESHP. In this thesis, we have studied the opportunities, challenges and reliability of the assessment methods for the donor heart in ESHP.

The first chapter is the introduction and provides some background about the preservation methods for the donor heart, limitations of the current standard of care for organ preservation, technology of ESHP, its potentials and limitations, and the need for optimizing devices and protocols.

In chapter two, we have introduced the ESHP apparatus including the operational software both designed in our lab. Using this apparatus, the heart can be perfused with minimal supervision in either empty-beating, non-working mode (NWM), or loaded left ventricle, working mode (WM). The custom-designed software system calculated and records the functional parameters interpreting the data collected from the pressure and flow sensors.

In most, if not all of experimental ESHP studies, and in the clinical setting, the heart has been perfused suspended from the aorta (hanging), in a position considerably different from the *in-vivo* position in the mediastinum. In chapter three, we showed that while the cardiac function preservation and recovery were both significantly better in the hearts perfused in a semi-anatomical supported position compared to the hearts that were perfused in a suspended position, the perfusate marker of cardiac damage (cardiac

troponin-I) was significantly lower in the supported-perfused hearts compared to hanging-perfused hearts.

In chapter four, we demonstrated that the percentage of apoptotic cells only increases very slightly during ESHP, thus it is not explaining the significant decline of the cardiac function occurring during ESHP. However, we showed significant alterations of the metabolic metrics in the ex situ-perfused hearts, suggesting that the energy-yielding metabolism is impaired during perfusion. The alterations in energy-yielding metabolites/metabolism may play a key role in the functional decline of the heart during ESHP.

In chapter five, we demonstrated that the markers of inflammation are considerably elevated in the perfusate during ESHP, which occurred regardless of the left ventricular workload. Also, in general, the endoplasmic reticulum stress responses (ERS, one of the important stress-sensing organelles) were induced during ESHP, however, both ERS and inflammation were significantly higher in the left ventricular tissue of the hearts perfused in WM compared to NWM.

In the setting of extracorporeal circulation, oxidative stress may occur in a persistent fashion, going beyond the ischemia-reperfusion injury-related oxidative stress, and may significantly alter energy metabolism in the cell, especially if the cellular protective systems are impaired. In chapter six, we showed that various markers of oxidative stress were induced in both perfusate and left ventricular tissue of the perfused hearts, while the natural antioxidative defences of the cell decline. The hearts perfused in WM showed better functional preservation, and similar values of some of the enzymes that play important roles in maintaining the metabolic reserve and/or exert protective effects

against oxidative stress when compared to *in vivo*. However, the NWM hearts showed significantly lower activity of those enzymes and poor functional preservation. On the other hand, leukocyte depletion of the perfusate did not attenuate inflammation, oxidative stress, or functional decline of the heart during ESHP.

While the ESHP technique and the related protocols for the protection of the donor heart in this setting has significantly improved during the last decade or so, there are still limitations for this method. This work introduces some areas that can be targeted for improving preservation of myocardial viability during ESHP.

Preface

Most of the research shown in this thesis are either published in peer reviewed journals, in press, or submitted as provided below.

A part of the first chapter is published in Current Transplantation Reports as “Hatami S, Freed DH: **Machine Perfusion of Donor Heart**: State of the Art. Current Transplantation Reports 2019, **6**(3):242-250.

The other part of the first chapter is being drafted for submission as Hatami S, Himmat S, Freed D: **Inflammation and oxidative stress in context of cardiac and pulmonary –supporting extracorporeal circulation.**

The second chapter is published at the Journal of visualized experiments as “**Hatami S**, White CW, Ondrus M, Qi X, Buchko M, Himmat S, Lin L, Cameron K, Nobes D, Chung HJ *et al*: **Normothermic Ex Situ Heart Perfusion in Working Mode: Assessment of Cardiac Function and Metabolism**. Journal of visualized experiments *:* JoVE 2019(143).

The third chapter is submitted to the American Society for Artificial Internal Organs as :Hatami S, Qi X, White C.W, Bozso S.J, Himmat S, Sergi C, Nagendran J, Chung H.J, Nobes D.S, Freed D.H: **The Position of the Heart During Normothermic Ex-situ Heart Perfusion is an Important Factor in Preservation and Recovery of Myocardial Function**. The American Society for Artificial Internal Organs journal, Aug 2020.

The fourth chapter is published at The Annals of thoracic surgery as “Hatami S, White CW, Shan S, Haromy A, Qi X, Ondrus M, Kinnear A, Himmat S, Michelakis E,

Nagendran J *et al*: **Myocardial Functional Decline During Prolonged Ex Situ Heart Perfusion**. The Annals of thoracic surgery 2019, 108(2):499-507.

The fifth chapter is in press in the journal of Circulation: Heart Failure as "Hatami S, White C, Qi X, Buchko M, Ondrus M, Kinnear A, Himmat S, Sergi C, Nagendran J, Freed D: **Immunity and Stress Responses Are Induced During Ex-Situ Heart Perfusion**. Circulation :Heart Failure 2020, 13(6):e006552.

The manuscript reflecting the findings of the sixth chapter is ready for submission as: Hatami S, Qi X, White C.W, Himmat S, Khan M, Tkachuk B, Sergi C, Nagendran J, Freed D.H

Dedications

I dedicate this work to the love of my life, Nariman Sepehrvand, to my Mother, Behjat Hatami, and my late father, Javad Hatami, my sister, Sepideh Hatami, and my late brother, Siamak Hatami and to all the mentors who shaped my life and brought me here.

I would also like to dedicate this thesis to precious research pigs, whom without them, none of the achievement in this thesis would be possible.

Acknowledgements

I would like to sincerely thank my supervisor, Dr. Darren H. Freed, for his invaluable mentorship and guidance over the last several years. I have learned a lot from him about heart failure, mechanical perfusion systems, and the physics of the circulation.

Dr. Freed has been a true advocate, and a great mentor who helped me a lot through developing the ability to critical thinking and has been a very inspiring role model for me both as a scientist, and as a dedicated physician. He is a world-renowned inventor and scientist, and a fantastic mentor and I look forward to benefiting from his expertise in future collaborative works.

I would also like to thank my committee members Dr. Jayan Nagendran and Dr. Consolato Sergi, and also to Dr. Richard Schulz for their kind support, suggestions and valuable feedbacks during my PhD studies.

This work would not have been possible without the support of my colleagues at Drs Freed and Nagendrans' lab, including the lab manager Dr. Xiuhua Wang, Dr. Christopher White, Dr. Xiao Qi, Dr. Sayed Himmat, Dr. Nader Aboelnazar, and many more who I have been privileged to work with over these years.

I would like to also thank the colleagues at the Surgical Medical Research Institute including Deb Dixon, Katie-Marie Buswell, Ryan Edgar, and Greg Olson for their kind support and help with our animal surgery projects during these years.

I am grateful to my parents, my mother Behjat Hatami, and my late father Javad Hatami for being the wonderful parents they have been, for always encouraging me and teaching me to overcome any limitation by working hard and aiming high. I have been very lucky

to have my siblings growing up, and I would like to thank my sister Sepideh Hatami, and my late brother Siamak Hatami for their great support and love.

I am thankful to my in-laws Saeid Sepehrvand and Seyedeh Tayebeh Motie-Langroudi and my brother-in-law Aslan Sepehrvand for their invaluable supports during all these years.

Finally, I would like to thank my mentors in Iran including Dr. Ali Ardalan, Dr. Kamran Shateri, and Dr. Ali Ghafari Moghadam, who all had a great impact on shaping who I am today.

Table of Content

List of Tables

List of Figures

List of Abbreviations

ACC: American College of Cardiology

ACE: Angiotensin Converting Enzyme

ADP: Adenosine Diphosphate

AF: Atrial Fibrillation

AHA: American Heart Association

AHF: Acute Heart Failure

AIF: Apoptosis Inducing Factor

AMPK: Adenosine Mono Phosphate Kinase

ANOVA: Analysis Of Variance

Ao: Aorta, Aortic

AP: Alternative Pathway

ARDS: Acute Respiratory Distress Syndrome

ATF-6: Activating Transcription Factor 6

ATP: Adenosine Triphosphate

BAL: Bronchoalveolar Lavage

BNP: Brain Natriuretic Peptide

C: Complement Compartment

cAMP: cyclic Adenosine Monophosphate

CaO_2: Arterial Content of Oxygen

CBF: Coronary Blood Flow

CD: Cluster of Differentiation

cGMP: cyclic Guanosine Monophosphate

CHOP: CCAAT-enhancer-Binding Protein Homologous Protein

CI: cardiac index

CIT: Cold Ischemic Time

CK-MB: C**reatine Kinase** Myocardial Band

CK: Creatine Kinase

CMRI: Cardiac Magnetic Resonance Imaging

CO_2: Carbon Dioxide

CoQ10: Coenzyme Q10

CP: Classic Pathway

CPB: Cardiopulmonary Bypass

CSS: Cold Static Storage

cTn-I: Cardiac Troponin-I

CvO_2: Venous Content of Oxygen

DCD: Donated after Circulatory determination of Death

dp/dt max: Maximum rate of Pressure change

dp/dt min: Minimum rate of Pressure change

ECG: Electrocardiography

ECLS: Extracorporeal Life Support

ECMO: Extracorporeal Membrane Oxygenation

EIF-2α: Eukaryotic translation Initiation Factor 2 alpha

ER: Endoplasmic Reticulum

ERO-1α: Endoplasmic Reticulum Oxidoreductase 1 alpha

ESHP: Ex-situ Heart Perfusion

ESTP: Ex-situ Thoracic organ Perfusion

FFA: Free Fatty Acid

FIO_2: Fraction of Inspired Oxygen

F: Factor

H*:* perfused in a hanging position

Hb: Hemoglobin

HBP: Hexosamine Biosynthesis Pathway

HF: Heart Failure

HMWK: High Molecular Weight Kininogen

HO: Haem Oxygenase

ICAM: Intercellular Adhesion Molecule

ICP: Intracranial Pressure

ICU: Intensive Care Unit

IHD: Ischemic Heart Disease

IL: Interleukin

IQR: Interquartile Range

IQR: interquartile range

IRE-1α: Inositol-Requiring Enzyme

IRI: Ischemia-Reperfusion Injury

ISHLT: International Society for Heart and Lung Transplantation

IVC: Inferior Vena Cava

JVP: Jugular Vein Pressure

KH: Krebs-Henseleit

LA: Left atrium, Left atrial

LP: Lectin Pathway

LSD: Least Significant Difference

LV: Left Ventricle, Left Ventricular

LVEF: Left Ventricular Ejection Fraction

LVSW: Left Ventricular Stroke Work

MAC: Membrane Attack Complex

MAPK: Mitogen-Activated Protein Kinase

MCP: Monocyte Chemoattractant Molecule

MDA: Malondialdehyde

ME: Mechanical Efficiency

MECC: Minimised Extracorporeal Circuit

MMP: Matrix Metaloproteinase

MPO: Myeloperoxidase

MVO_2: Myocardial oxygen consumption

NAC: N-Acetylcysteine

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

NDD: Neurologic Determination of Death

NFkβ-p65: p65 subunit of Nuclear Factor kappa-light-chain-enhancer of activated B cells

NO: Nitric Oxide

NOS: Nitric Oxide synthase

Nrf2: Nuclear Factor erythroid-2-related factor 2

NRP: Normothermic Regional Perfusion

NT-proBNP: N-terminal pro Brain Natriuretic Peptide

NWM: Non-working Mode

NYHA: New York Heart Association

O-GlcNAc: O-linked β-N-acetylglucosamine

O_2: Oxygen gas

OCS: Organ Care System

OCT: Optimum Cutting Temperature

OH^-: Active Hydroxyl radical

OI: Oxygenation Index

Ox-LDL: Oxidized Low-density Lipoprotein

p-PKM2: phosphorylated Pyruvate Kinase isozyme M2

p-AMPK: phosphorylated Adenosine Monophosphate Kinase

P_aCO_2: arterial partial Pressure of Carbon dioxide

PaO_2: arterial partial Pressure of Oxygen

PAR: Protease-activated Receptors

PDEs: Phosphodiesterases

PDI: Protein Disulfide Isomerase

PERK: Protein kinase R (PKR)-like Endoplasmic R*eticulum* Kinase

PK: Pyruvate Kinase

PKM1: Pyruvate Kinase isozyme M1

PKM2: Pyruvate Kinase isozyme M2

PMN: Polymorphonuclear

PPP: Pentose Phosphate Pathway

pRBC: packed Red Blood Cell

PRSW: Preload Recruitable Stroke Work

PUFA: Poly Unsaturated Fatty Acids

PvO_2: venous partial Pressure of oxygen

QPCR: quantitative polymerase chain reaction

R2: coefficient of determination

RBC: Red Blood Cell

RONS: Reactive Oxygen and Nitrogen Species

RPM: Revolutions Per Minute

S*:* perfused in a supported position

SD: standard deviation

Ser473: Serine 473 position

SIRS: Systemic Inflammatory Response Syndrome

sO_2: Oxygen saturation

SW: Stroke Work

Tg: Triglyceride

Thr308: Threonine 308 position

TNF- α: Tumor Necrosis Factor alpha

TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP-biotin Nick End Labeling

UNOS: United Network for Organ Sharing and Storage

UPR: Unfolded Protein Responses

VA: Venoarterial

VCAM: Vascular Cell Adhesion Molecule

VV: Venovenous

VWF: Von Willebrand Factor

WM: Working Mode

XO: Xanthine Oxidase

Chapter 1: Introduction

1.1 Heart Failure: Burden and epidemiology

After being introduced as an emerging epidemic in the late 1990s [1], heart failure (HF) remains a growing, costly and disabling health problems worldwide [2-4] and affects more than 6 million patients in the United States (USA), more than 0.6 million in Canada, and approximately 15 million patients in European countries, predominantly elderly population with age > 65 years [5-9]. Considering the recent advances in the diagnosis and treatment of patients with coronary disease as well as an increase in the incidence of HF in the elderly population, it is the only cardiac illness with an increase in prevalence [2, 10, 11]. The lifetime risk of developing HF is 20% [12] and its prevalence is estimated to be 1-2% in the USA and 2-3% of the general population worldwide, with 5-10% of HF patients having advanced stage of disease [3, 8, 13]. Advanced HF is associated with increased morbidity and mortality, recurrent hospitalization, and poorer quality of life [13, 14]. Almost half of the patients with HF die within 5 years after diagnosis.

Despite novel treatments, HF-related hospitalization has been increased 155% over the past two decades and HF has become the most common cause for hospital admission and re-hospitalization [4, 15-17]. More than 1 million hospitalizations occur annually because of heart failure [2, 18, 19], so an important economic burden was incurred by this disease on our limited healthcare resources [8, 20] with a total annual cost (direct and indirect) of over $33 billion in USA [21], which means more than $8,000 per person per year. In most westernized economies, HF is responsible for 1-2% of total healthcare expenditure. The bulk of these costs are driven by frequent, prolonged and repeated HF-related hospitalizations [21, 22].

1.2 Heart failure: definition, aetiology, and classification

About three decades ago, HF used to be defined as a clinical syndrome associated with reduction in left ventricular ejection fraction (LVEF) due to left ventricular systolic dysfunction [23]. It is a complex syndrome and can be the result of any cardiac disorder (structural or functional) that impairs the ability of the ventricle to be effectively filled and/or eject the blood [19]. Regarding patient characteristics, etiology, and associated co-morbidities, the clinical presentation of HF can be highly variable [8].

Patients with left ventricular (LV) dysfunction generally present with a syndrome of decreased tolerance to exercise and fluid retention; or patients with no symptoms but an incidentally discovered left ventricular dysfunction [19].

left ventricular systolic dysfunction (LVSD) is the most common cause of heart failure (60%) and in most cases, is a consequence of end-stage coronary artery disease (either with a history of myocardial infarction or with a chronically under-perfused but viable myocardium). Other common causes of LV dysfunction include idiopathic dilated cardiomyopathy, valvular heart disease, hypertensive heart disease, toxin-induced cardiomyopathies (e.g. doxorubicin, herceptin, alcohol), and congenital heart diseases [24]. Since some causes of left ventricular dysfunction are reversible or treatable, identifying the underlying etiology of HF is very important. Although it may not be always possible to discern the cause of HF, or the underlying condition may not be always treatable, it would be more beneficial to focus on diagnosing cases who have some potential for improvement after diagnosis/treatment of underlying disease [19]. Some initial epidemiological studies suggested hypertension as the leading cause of HF but this pattern changed significantly over the past four decades and now ischemic heart disease

(IHD) is considered as the leading cause [25-27]. The results of epidemiologic studies vary among different regions that may reflect the different susceptibility of these populations to IHD [25, 28, 29].

Heart failure is characterized by shortness of breath (dyspnea), fatigue, exercise intolerance, fluid retention [30] and sometimes pain [2]. The classification system has been most commonly used to quantify the degree of functional limitation in heart failure is the one first developed by the New York Heart Association (NYHA) [19]. The NYHA classification system reflects a subjective assessment, applicable by both the care provider and patient. Individuals in class I have no functional limitation and no symptoms of HF with ordinary physical activity. Patients in class II experience some functional limitation with HF symptoms with ordinary physical activity. These patients have no symptoms at rest. Patients in class III have significant functional limitations with HF symptoms with less than ordinary activity. They also have no symptoms at rest. Patients in class IV experience severe functional limitation with symptoms at any level of physical activity (Table 1.1) [2]. The American Heart Association (AHA) and American College of Cardiology (ACC) update of HF clinical practice guidelines in 2005, moved beyond the NYHA classification system and included four new stages of heart failure A-D (Table 1.2) [24].

1.3. Heart Failure: Diagnosis

1.3.1. Symptoms and signs

The diagnosis of heart failure is mostly based on symptoms and signs but in early stages, symptoms may be neglected or missed. Furthermore, most of the symptoms in HF are non-specific and this makes it more difficult to differentiate between HF and other possible

underlying problems. More specific symptoms such as orthopnea, or paroxysmal nocturnal dyspnea and signs such as elevated jugular venous pressure (JVP) and displacement of the apical impulse are less common or harder to detect [31-33]. Some patients are asymptomatic but still at high risk of developing heart failure. This includes patients with hypertension, valvular heart disease, diabetes mellitus, sleep disorders and history of chemotherapy, alcohol and familial history. These patients should be aggressively treated to prevent or defer the incidence of HF [34].

1.3.2. Laboratory tests, biomarkers:

1.3.2.1. Natriuretic Peptides

AHA/ACC heart failure guidelines and the Heart Failure Society of America guidelines recommend evaluation of B-type natriuretic peptide (BNP) or amino-terminal pro-B-type natriuretic peptide (NT-proBNP) in patients presenting with symptoms of HF [35]. BNP is especially helpful in excluding HF in the presence of low clinical suspicion [36]. Both BNP and NT-proBNP are driven from the same precursor molecule (pro-BNP). The BNP gene natriuretic peptide B is upregulated in hemodynamic changes such as cardiomyocyte stretch, injury, and hypoxia [36, 37]. These biomarkers are useful to support the clinical judgment in diagnosing and identifying prognosis in acutely decompensated heart failure [35]. Several new biomarkers have been suggested to be useful in the diagnostic evaluation of HF but yet needed to be further studied [37].

1.3.2.2. Cardiac Troponins

Sensitive cardiac troponin tests, including cardiac troponin-I (cTn-I) can be useful in establishing prognosis or disease severity in acute heart failure [36, 38, 39].

1.3.3. Non-invasive Imaging

Electrocardiogram and Echocardiography

A 12-lead electrocardiogram (ECG) is useful to determine any abnormality in heart rhythm, heart rate, QRS morphology, QRS duration, etc. ECG is also important in prognostication and treatment planning. A completely normal ECG is rare in presence of HF [40-42].

Echocardiogram is widely considered as the most useful diagnostic modality in patients with suspected heart failure [41]. It provides immediate information on ejection fraction, chamber size and valve function. Left atrial and left ventricular filling pressures, pulmonary pressures, central venous pressure, and stroke volume can also be estimated [35, 36, 41, 43]. Serial echocardiographic evaluation is recommended in the case of notable change in clinical status, experiencing a clinical event, receiving treatments that may affect ventricular function, and in patients who may be a candidate for device therapy [36], but routine repeated assessment of ventricular function is not indicated otherwise [44-46].

Chest radiograph

Cardiomegaly and pulmonary congestion can be assessed by chest X-ray in patients suspected of HF. Of note that even in the presence of LVSD, cardiomegaly may be absent [35, 36, 41].

Cardiac magnetic resonance imaging

Cardiac magnetic resonance imaging (CMRI) can provide high anatomical resolution and valuable information on the function of the heart including ischemia, viability and fibrosis. Taking into account its accuracy and ability to reproduce right and left ventricular volume, ejection fraction and wall motion, it is regarded as the gold standard in diagnosing heart failure by European guidelines for heart failure especially in patients with non-diagnostic echocardiography findings and it is useful for differentiation of particular causes of heart failure including myocarditis, infiltrative diseases (e.g. amyloidosis or hemochromatosis), and cardiomyopathies [35, 36, 41].

Computed tomography

Computer tomography is a non-invasive method to visualize coronary anatomy but like CMRI, its accuracy is reduced in high heart rates. The only advantage of computed tomography over echocardiography is its ability to characterize the myocardium [35].

1.3.4. Invasive studies

Complete right heart catheterization (assessment of pulmonary vascular resistance) [35], Coronary angiography (left heart catheterization) [35, 47, 48], endomyocardial biopsies [36, 41] are some invasive methods of assessment in HF.

1.4. Heart Failure: Management

Although medications such as angiotensin-converting-enzyme inhibitors, β-blockers, aldosterone antagonists, and angiotensin-receptor has significantly improved outcomes in heart failure but in many cases, treatments fail to control the disease progression [12, 49]. In treating acute heart failure (AHF), the goal is to relieve symptoms and reverse the

ventricular dysfunction, if possible. When it is not possible to resolve underlying ventricular dysfunction that has lead to AHF, targeting ventricular preload and afterload, and the neurohormonal response to cardiac dysfunction, may be helpful in the resolution of morbid conditions such as pulmonary edema and/or improvement in vital organ perfusion [50].

Control of systolic and diastolic hypertension, treatment of lipid disorders, angiotensin-converting enzyme (ACE) inhibition in patients with a history of atherosclerotic vascular disease, diabetes mellitus, or hypertension and associated cardiovascular risk factors, control of ventricular rate in patients with supraventricular tachyarrhythmia, treatment of thyroid disorders, changing lifestyle such as avoiding smoking, alcohol consumption, illicit drug use, reduction of dietary salt, having exercise, and periodic evaluation for signs and symptoms of HF are some examples of effective measure could be taken to mitigate the risk of underlying conditions in HF [19, 51].

1.4.1. Management of acute heart failure

Development of AHF leads to a significant increase in hospital mortality and is associated with recurrent hospitalization [49]. Improving myocardial contractility, along with maintaining adequate peripheral perfusion and reducing fluid overload are the main goals in the management of AHF [52]. Supplemental oxygen, diuretics in cases with fluid overload, and vasodilators should be considered in the treatment of acute heart decompensation.

1.4.2. Management of chronic heart failure

The management of chronic heart failure (stage A-D) according to the 2013 ACC/AHA Guideline [35] is summarized in Figure 1.1

1.5. Heart Transplantation

During the decades after the first clinical heart transplantation by Christian Barnard in 1967, clinical heart transplantation has transformed into a highly integrated and efficient intervention considered as the “gold standard” treatment for end-stage heart failure [53]. However, despite numerous policies that have been taken to increase the number of available organs for transplantation, according to the report of the International Society for Heart and Lung Transplantation (ISHLT) registry, the number of heart transplantations has not increased during the past two decades and has reached a plateau state [14, 54]. Moreover, only 36%-39% of the available donor hearts are transplanted and the majority are discarded [55, 56]. Thus, there is a significant gap between organ supply and demand for the donor heart [57, 58]. Development of new strategies to extend the donor pool seems to be necessary in both adult and pediatric patients in need of a heart transplantation [59]. Accepting the extended-criteria donor hearts, including the hearts donated after circulatory determination of death (DCD) can potentially help to expand the donor pool [60]. Among the most important detriments of success in heart transplantation is the original quality of the graft. Not only different donation methods directly affect the quality of the donated heart, efficiency of the organ preservation method also plays a pivotal role in this matter [61].

1.5.1. Donation after Neurologic Determination of Death (NDD, DBD)

The term “Neurologic Determination of Death” or “Brain Death” refers to irreversible loss of all functions of the brain, including the brainstem. Coma, apnea, and complete loss of brainstem reflexes are known to be the three findings that should be present for the neurologic determination of death [62]. Traumatic head injury, stroke, subarachnoid and

intracerebral hemorrhage, and encephalopathy are among the causes of brain death occurrence. Brain injury leads to edema and increased intracranial pressure (ICP) which in turn, will cause a reduction in cerebral blood flow through decreasing cerebral perfusion pressure and these events together make a vicious cycle that eventually stops cerebral blood flow and culminates in transtentorial herniation through the foramen magnum with resultant brainstem compression and brain death [63]. A patient who is clinically diagnosed as brain dead, may be considered as a potential donor since he is clinically and legally dead [62].

The majority of organs used for transplantation are obtained from the heart-beating donors after neurologic determination of death (NDD) and these donors are currently the main, if not the only accepted source for cardiac transplant in most of the world. Maintaining the condition of the organ is considered to be possible in brain death donors by proper management and minimizing retrieval-related warm ischemia [64]. Nevertheless, it is well known that the process of brain death can negatively influence donor heart function [65, 66]. Cardiac hemodynamic perturbations occur more in cases of a rapid increase in ICP. Abrupt brainstem compression will also cause a catecholamine surge, characterized by hypertension and tachycardia (Cushing response). These events together with the inflammatory response due to the up-regulation of pro-inflammatory cytokines, adhesion molecules, and rapid leukocyte infiltration related to brain death, have been suggested to worsen myocardial injury and accelerate acute rejection of the heart graft post-transplant [67]. Increasing demand for transplantation concurrently with progress in the management of conditions that can lead to brain death made the gap between supply and demand wider and has raised the interest toward expansion of the

heart beating donor pool by “marginal” or “extended criteria” organs and organs “Donated after Circulatory Determination of Death (DCD) [64, 66].

1.5.2. Marginal donor grafts in heart transplantation

Since cardiac transplantation was accepted as a promising treatment in end-stage heart failure, suitability criteria were established and have been used for the evaluation of potential donor hearts. Standard criteria for heart donation include age< 65 years, no known heart disease (including angina pectoralis, myocardial infarction, prior coronary bypass surgery, moderate to severe valvular disease, cardiomyopathy and important arrhythmias), cold ischemia time less than 2 hours, being non-dependent upon high-dose inotropes, and absence of chest trauma. Also untreated sepsis, malignancies and active infections are general contraindications for all organ donations [14, 68, 69]. The growing demand of heart transplantation and lengthening of the time spent on the waiting lists has raised the attempts to modify the donor criteria for the heart. Recent studies have suggested that such eligibility criteria modifications can increase the donor pool by including some of the donated hearts that have been identified before as "not suitable for transplantation", or so-called “Marginal donors” [70]. Factors that determine the usability of “Marginal Donors” in adults are summarized in Table 1.3.

1.5.3. Donation after circulatory determination of death

The phrase “Donation after Circulatory Determination of Death” (DCD) refers to organ donation from patients with permanent circulatory arrest [71]. “Donation after cardiac death” and “non-heart-beating donor” are deprecated terms for multi-organ donors who donate after the permanent cessation of circulation. Unlike brain death, circulatory death is defined on the basis of cardiopulmonary criteria rather than neurologic criteria [72].

Acceptance of NDD in western countries as the method of choice for organ donation (due to the absence of warm ischemia) led to complete negligence to the possibility of donation after circulatory death. During the early 1990s, the interest in DCD was renewed as a potential avenue to overcome organ shortage [73].

While a “no-touch” period is legally necessary between the onset of circulatory arrest and declaration of death, the duration of the “no-touch” period is variable between jurisdictions. Although a sufficient time period is required to ensure the permanency of circulatory cessation, this time period must be limited to minimize the insult to the ischaemic organs [71]. The modified Maastricht DCD categories are summarized in Table 1.4, in which controlled DCD is referred to patients with brain damages that not meet the neurologic criteria of death, but with such severe injuries that withdrawal from life-supporting treatments is pursued after careful consultation between the “Intensive Care Unit” (ICU) team and the patient’s family. An uncontrolled DCD refers to a patient, already bearing warm ischemia as a consequence of the cessation of circulation outside a suitable context for proceeding with organ procurement [74].

Despite all the potential benefits of DCD in expanding the donor pool, several limitations must be overcome for these donors to be considered acceptable for heart donation. Duration of warm ischemia and its adverse effects on the heart function, on-site access to efficient cardioprotective modalities, possibility of rapid procurement, proper organ resuscitation, storage and transport are some of the most important limiting factors for using the heart after DCD [71].

1.6. Organ preservation methods, potentials, and limitations

Many aspects of heart transplantations have significantly evolved over time, leading to a considerable improvement of the outcomes, yet the current standard of graft preservation is still cold static storage (CSS), the same as the early eras of transplantation. Cold storage although simple and inexpensive, exposes the donated heart to an episode of cold ischemia which is associated with a time-related graft injury and primary graft dysfunction that limits the safe preservation time to a maximum of 4-6 hours in ideal donor/donation conditions, and renders CSS unsuitable for preservation of sub-optimal, extended criteria donor hearts [54, 76]. Moreover, due to the static nature cold storage assessment of cardiac viability and function during preservation time is not possible with this method [60].

1.6.1. Cold Storage

Cold static storage (CSS) has been accepted as a simple, inexpensive and reliable method for preserving organ, thus it is the current standard of care for preserving donor hearts [77, 78]. This method is associated with low-level but persistent anaerobic metabolism (cold ischemia) [79, 80]. Cold ischemia is associated with a exposure time-dependent increased risk of post-transplantation primary graft dysfunction and failure [78]. Although preservation times under 4-6 hours restricts these consequences particularly in hearts from young donors [80-82], it will limit the opportunity of transporting a heart to a geographically remote but otherwise well-matched recipient [60]. Also, there have been concerns about the detrimental effects of exposing the organs from non-cold tolerant, non-hibernator mammals (including humans) to long episodes of cold as they lack the adaptive systems to sustain and redirect minimal essential energy supplies, and

to return to the normothermic condition (arousal) in a safe, cohesive way [53]. Moreover, due to the static nature of cold storage assessment of cardiac viability and function during preservation time is not possible with this method, which is believed to be a necessity for DCD hearts [60]. These limitations, render CSS unsuitable for preservation of sub-optimal, extended criteria donor hearts [54, 76] and a less than ideal cardiac preservation method even in ideal donor/donation conditions and suggests the need for improvement in preservation techniques [77, 82].

1.6.2. Cold ischemia

Cold ischemia is an inevitable part of the current standard organ preservation process [83]. It has been suggested that cold ischemia may have a relationship with chronic rejection of transplanted heart through stimulating an inflammatory response leading to pathological remodelling of graft vessels. Cold ischemia can also cause rupture of the outer mitochondrial membrane and may impair respiratory function that may eventually cause loss of cardiac contractile function [84]. It is generally thought that cold ischemia time (CIT) is well tolerated especially in young donors [80-82]. Yet, based on ISHLT/United Network For Organ Sharing (UNOS) database, CIT > 6 hours is a risk factor for mortality [83]. It is associated with a reduction in post-transplantation organ flow after only 4 hours, and reduction in both function and flow of the heart after 8 hours, suggesting the vasculature and parenchymal cells to be the first and second sites of cardiac injury during cold storage [85]. The acceptable period for cold storage of the heart is much shorter than other organs. Hypothermia suppresses basal metabolic rate and modifies signalling pathways towards the expression of protective genes, however, the consequences of ischemia will still be present [84]. Several studies suggest that the

mechanisms of cardiac injury in cold ischemia is different from warm ischemia and should be treated differently [85].

1.6.3. Warm ischemia

Episodes of warm ischemia in DCD donors can be categorized into two phases, 1) A variable period of warm ischemia before organ procurement and storage (pure warm ischemia), and 2) A shorter period of warm ischemia from the moment that the heart is removed from ice until transplantation (anastomosis time), during which the organ slowly warms up through body temperature [86]. These two periods of warm ischemia vary indeed in their pathophysiologic effects. During pure warm ischemia, the heart bears the ischemia at body temperature and without the protective benefits of a preservation solution. But, in warm ischemia of anastomosis time, the organ temperature is passively gradually raised from 4°C toward the recipient's body temperature at a rate of 0.5°C per minute and it rarely reaches the normal body temperature until reperfusion in the recipient. DCD organs, based on the nature of this kind of donation, may suffer from variable, unprotected periods of warm ischemia. The ischemic tolerance varies between different organs but the heart may be the most sensitive solid organ to warm ischemia because of its extremely high energy demands. Thus, long periods of warm ischemia can irreversibly damage the heart [71]. Studies on large animal models have suggested that to recover at least 60% of cardiac function, the warm ischemia duration should be no longer than 30 minutes (the function decreased to less than 10% after 30 minutes of warm ischemia) [87]. Taking into account the obligatory "no touch (stand-off)" period after declaration of death, and the time required to reperfuse the organ, achieving such timing

goals may not be easy, therefore limiting DCD heart donation to controlled Maastricht groups.

1.6.4. *Ex-situ* heart perfusion

Ex-situ heart perfusion (ESHP), also known as continuous machine perfusion of the heart, is a method in which the donated heart is perfused with oxygenated, energy substrate-containing solutions during preservation time. ESHP offers preservation of the heart in a beating, semi-physiologic status avoiding cold ischemia. As a dynamic method of preservation, ESHP provides the opportunity for evaluation of the myocardial viability and function during preservation time. It also has the potential to improve the reparative process in ischemic myocardium and provides the opportunity for assessment of organ function before transplantation. These beneficial attributes can also be extended to dysfunctional hearts from NDD donors [58, 60].

1.6.4.1. History of *ex-situ*, machine perfusion of donor heart and regaining interest in the new era

With the pioneering work of isolated perfused heart method by Cyon in 1866, and its further adaptation for perfusion of mammal heart a few decades later in 1895 by Langendorff, and finally by the introduction of isolated working heart preparation later in the mid 20th century by Neely and Morgan, isolated ESHP heart has been widely utilized for studying the heart [61, 88]. Interestingly in the first era of the human heart transplantation, the donated hearts preservation was achieved with hypothermic ESHP with either blood or crystalloid-based perfusate. Later on in 1984 Wicomb *et al* published a case series of donated hearts preserved with a portable hypothermic perfusion system [61, 89-91]. However, due to technical complexity and high costs of commercial

equipment for hypothermic ESHP, and the introduction of the CSS method in combination with cardioplegic arrest, which was considered efficient for the preservation of the heart for 4-6 hours, ESHP of donated heart was overlooked for a few decades [92].

One of the most important detriments of success in heart transplantation is the quality of the graft. The current most frequent cause of death in potential donors has changed from head trauma to hypoxic brain injury. The median age of donors has also increased leading to older, heavier donors with more comorbidities [61, 93]. Moreover, the success of heart transplantation, as a reliable treatment modality for patients with end-stage refractory heart failure is limited by the considerable shortage of eligible hearts from donors with brain death (DBD). Due to the existing supply-demand mismatch, a considerable percentage of potential recipients die while on the waiting list [75, 94]. Thus, during the recent few decades the field has been pushed to extend the donor acceptance criteria to include marginal, sub-optimal hearts/donors such as DBD hearts from older donors, or with some degree of malfunction, and hearts donated after circulatory determination of death (DCD) [95]. The inclusion of the extended criteria donated hearts, although considered to be able to expand the heart donor pool, raised concerns about the viability of such transplants. On the other hand, in only bout 10 years after the first human heart transplantation, CSS was already deemed as a less than perfect method for preservation of donated hearts, even under ideal donor/transplantation conditions. Hypothermia, slows the process of ischemia-related tissue damage, however, 5-10% of the metabolism and tissue damage will still occur during CSS, leading to time-related damage of the donated heart. CSS is associated with primary graft dysfunction and failure and limits safe preservation times [53, 54, 96].

The deleterious effects of the cold ischemia in CSS make it a non-suitable method for preservation of either suboptimal DBD, or DCD hearts, which are already damaged and may not be able to tolerate an extra episode of cold ischemia [54, 76]. On the other hand, the static, non-physiologic status of the heart during cold storage precludes the possibility of function and viability assessment during the preservation time. This is an important limitation of CSS particularly in DCD hearts as they need to be evaluated after undergoing a severe ischemic insult during the hypoxic circulatory arrest and ”hands-off” period before organ procurement. Thus the need for expanding the donor pool, led to a rising interest in ESHP as a dynamic method of preservation, which prevents extra ischemia and potentially protects the donated heart better during preservation, and provides an opportunity to assess metabolic and/or functional viability of donated heart [94, 97, 98].

1.6.4.2. Hypothermic versus normothermic *ex-situ* perfusion of donor heart

The setting of hypothermic ESHP was launched based on the idea of using the benefit of hypothermia to 1) reduce the metabolic requirements of procured heart, with an optimal, homogenous cooling of the heart, and 2) providing metabolic support by continuous perfusion of coronary vasculature with perfusates containing nutrients and low demand oxygen [99]. The early experimental studies of hypothermic ESHP performed more than three decades ago provided robust evidence for the feasibility of longer safe preservation times with ESHP compared to conventional CSS. The more recent experimental studies performed in the 21st century by Steen, Rosenbaum, Michel, and others on large animal model (mainly porcine and canine) of hypothermic ESHP at 0-8° C have suggested that in comparison to CSS, hypothermic ESHP provides superior myocardial function and attenuates tissue injury in minimally damaged, or DBD model [78, 100-103]. Similarly,

hypothermic ESHP studies in large mammal and human DCD heart by Van Caenegem, Rosenfeldt, and others suggest that functional and metabolic recovery of DCD heart is achievable with hypothermic ESHP, and the recovery is superior with hypothermic ESHP compared to CSS [104-106]. Additionally, the technical device required for the hypothermic perfusion with an oxygenated crystalloid solution is considered less complicated and expensive in comparison to a device to carry continuous warm perfusion [76]. The technology of hypothermic ESHP, has shown promising results and abilities including the successful first human heart transplant using Steen's perfusion system in Sweden [106], and the possibility of some viability and functional assessment during preservation time using methods such as diffusion tensor magnetic resonance imaging, myocardial pH tracing, or by placing an inflatable flexible balloon inside LV to measure its contractility [54, 105]. However, adoption of this technology has been minimal, possibly due to the growing concerns about diastolic dysfunction that may occur as a result of significant myocardial edema formation during hypothermic ESHP, and the infeasibility of a less invasive, reliable functional assessment with the more widely and universally used myocardial function parameters under hypothermic conditions [75, 95, 106]. Also, there have been concerns about the detrimental effects of exposing the organs from non-cold tolerant, non-hibernator mammals (including humans) to long episodes of cold as they lack the adaptive systems to sustain and redirect minimal essential energy supplies, and to return to the normothermic condition (arousal) in a safe, cohesive way [53].

Normothermic ESHP on the other hand, allows for metabolic and functional assessment of the heart in a semi-physiologic status during preservation time, while it is perfused with nutrient-rich, oxygenated, solutions [97, 107]. Our group alongside others have published

several reports on experimental normothermic ESHP in large mammals, in either a minimally damaged heart, or a DCD model. These studies, regardless of the different devices and different settings applied (including Langendorff perfusion versus perfusion in working mode, and various compositions of blood-based perfusates) generally suggest that normothermic ESHP provides a dynamic platform for an accurate metabolic and/or functional assessment of donated hearts, and potentially reconditioning of damaged, dysfunctional hearts through pharmacological interventions. Normothermic ESHP may be able to offer a superior myocardial preservation, resuscitation of dysfunctional, and DCD hearts, and extension of the safe preservation time for the donated heart by maintaining efficient aerobic metabolism and avoiding cold ischemia [94, 98, 108-111]. Thus, in this review although hypothermic machine perfusion has also been discussed in regard to different aspects of machine perfusion of donor heart preservation, we have focused mainly on normothermic machine perfusion of the donor hearts.

1.6.4.3. Reconditioning of sub-optimal hearts with ex-situ heart perfusion

During the last two decades, the possibility of expanding the donor pool by reconditioning of the sub-optimal, extended criteria donor hearts, and DCD hearts in particular has been a topic of debate and widely studied in both hypothermic and normothermic ESHP.

In a pig model of DCD heart, Van Caenegem et al. showed that four-hour preservation of DCD hearts with hypothermic ESHP led to lower lactate levels, together with better preservation of high energy phosphate levels and contractility compared to four-hour cold-stored DCD pig hearts [105]. In a canine model of DCD orthotopic heart transplantation, Brant et al. observed a significantly better post-transplantation systolic function and lower cardiac enzyme leak and rate of cell death occurring in the hypothermic ESHP in

comparison with cold-stored DCD hearts [112]. Similarly, Rosenfeldt et al. showed that hypothermic ESHP (8-12° C) led to the reestablishment of oxidative metabolism during perfusion, and significant post-transplantation recovery of myocardial function in DCD canine hearts witch had tolerated 30 minutes of warm ischemia [113]. Choong et al. reported that following a 30-minute period of warm ischemia, the canine hearts preserved with hypothermic ESHP, not only showed lower levels of lactate in the perfusate during preservation time, but compared to cold storage, hypothermic ESHP of DCD hearts was associated with a superior post-preservation cardiac functional state (assessed in a working heart rig) [76]. Although these studies provide robust evidence for the possibility of resuscitating sub-optimal, routinely discarded extended criteria donor hearts, they all meet at the same limitation, which is the lack of opportunity to assess metabolism and functional state of the heart in a semi-physiologic condition. In these studies in general, the function and viability of the heart has been assessed mainly after completion of hypothermic ESHP either by performing an experimental orthotopic transplantation, on a working mode perfusion system, or with other non-routine methods [54, 76, 112]. Such assessments may not be adaptable to individual hearts in the clinic, cost much more and expose the heart to frequent episodes of ischemia and manipulation. Moreover, it may not be possible to assess the function of the machine-perfused donor heart using universally applied assessment approaches.

Normothermic ESHP provides a dynamic bridging period between the organ procurement and transplantation which offers the opportunity to deliver cardiac-reconditioning agents/therapies, and also to evaluate the effects of therapeutic interventions delivered through the initial reperfusion/cardioplegic solution. Several studies have been utilizing

normothermic ESHP as a platform for experimental reconditioning of sub-optimal hearts. The main focus of these studies has been rehabilitating those hearts with extended ischemic time, including DCD hearts. Considering the time the heart spends on the normothermic ESHP apparatus in a metabolically dynamic condition, by using controlled perfusion modalities (including flow and pressure), perfusate composition and/or pharmaceutical interventions, the IRI that traditionally would manifest after transplantation may potentially be attenuated [75, 114]. Lucchinetti et al. reported that post-conditioning of porcine DCD hearts with intralipid during initial normothermic ESHP, may reduce cell membrane damage, and induce cardioprotective pathways, and may improve contractile function [115]. We have also demonstrated that a moderately acidic, hypocalcemic initial reperfusion leads to less edema formation and improved recovery of cardiac function during normothermic ESHP in a porcine DCD heart model [116]. Iyer et al. reported that the ischemic tolerance of porcine DCD hearts could be enhanced with the addition of erythropoietin, glyceryl trinitrate, and Na+/H+ exchanger inhibitor (Zoniporide) to the cardioplegic solution, leading to a better functional recovery observed during normothermic ESHP [87]. Our group has also reported that the addition of pyruvate to the normothermic ESHP perfusate leads to rapid recovery of function in a porcine model of minimally damaged donor heart [107]. The antemortem cardioprotective interventions that would not directly benefit the donor are either discouraged, or admitted in a very limited range by the ethic boards [117] , thus the dynamic platform of normothermic ESHP may provide the opportunity for post-conditioning of sub-optimal and/or damaged hearts and to evaluate the efficacy of such interventions before transplantation. Due to the near physiologic conditions that can be achieved in normothermic ESHP, functional and

metabolic viability of the heart can be assessed elaborately with minimally invasive methods and widely used parameters [94].

1.6.4.4. *Ex-situ* perfusion of donor heart, setting and perfusion parameters

The setting for hypothermic ESHP of the heart is generally considered to be of low complexity due to low metabolic and coronary perfusion pressure requirements in a hypothermic condition [76]. While hypothermic perfusion must take place in non-working mode, there have been different methods described in literature for hypothermic ESHP of heart including applying different devices (e.g. pump-perfused or gravity-feed), coronary perfusion with or without submerging the heart in perfusate, and varying temperatures (4-33°C), perfusion pressures (3-15 mmHg), flow rates (4- 250 mL/100g/min), and partial oxygen pressures (150-600 mmHg) [76, 105, 113, 118, 119]. Normothermic ESHP systems on the other side, needs a more sophisticated setting to be able to provide the semi-physiologic conditions for the beating heart. Experimentally, normothermic ESHP of heart may take place in non-working mode and/or working mode in a single-mode or multi-mode operating apparatus. These devices may be consisted of either one pump, or two pumps (for the active, controlled perfusion of the aorta and left heart). Similar to experimental hypothermic ESHP, different perfusion parameters have been applied during experimental normothermic ESHP, with the target normothermic aortic perfusion pressure being mainly 40-80 mmHg [87, 94, 107, 120-122]. In clinical normothermic ESHP of heart with Organ Care System device (OCS, the only commercially available device for clinical normothermic ESHP) coronary flow is adjusted to 650-850 mL/min, and aortic perfusion pressure of 65-90 mmHg. Currently, OCS can only support non-working (Langendorff) perfusion [97, 123].

1.6.4.5. Perfusate composition in *ex-situ* perfusion of donor heart

The perfusate is an important element in ESHP and has a direct effect on the myocardial function. Whole blood from the donor is originally believed to be the optimal perfusate. In the setting of hypothermic ESHP, early attempts to apply whole blood were unsuccessful because of 1) increased viscosity even after hemodilution; 2) deep hypothermia-induced damage and deformity of red blood cells' (RBCs); 3) ceased dissociation of oxygen from oxyhemoglobin in temperatures below 12° C [124]. Replacing blood-based perfusates with synthetic crystalloid/colloid solutions lead to better results [101, 124].

In a setting of normothermic ESHP of pig hearts, White *et al.* reported that a whole blood-based perfusate leads to better preservation of myocardial function and less tissue damage when compared with either packed RBC–based perfusate, or a perfusate containing hemoglobin-based oxygen carrier. They concluded that plasma may carry nutritional substrates (such as free fatty acids, FFAs, and amino acids) and cardioprotective properties (such as vitamins and antioxidants) which alongside RBC may improve myocardial preservation in normothermic ESHP [111]. Church *et al.* in an experimental study in a porcine model, compared the effects of cross-circulated leukocyte-free blood, with cross-circulated plasma from another live pig on machine-perfused hearts. They reported no significant difference in myocardial function, lactate profile, and tissue injury between the two groups. They concluded that an efficient normothermic ESHP may be possible with a plasma-based perfusate [121].

Although the clinical normothermic ESHP with the TransMedics Organ Care System device (OCS) is based on perfusion of the heart with leukocyte-free blood of the donor, according to the findings of experimental studies on normothermic ESHP, and clinical

studies on other extracorporeal circulation systems the optimal perfusate is still a matter of debate. Clinical extracorporeal circulation systems (such as cardiopulmonary bypass) that are common in many artificial parts with ESHP devices (including oxygenator, filters, and tubes), cause hemolysis, and induce significant changes in the mechanical properties of RBCs such as decreasing their elasticity and surface charge, decreasing 2, 3 diphosphoglycerate concentrations that lead to increased hemoglobin-oxygen affinity and impaired tissue oxygenation [125, 126]. These conditions may also happen during normothermic ESHP, significantly impairing the efficiency of the perfusate. More studies are warranted to 1) identify the optimal composition of blood-based perfusate; 2) to design bio-friendly lining to reduce the deleterious effect of blood elements/cells coming in contact with artificial parts during normothermic ESHP.

Another important factor in maintaining the efficiency of the perfusate, and normothermic ESHP in general, is providing hormonal support for the heart during perfusion. Generally, insulin and catecholamines are utilized during normothermic ESHP for metabolic and inotropic support respectively [97, 98, 107]. These hormones are delivered at constant rates/doses during the perfusion, thus lacking the in-vivo real-time, demand-based regulation, and the interaction with other hormones which take place under a physiologic condition. A better understanding of hormonal requirements of the DBD and DCD hearts during normothermic ESHP is warranted.

1.6.4.6. Clinical experience of normothermic machine perfusion of the donor heart

While not yet FDA approved, The (OCS) is the only commercial, portable device available for clinical normothermic ESHP which preserves the donated human heart in a beating, dynamic state via perfusion of coronary vasculature in the non-working mode. The OCS

device consists of a portable console equipped with a wireless monitor, a disposable perfusion module, a diaphragmatic pump able to produce pulsatile flow, and a plate heater to maintain normothermia. The priming solution is 1.2-1.5 L of leukocyte-depleted donor blood, insulin, methylprednisolone, multivitamins, and sodium bicarbonate. During the period of perfusion, the cardioprotective property of the perfusate is maintained by infusion of electrolytes, dextrose, adenosine, and catecholamine (epinephrine) [95, 97]. A few centers in the world have been performing research in clinical normothermic ESHP using the OCS device. After two non-randomized clinical trials (PROTECT I, and PROCEED I) by Ardehali *et al.* in the US and promising results of 30-day post-transplantation survival using normothermic ESHP, they pursued a prospective, randomized, multi-center trial comparing DBD hearts preserved with OCS, with hearts preserved with CSS. The preliminary results published in 2015, suggested non-inferiority of normothermic ESHP with the OCS, compared to CSS for the preservation of DBD hearts. There was not a significant difference in either of 30-day patient and graft survival (94% in OCS versus 97% in CSS), or cardiac-related serious adverse effects (13% in OCS versus 14% in CSS) between OCS and CSS. Despite significantly longer total "outside of body" preservation time in OCS group, the duration of cold ischemia was significantly shorter in this group compared to the CSS hearts [95]. Recently, Schroder *et al.* in their clinical trial (EXPAND), showed that OCS may be efficiently used to resuscitate and preserve extended criteria donor hearts, leading to excellent post-transplantation function and low primary graft dysfunction rates [127]. In the UK, Messer *et al.* compared the outcomes of transplantation of hearts obtained from DCD and DBD donors preserved with OCS, and in 2017 reported a comparable one-year survival (86% in DCD versus

88% in DBD, p=0.98), length of hospital stay (20 days in DCD versus 27 days in DBD, p=0.09), and number of treated rejection episodes in DCD and DBD hearts [123].

Garcia Saez *et al.* investigated the outcomes of transplantation of hearts preserved with OCS in a group of recipients who had been categorized as “high risk” due to either presence of left ventricular assist device and/or intraaortic balloon pump, or high pulmonary vascular resistance. In the patients who received the hearts that were recognized as transplantable according to the assessments during OCS, the survival rate was 96% in the follow-ups performed between 109-445 days after transplantation [109]. In Australia, Dhital *et al.* in a series of three cases were able to successfully resuscitate human DCD hearts of Maastricht category III (controlled DCD) with OCS and transplant them, with all the three recipients gaining normal cardiac function within a week [128].

1.6.4.7. Assessment of the viability of donated heart in OCS, strengths and weaknesses

Assessment of quality and viability of donated heart is a critical step in heart transplantation, directly affecting the outcomes [61]. Viability assessment is of particular importance in the attempt to include the extended-criteria DBD, or DCD hearts in the donor pool. In OCS, the assessment and prediction of post-transplant viability is based on metabolic findings, mainly lactate concentration during perfusion. The OCS device currently operates only in non-working mode, which prevents the functional assessment of the donor heart during normothermic ESHP [94, 123]. Arterial and venous gas status, lactate concentration, coronary blood flow, and aortic pressure can be monitored on this platform. Effective perfusion on the OCS is identified as venous lactate lower than the arterial lactate concentration during the run and a concentration of less that 5 mmol/L at

the completion of the perfusion [97]. Lactate concentration during normothermic ESHP has been suggested to be a reliable predictor of the viability of the donated heart. Hamed *et al.* evaluated the correlation of lactate levels during OCS perfusion of human donor hearts, with 30-day outcomes of transplantation and reported that the “end of perfusion” lactate concentration to be the most powerful predictor of post-transplantation graft failure (63% sensitivity and 93% specificity) [129]. This cut-off value of 5 mmol/L was recently challenged in the context of hearts donated after circulatory determination of death wherein in spite of a final lactate concentration greater than 5mmol/L, twelve DCD hearts were transplanted with 100% survival in the recipients (35). Ardehali *et al.* in PROCEED II randomized clinical trial reported that four of the initially acceptable donated hearts were eventually discarded due to the impaired total perfusate lactate profile during the perfusion on OCS. One of these hearts had a history of cardiac resuscitation and chest compressions, and later pathologic findings suggested a clinically significant cardiac contusion. The other three hearts belonged to donors with a history of substance abuse, congenital cardiac anomaly, or overlooked left ventricular hypertrophy [97]. Thus, they suggested that lactate concentration can be used as a tool in the setting of OCS to determine the hearts with a high risk of post-transplantation failure, and to prevent transplantation of non-optimal hearts. However, the possibility of perfusion with OCS causing myocardial damage, leading to impaired metabolic profile was also suggested [97]. Furthermore, Stamp *et al.* reported a case of extended perfusion (>8 hours) of an ideal human heart on OCS which despite normal lactate concentration during the run, developed significant edema and primary graft failure, and needed mechanical circulatory support post-transplantation [130]. These findings highlight the need for the functional,

alongside the metabolic assessment of the hearts perfused with OCS, to determine the eligibility of the heart for safe transplantation [98, 131].

Our group has reported that in the setting of experimental normothermic ESHP of pig hearts, while the lactate concentration and myocardial oxygen consumption (MVO_2) showed a weak correlation with myocardial performance (MVO_2: R^2= 0.28; Lactate: R^2= 0.02), functional parameters such as left ventricular stroke work (LVSW) and the minimum rate of pressure change (minimum dP/dt) demonstrated an excellent correlation with myocardial performance, and high sensitivity and specificity (LVSW: R^2= 0.76, sensitivity= 1.00, specificity= 0.77; dP/dt min: R^2= 0.74, sensitivity= 1.00) [94]. The opportunity for an elaborate, reliable functional assessment of the donated heart during normothermic ESHP will only be possible in working mode. We have previously introduced the device and related protocol developed in our lab for normothermic ESHP in a large mammal (pig) model, which is easily reproducible for different animal models and heart sizes, and provides the possibility of performing echocardiographic evaluation as well. The software program supporting this apparatus allows for real-time and automated control of the pump to maintain desired aortic and left atrial pressure and evaluates and record a variety of functional and electrophysiological parameters with minimal need for supervision or manipulation [98], suggesting that such modifications may be possible in the clinical devices for normothermic ESHP too. However, such an adaptation seems inevitable to get the full benefits of normothermic ESHP to evaluate biochemical, anatomical, and mechanical characteristics of the donated heart in a semi-physiologic working mode [95, 98].

On the other hand, despite the non-inferiority of OCS perfusion to CSS, and significantly shorter cold ischemic time with OCS in PROCEED II trial, OCS did not lead to an improvement in post-transplant early outcomes, suggesting that avoiding extra ischemia is not enough for optimal preservation of donated heart [132].

1.6.4.8. Normothermic *ex-situ* perfusion of the donor heart, cost versus effectiveness

When compared to CSS, which is simple and quite inexpensive, the expenditures of heart perfusion with OCS are significantly higher. While the reusable OCS heart console alone costs ~ $300,000, the disposable single-use perfusion kit costs ~ $50,000 [133]. In addition, OCS needs more technical support and training. The results of recent clinical trials on OCS does not support the cost-effectiveness of this method yet as they have not been able to prove its superiority over CSS, however, the expenditures of normothermic ESHP may be justified by the reduction in the lifetime costs of the graft through shortening of the waiting times (less need for mechanical circulation support devices and/or hospitalization), and better graft survival, which may be possible with normothermic ESHP [75, 114, 132]. Larger clinical trials with longer follow-ups and associated cost-effectiveness analysis are warranted.

1.6.4.9. Effects of semi-physiologic left ventricular load

The maintenance of semi-physiologic heart chamber pressures may exert cardioprotective effects during normothermic ESHP. We have shown that the myocardial decline occurring during ESHP of pig hearts, regardless of perfusion mode (non-working and working) but surprisingly, those hearts perfused in working mode (WM) had significantly better preservation of function compared to the hearts perfused in non-

working mode (NWM) [107, 111]. The mechanism for superior functional preservation of hearts perfused in WM has not yet been determined and is under investigation in our group. These findings suggest that normothermic ESHP in the more physiologic setting of a loaded left ventricle (WM) is not only essential for an elaborate assessment of the donated heart, but may also have cardioprotective effects.

1.6.4.10. Normothermic *ex-situ* heart perfusion, challenges

1.6.4.10.1 Preservation of function

As reported previously by our group and others, myocardial systolic and diastolic function decline during normothermic ESHP. Preservation of myocardial function and viability is an important element for maximizing the potential advantages of normothermic ESHP for the purpose of 1) expanding the donor pool, 2) enhancing safe preservation times for better evaluation, reconditioning, and allocation. Experimental studies have shown a significant decline in myocardial function during normothermic ESHP in either a setting of short perfusion in a rat model, or 6-hour and 12-hour perfusion in a pig model in the presence of acceptable lactate concentration range [107, 134, 135]. The observed functional decline did not appear proportionate to either rate of cell death happening during normothermic ESHP, or severity of myocardial injury observed histologically [58, 107]. An alternative explanation for the functional deterioration of the heart during ESHP may be inadequate energy substrate or inefficient energy-yielding metabolism. In general, the main source of energy during clinical or experimental normothermic ESHP has been exogenous glucose, as the free fatty acids (FFAs) tend to rapidly deplete during the perfusion [107]. However, Experimental ESHP performed by our group and others, in either pig or rat hearts have shown that catabolic, energy-producing metabolism of

glucose may not take place efficiently during ESHP regardless of temperature [107, 136]. Therefore, the functional decline during normothermic ESHP may be preventable and/or reversible. Therefore, the functional decline during ESHP may be preventable and/or reversible. We have shown that the myocardial function improved partially but significantly at 12 hours of normothermic ESHP of pig hearts by providing added energy substrate (pyruvate) [107]. As the heart has very high energy demands, altered energy-yielding metabolism during normothermic ESHP can cause functional decline. The subject of efficient energy substrate support for the heart in the setting of normothermic ESHP has been overlooked. More studies are warranted to identify the efficient energy substrate support for the heart during MP for the aim of optimal functional preservation.

1.6.4.10.2. Edema formation in myocardial tissue

Edema formation may also play role in the functional decline of the heart. Edema, although was reported to be a significant, limiting factor in hypothermic ESHP, has been also frequently reported in normothermic ESHP studies. Although edema formation has been significantly lower with blood-based perfusates, it still raises concerns as edema can impair diastolic function/relaxation, and may also lead to capillary collapse and impairment of myocardial perfusion during normothermic ESHP [95, 137]. Interestingly edema formation has been frequently reported, even with the perfusates holding adequate colloid properties (blood and/or colloid-added crystalloids) [107]. Induction of inflammatory responses triggered by exposure to the extracorporeal circuit may be an important underlying reason for edema formation during normothermic ESHP. Sandha et al. reported that in a pig model of normothermic ESHP, the addition of steroids *(*methylprednisolone) to the perfusate during perfusion significantly reduced the

concentration of pro-inflammatory cytokines, and myocardial edema, however, reduced edema was not accompanied by an improved myocardial functional state [134]. Experimental observations suggest that in the hearts perfused with blood-based perfusates, only approximately 10% of the functional decline is attributable to edema formation [95].

1.7. The extracorporeal perfusion-related effects and their implications on patient/organs

Extracorporeal life support (ECLS) systems are routinely utilized to either support the body circulation during cardiac surgery (cardiopulmonary bypass, CPB), or to support blood ventilation (extracorporeal membrane oxygenation, ECMO) in patients with critical heart (Venoarterial ECMO, VA ECMO) or lung conditions (Venovenous ECMO, VV ECMO). These systems are lifesaving, however, the extracorporeal circulation systems in general are associated with many alterations induced to cellular and non-cellular content of circulation, and exposed tissues which negatively affect patient outcomes [138, 139]. The apparatuses for *ex-situ* thoracic organ perfusion (ESTP), including ESHP apparatus, while different from ECLS apparatuses, share many aspects with those, particularly with CPB system. Thus, similar alteration occurring to the blood and organs during ECLS may occur during ESTP (e.g. ESHP) which may affect optimal preservation of the donor organs in these settings. The similarities and differences of ESHP with ECLS systems are summarized in Table 1.5 [98, 139-141].

1.7.1. Inflammation and oxidative stress in the setting of thoracic organ support-oriented extracorporeal circulation

The mechanical extracorporeal circulation systems (including CPB and ECMO) are associated with induction of systemic inflammatory response (SIRS) and systemic oxidative stress in exposed patients. Induction of these responses which are associated with adverse patient outcomes, has been a limiting challenge for these otherwise life-saving technologies [138, 139]. The alterations induced to various aspects of circulation/blood during extracorporeal circulation (ECC) play a central role in the induction of inflammatory and oxidative stress responses in this setting [138, 139, 142].

1.7.1.1. Inflammation and oxidative stress in cardiopulmonary bypass and extracorporeal membrane oxygenation

Although in general these phenomena has been observed in both experimental and clinical setting, the study designs and response patterns have been very variable between the studies. McILwain *et al.* reported a rapid rise in plasma levels of pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interleukin (IL) 8, in healthy neonatal pigs subjected to venoarterial ECMO for eight hours. [143]. Chen *et al.* showed that in a setting of 24 hours of ECMO in healthy pigs, the circulating concentrations of malondialdehyde (MDA, a marker of oxidative stress) increased, while the concentrations of superoxide dismutase (one of the key antioxidantive enzymes), glutathione (the mother antioxidant), and in general the total antioxidant capacity decreased only in two hours after starting ECMO [142]. Graulich *et al.* reported that perfusion of fresh human blood at normothermia in an ECMO circuit for 12 hours lead to activation of the leukocytes and increased plasma levels of neutrophil elastase, and expression of surface interleukin-18

in polymorphonuclear cells (PMN) and monocytes without a change in the number of the leukocytes [144]. These findings suggest that oxidative and systemic inflammatory responses are stimulated very early during ECMO. On the other hand, Chen *et al.* reported that six hours of ECMO treatment in a piglet model of acute respiratory failure was not associated with increased plasma pro-inflammatory cytokines in either VV or VA ECMO. However, IL-1β and IL-8 cytokines were significantly increased overtime in the bronchoalveolar (BAL) lavage of only VA ECMO group [145]. In an ovine model of smoke-induced acute lung injury placed on venovenous (VV) ECMO, Passmore *et al.* reported increased levels of circulating IL-6, 1-2 hours after starting of ECMO alongside increasing levels of active matrix metalloproteinase-2 (MMP-2) in BAL during ECMO in both smoke-injured, and healthy control lungs. Tissue infiltration of leukocytes, upregulation of MMPs, and circulating interleukin (IL)-6 decreased the pulmonary compliance in smoke-injured lungs compared to healthy control lungs placed on VV ECMO. They concluded that the pre-existing injuries exacerbate the inflammatory response induced by VV ECMO [146]. In an experimental model of freshly donated human blood circulated for 12 hours with neonatal ECMO *circuit,* plasma levels of TNF-α, IL-6 and IL-8 all showed a trend for increasing during the first 6 hours compared to baseline. However, compared to the baseline, only TNF-α and IL-8 were significantly higher after 12 hours of circulation [147].

Thangappan *et al.* in a retrospective study, reported the incidence of SIRS in 24% of the patients who had undergone ECMO due to cardiac or respiratory failure, without any evidence of infection despite extensive investigation [148]. Hirthler *et al.* reported increasing of plasma TNF-α, IL-1, and IL-2 during 36 hours of ECMO in infants, with only TNF-α showing a significant increase overtime during ECMO among the non-survivors,

suggesting that life -hreatening complications associated with ECMO (including cardiac complications) may be related to the activation of inflammatory responses [149]. In a mixed group of neonates undergoing ECMO due to respiratory or cardiorespiratory failure, Fortenberry *et al.* reported a significant increase in the circulating IL-8 which occurred in only 15 minutes after starting of ECMO, and stayed elevated for 24 hours. They also observed a significant activation of neutrophils (evaluated by assessment of plasma neutrophil surface adherence protein [cluster of differentiation (CD) 11b] which initiated in the first 15 minutes and persisted for 12 hours after starting of ECMO. However, the levels of TNF-α and IL-1β did not significantly increase during ECMO. In the same study, the radiographic lung injury scores deteriorated during ECMO suggesting a negative association between activation of neutrophils and inflammatory responses, and lung viability [150]. Vitkova *et al.* reported increased plasma values of IL-1B, IL-6, and IL-22 during application of ECMO in newborns [151]. Vallhonrat *et al.* reported a case of significant activation of the complement system (predominantly via the alternative pathway) one hour after the starting of the ECMO [152].

Similar to ECMO, the patterns of development of inflammation and oxidative stress have been variable in the studies involving CPB. Ohata *et al.* reported increased plasma values of plasma IL-6, IL-8 and PMN elastase during CPB [153]. Halter *et al.* also showed elevated plasma IL-6, IL-8, and IL-10 during application of CPB in elective cardiac surgeries [154]. Ito *et al.* reported a significant and immediate increase in IL-6 and IL-8 and granulocyte elastase after the starting of the CPB, with the values being higher in the group with the oxygenation index (OI) of <250 in the post-operation day-one compared to those with OI>250 suggesting that the level of the inflammatory molecules may be

negatively associated with respiratory function [155]. In a group of high-risk patients who undergo elective myocardial revascularization surgery using CPB, Stassano *et al.* reported a significant increase in plasma IL-1B, IL-6, and TNF-a during, and early post-operation [156]. Similarly, Chen *et al.* reported a significant increase in IL-8, MDA, and adhesion molecules involved in neutrophil-endothelial cell interactions (including intercellular adhesion molecule-1) both during, and after the termination of CPB in a group of patients undergoing elective cardiac surgery [157]. Liu *et al.* reported a significant increase in the levels of MDA, TNF-a, IL-6 as well as anti-inflammatory IL-10 in adult patients undergoing cardiac valve replacement surgery [158]. Clermont *et al.* using Electron Spin Resonance technology showed that the concentration of nitroxide spin adducts (more stabilized reactive species) considerably increases in the circulation after starting of CBP, and remains high during the period of CPB [159].

Chenowetch *et al.* reported a significant increase in the plasma levels of cleaved complement compartment-3 (C3a) occurring soon after the start of the CPB, which continued throughout the CPB, reaching to values five times higher than preoperative values, however the plasma C5a did not change during CPB [160]. In a group of adult patients undergoing CPB Steinberg *et al.* also reported a significant elevation in the plasma IL-6, and the products of complement compartment cleavage and activation C3a, C4a, and C5b-9 suggesting the involvement of inflammatory cytokines and the complement system in the CBP-adverse systemic effects [161].

On the other hand, there are also observations opposing early, fulminant activation of the inflammatory responses during CPB. Deng *et al.* in a low risk group of patients undergoing coronary artery bypass graft, reported that the venous and arterial plasma levels of IL-6

and TNF-a, while stayed within normal ranges during the surgery and CPB, significantly increased after the termination of the CPB, with the levels of IL-6 still remaining high at 24 hours after termination of CPB [162]. In a study by McBride *et al.* in a group of pediatric patients undergoing cardiac surgery, while levels of TNF-a and IL-1B did not change during, and after the termination of CPB in either of the plasma and BAL, IL-8 and anti-inflammatory IL-10 significantly increased in plasma and BAL during and after CPB [163]. Haeffner Cavaillon *et al.* showed that the monocyte production of IL-1B increases 24 hours after CPB, with no significant change in IL-1B during or early after the termination of CPB [164].

1.7.1.2. Inflammation and Oxidative stress in *ex-situ* thoracic organ perfusion

Adoption of *ex-situ* perfusion technology to clinical transplantation of thoracic organs has provided a dynamic method for preservation of donated organs in a more physiologically-relevant condition when compared to cold storage [165]. Moreover, it allows for functional assessment, and potentially improvement of the donor organ during preservation time. Although this technology has facilitated transplantation of the sub-optimal, extended-criteria hearts, the optimal *ex-situ* preservation of the myocardial function and viability has been a matter of debate as a result of the observed significant edema alongside declined functional status during extended *ex-situ* perfusion periods. This observation has limited the successful outcomes to shorter perfusion times, creating an obstacle for potential advantages of this preservation/ evaluation method [107, 141, 166]. The *ex-situ* organ perfusion systems share many aspects with ECLS systems, including the artificial material used in the circuit. In fact, the application of this technology itself is associated

with the induction of the pro-inflammatory mediators in both tissue, and perfusate [141, 167].

Ex-situ heart perfusion (ESHP) on the other hand, has been more challenging compared to *ex-situ* perfusion preservation of other organs because of its higher metabolic demands, and its sensitivity to different stresses it may endure before/during procurement and preservation (including ischemia-reperfusion injury, IRI). There is a significant gap in data on the incidence of inflammatory and oxidative stress responses during ESHP. The limited available data from studies performed by our team and others have mainly focused on mitigation of the inflammatory responses in the DCD, or cold-stored hearts, which have tolerated a significant warm or cold ischemic time respectively [87, 134, 168], and face severe degrees of IRI during ESHP which may mask the inflammation and oxidative stress related to extracorporeal circulation/device itself. In the recent experimental studies of ESHP by our team in a porcine model, we observed that perfusion of healthy hearts, which had not endured the insults related to brain death or circulatory death (such as a significant ischemic time), was also accompanied by severe induction of various inflammatory mediators (including interleukins), and oxidative stress. Activation of these responses may play a key role in functional decline of the hearts during ESHP [141, 169].

1.7.2. The pathophysiology of inflammatory and oxidative stress responses during extracorporeal circulation and perfusion

The CPB, ECMO, and ESHP systems are different in many ways, but they share a very important aspect which is the artificial material used in building up the circuit, and they all expose the organs to various types of non-physiological conditions which may have negative effects on organ's function [138, 139, 170]. Soon after contacting of the patient's

blood (or the blood-based perfusate) the synthetic extracorporeal circuit, the inflammatory and oxidative stress responses are activated as a result of the various alterations induced to both cellular and humoral elements of the blood/perfusate. In the next sections the pathophysiology of these phenomena are being discussed and are summarized in Figure 1.2.

1.7.2.1 Complement and Contact activation system

Activation of the contact system is central to the interaction between inflammation and coagulation. Contact system includes serine proteases factor XII (FXII), prekallikrein, coagulation factor XI (FXI), and high molecular weight kininogen (HMWK) [171]. According to the “Vroman effect”, within minutes after the contact between blood and artificial material (biomaterial) of the circuit, the sequential absorption of plasma proteins starts with fibrinogen, which then acts as a surface for other proteins of plasma to bind to the biomaterial, including but not limited to the contact activation pathway molecules, albumin, and complement component-3 (C-3). With the formation of the protein layer on the biomaterial surface platelets and PMNs will interact with each other, leading to releasing of different pro-inflammatory cytokines. Activation of the complement system will boost these interactions [172]. Activation of the contact system leads to cleavage of factor XII to XIIa and XIIf (that will induce the intrinsic coagulation pathway). Factor XIIa converts prekallikrein into active kallikrein and HMWK into bradykinin. Increased concentrations of kallikrein and bradykinin activate neutrophils and stimulate the release of cytokines and nitric oxide, promoting inflammation [139, 173]. In a sequential order, the intrinsic pathway of coagulation is induced via factor XIIa converting factor XI to XIa, which activates factor IX to IXa. Then IXa turns factor X to its active form Xa. Being the common

factor between intrinsic and extrinsic coagulation pathways, activated factor X (Xa) converts prothrombin to thrombin. Thrombin then cleaves fibrinogen to fibrin, which leads to the formation of clot. Thrombin is formed during extracorporeal circulation, despite administration of heparin, and is a link between coagulation and inflammation since it activates endothelial cells and induces production of reactive oxygen and nitrogen species (RONS), selectins (on platelets and endothelial cells) and platelet-activating factor, activates neutrophils, induces production and release of pro-inflammatory cytokines, and promotes platelet activation [139, 174, 175].

Of the three initiating pathways of complement activation, while the classical pathway (CP) and lectin pathway (LP) are induced by antigen-antibody complex formation, alternative pathway (AP) can be directly induced through contacting with the biomaterial of the circuit. Activation of these pathways is followed by activation of the common pathway and activation of the C-3 that leads to the formation of the membrane attack complex (MAC). Activation of the complement system will significantly trigger inflammatory responses and increase capillary permeability and alter the vascular tone. Circulating products of the complement system activation and cleavage including anaphylatoxins (C3a, C4a, and C5a) activate platelets and endothelial cells and trigger release of Von Willebrand factor (VWF). The AP seems to be the main pathway activated during or after CPB as a result of the contact of the blood with biomaterial of the circuit and hydrolysis of C3 to C3a and C3b (activation of the common complement pathway). However, the CP can also be activated during CPB due to the activation of the complement protein C1 by factor XIIa of the activated contact system, protamine-heparin complex formation after administration of protamine at the end of CPB (to prevent

bleeding), and endotoxin released from the intestine during ischemia and reperfusion [172, 176]. Compared to CPB, there is a significant gap in the scientific literature about activation of pathways of the complement system during ECMO and its effects on patients' outcome. Most of the existing literature is from the 1990s during which ECMO was applied with much older technology, including older versions of pumps and oxygenators. In the few existing, more recent studies on ECMO, a fairly rapid elevation in the concentrations of the complement system element has been reported [152, 177].

To our best knowledge, although the ESHP technology has been utilized to study the effects of the complement system activation and inhibition in the pathophysiology and outcomes of myocardial IRI, there is not any scientific report on the activation complement during ESHP. Studies are warranted to assess activation of the complement system during ESHP, and the possible effects of it on the preservation of the organs' viability and function in this setting.

1.7.2.2. Endothelium and blood cells

1.7.2.2.1. Platelets

Platelet activation has a major role not only in coagulative responses induced during extracorporeal circulation but also in the systemic inflammatory responses in this setting. Gemmell *et al.* in the beginning of the 21st century showed that the contact of whole blood with biosynthetic surfaces massively activated platelets in the blood even with using anticoagulant [178]. The limited amount of thrombin production triggered by tissue factor released from activated/injured endothelial cells, will promote activation of platelets through the protease-activated receptors (PAR) on the platelet surface. The massive production of thrombin by thrombin-activated platelets can further induce inflammatory

and oxidative stress responses [172]. In addition to thrombin (as the main platelet-activating factor), complement activation (through CP), and physical characteristics of the circuit also contribute to the activation of platelets [139, 179]. Platelet activation has a major role not only in coagulation responses induced during extracorporeal circulation, but also in the systemic inflammatory responses in this setting [140]. A strong body of evidence suggests that adhesion of the activated platelets to the endothelial layer, serves as an attachment spot for the neutrophils, thus facilitating their migration. The binding of the platelet to the endothelium may take place through the interaction of CD40 on endothelial cell with CD40 ligand (CD40L), expressed on the activated platelets. On the other hand, CD40L can also induce production and release of chemokines and adhesion molecules in the endothelial cells. Secretion of IL-8 and monocyte chemoattractant molecule-1 (MCP-1) from the activated platelets lead to chemoattraction of neutrophils and monocytes respectively, and them attaching to the activated platelets. It has been also shown that the number of circulating activated platelets bound to PMNs and monocyte increase during CPB, which may trigger the pro-inflammatory effect of these immune cells [180, 181]. The activated platelets also experience alterations in their shape which leads to platelets to release the content of their granules including various chemokines, pro-inflammatory cytokines, and adhesion molecules into the circulation, which can further promote the inflammatory and oxidative stress responses to extracorporeal circulation [139]. While in general the existing studies about the specific role of platelet activation and dysfunction on the induction of the inflammatory responses during extracorporeal circulation in very limited, to our best knowledge there is not any

available clinical or experimental data on the effect of platelet activation and its role in systemic inflammation in the context of ESTP including ESHP.

1.7.2.2.2. Red blood cells and hemolysis

ECLS involve transfusion of significant amounts of packed red blood cells (pRBC). Transfusion of blood products is associated with increased morbidity and mortality in critically ill patients. This may be associated by the oxidative stress that is induced by the transfusion of pRBC, particularly aged pRBC that may contain low levels of Selenium (Se), necessary for the antioxidative defence of the cell [174]. During ECC, hemolysis occurs inevitably due to the blood passing through the different artificial compartment of the circuit including the oxygenator, pump, and the reservoir at varying speeds [182]. It has been reported that hemolysis increases during CPB in a time-related fashion, and the concentration of plasma lactate dehydrogenase (LDH) as a marker of hemolysis, has a positive correlation with inflammatory mediators induced in CPB [183]. Lysed RBCs will release cell- free hemoglobin, haem and iron. Being potent damage-associated molecular patterns (DAMPs), the cell-free hemoglobin and heme will induce/exacerbate the inflammatory responses and oxidative stress through affecting different systems and cells (including innate immunity, complement system, and endothelial cells) and may cause damage to the kidney, cardiovascular, pulmonary, and gastrointestinal dysfunction. Moreover, the Fenton reaction, converting haem- iron from a ferrous to ferric state generating highly active hydroxyl radicals (OH^-), exacerbates the situation with further promoting oxidative stress-related modifications including lipid peroxidation and protein alterations. These modifications will alter the cellular membrane polarity and permeability, making the cells more susceptible to lysis, thus exacerbate hemolysis [174, 184, 185].

Different organs and systems in the body actively participate in scavenging the pro-oxidant products of hemolysis. These systems include the liver and spleen that uptake and metabolize the hemopexin-heme complex, monocytes and tissue macrophages that uptake hemoglobin-haptoglobin complex, and various antioxidative enzymes (e.g. haem oxygenase-1, HO-1). Although these systems compensate hemolysis-related pathophysiologic conditions at least partially, when the hemolytic insult is sever, the defensive systems of body/cell may become exhausted or diminished, and not capable of preventing pathologic conditions related to hemolysis [184, 186]. Potentially, *ex-situ*-perfused organs may undergo more severe hemolysis-related redox and inflammatory alterations due to the absence of the organs involved in scavenging hemolysis products, and possible depletion or decreased activity of antioxidants and antioxidative enzymes in the perfusate particularly in longer *ex-situ* perfusion times. In the setting of *ex-situ* thoracic organ perfusion there has been an effort going on to replace blood-based perfusate, with optimized acellular ones, with one of the important objectives to be bypassing hemolysis and related problems [187-189]. However, there is a considerable lack of data on the severity of hemolysis occurring in the setting of *ex-situ* thoracic organ perfusion and its possible effects on preservation of the viability of the perfused organs. To our best knowledge there is only one study by Kappler *et al.* on four-hour normothermic ESHP in a porcine model in which the free hemoglobin in the perfusate increased throughout the perfusion by approximately 0.02 mmol/L per hour [190]. Moreover, it has been reported recently that RBCs are active players in cytokine signalling and produce numerous inflammatory mediators including cytokines (e.g. IL-1B and TNF-a), chemokines, and growth factors. These factors release into the circulation due to hemolysis, or secretion

from intact cells [191]. Thus more studies are warranted to identify and target different roles of RBCs in triggering inflammatory responses in ECC, and more importantly in the setting of *ex-situ* thoracic organ perfusion (e.g. ESHP).

1.7.2.2.3. Leukocytes

Similar to the other pathologic inflammatory conditions, leukocytes are considered as the main players in the inflammatory and oxidative stress responses induced during ECLS and activation of leukocytes, mainly neutrophils is a major contributor to the organ damage in this setting. During circulation of blood in the extracorporeal circuit, continuous leukocytes activation has been witnessed by the significant increase in expression of the different markers of leukocyte activation such as CD18, CD11b, and CD62L. Thus, preventing them from coming in contact with the artificial circuit by filtering them or inhibiting their activation may attenuate the inflammatory responses and oxidative stress related to ECC [192-194]. During ECLS, neutrophils are activated mainly as a result of the activation of the complement system but activation of contact system, thrombin, histamine, heparin, cytokines, neutrophil-activating peptide 2 released from activated platelets, and interactions between platelets and neutrophils via CD40-CD40L and Mac-1 (CD11b/CD18) also participate. Activated neutrophils release the content of their granules with potent inflammatory, oxidative, and cytotoxic effect including lysozymes, myeloperoxidase, elastase, hydrogen peroxide, and RONS [150, 170, 176, 180]. Experimental studies have suggested that activation of neutrophils occurs very early during ECLS, peaks in a few hours, and then declines after that. It seems that hemodilution that occurs in CPB may lead to a more severe neutrophil activation [140]. Although there is not any scientific report about the activation of neutrophil or in general

leukocyte as a direct result of the extracorporeal thoracic organ perfusion, in clinical ESHP, leukocyte-depleted blood is utilized for the perfusate to avoid or minimize activation of the immune system [97]. In many of the experimental settings for extracorporeal thoracic organ perfusion, the perfusate in either acellular, or the blood is mixed with different buffers to achieve the final volume of the perfusate, which leads to hemodilution that can potentially increase neutrophil activation [60, 111, 140, 195]. On the other hand, in ESTP, high dose heparin administration to the perfusate (that is not followed with protamine administration at the end of perfusion) may have inhibiting effects on neutrophil activation [196]. More studies are warranted for characterization of neutrophil activation and related effects on the preservation of the organ and outcomes of transplantation in ESTP.

During application of ECLS, the increasing number of circulating monocytes alongside the downregulation of the monocyte surface adhesion molecules, and loss of myeloperoxidase (MPO) suggest a significant activation of monocytes (although occurring slower than neutrophil activation), accompanied with production of pro-inflammatory cytokines TNF-a, IL-1B, IL-6, IL-8, and prostaglandin [197, 198]. During ECC, the complement system is the major factor in the activation of monocytes directly, and also through promoting the formation of platelet-monocyte conjugates that trigger inflammation as well. Additionally, monocytes also express receptors for thrombin, and thrombin boosts the inflammatory activity of monocytes. Thrombin can also trigger activation of monocytes indirectly, through bonding of the platelets activated by thrombin to monocytes [199, 200]. The circulating monocytes are divided into different sub-population (classical, intermediate, and non-classical) according to markers expressed

on the cell surface. Evidence suggest that while the non-classical monocytes may play role in induction of oxidative stress in vascular system and endothelial dysfunction, intermediate monocytes may be involved in the induction of systemic inflammatory responses related to ECC [201]. Paradoxically, some monocytes that are also induced during ECC, express hemoglobin scavenger receptor (CD163). This group of monocytes exert anti-inflammatory effects by removal of the potent pro-oxidant hemoglobin-haptoglobin complexes formed due to hemolysis [202].

ECC is also associated with alterations in lymphocytic sub-population and activity. It is a common belief that the number of different types of lymphocytes including T-cells, B-cells, helper T-cells, and natural killer cells together with their immunity-inducing potential decrease during application of CPB. However, there are some controversies. While some studies have reported no change in either number of lymphocytes, or their activation during application of ECLS [144, 176, 203], some others report evidence for the number of absolute T-cells, natural killer cells, and suppressor T-cells increasing during ECLS, and then reducing to lower than normal values in only one day after terminating the application [204].

1.7.2.2.4. Endothelial cells:

The endothelium is an active player in different physiologic functions including controlling vascular tone, hemostasis, vascular permeability, and immune system responses. Conditions related to inflammation, activation of coagulation and complement system, production of pro-inflammatory cytokines and RONS, such as the events in ECLS lead to stimulation/activation of the endothelial cells similar to SIRS [139, 151]. Vascular endothelial irritation and damage not only leads to the production of cytokines and RONS

by endothelial cells, may lead to permeability edema and impairment of the oxygen exchange causing multiple organ dysfunction and failure [139, 140].

Activation of the endothelium has been reported in the clinical and experimental ECLS, observed with a significant change in the plasma markers of endothelium such as P-selectin, E-selectin, and VWF, which are preformed and stored in the granules of the endothelial cells (as well as platelets), and are released into the circulation during application of ECC [205].

Stimulated by many of the factors released in the circulation during ECLS including anaphylatoxins, thrombin, and pro-inflammatory cytokines (most potently TNF-a and IL-1B), the expression of P-selectin and E-selectin on the surface of the endothelial cells, and L-selectin of the neutrophils increase, mediating the low-affinity, reversible rolling of the neutrophils along the vascular endothelium. The CD 11b/CD 18 (Mac-1) integrin expressed highly in activated neutrophils during ECC interacts with endothelial intercellular adhesion molecule-1 (ICAM-1), ICAM-2, and platelet endothelial cell adhesion molecule-1 to form a firm binding between neutrophil and endothelial cell. These interactions between the endothelial cells and leukocytes mediate transmigration of the leukocytes into the extravascular compartment that exacerbates the inflammatory responses related to ECC [144, 176]. On the other hand, with the adhesion of neutrophils to the endothelial cell, the elastase is introduced into the endothelial cell. The neutrophil elastase converts xanthine dehydrogenase enzyme into xanthine oxidase (XO), and the reaction of XO with xanthine generates superoxide anion that in turn reduces intracellular, transferrin-bound Fe^{3+} to unstable Fe^{2+}. It is believed that this process is at least partially compensated for with the activity of the nitric oxide synthase (NOS), producing nitric oxide

(NO) that can decrease the adhesion between neutrophil and endothelial cell, and scavenge superoxide to peroxynitrite anion. However, in high concentrations or longer exposure times, peroxynitrite may directly nitrate the tyrosine residues of enzymes, inhibiting their enzymatic functions, negatively affecting the function of the exposed organs [206, 207]. Moreover, the endothelial cells can also be stimulated directly by the circulating pro-inflammatory cytokines, leading to a pathological increase in vascular permeability that may cause tissue edema, and endothelial cell production of cyclooxygenase [206].

The technology of ESHP has been widely utilized to study the endothelial health and reactivity in the presence of different stimuli (e.g. IRI), and also to assess the effect of different agents and/or strategies to protect or treat the coronary endothelial layer. Interestingly, there has been a significant lack of information about the effects of the ECC itself on the coronary endothelial cells during ESHP. In a recent study by our team, we reported that during 12-hour ESHP of healthy porcine hearts, the cardiac function and vascular tone (measured by increased coronary flow) decreased overtime. In the same hearts the soluble ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) significantly increased in the perfusate alongside different pro-inflammatory cytokines. The hearts also developed significant edema during ESHP suggesting that ECC induces inflammation, and activation and damage of the coronary endothelial cells while the function of the heart gradually declines during ESHP [141]. Exploring the interaction of the inflammatory responses induced during ESHP with endothelial activation and damage, and related effects on the quality of the perfusion-preserved donor organs (e.g. tissue edema), and on the outcomes of transplantation is highly warranted.

1.7.2.3. Hyperoxia

Hyperoxia is considered as one of the main reasons behind the generation of RONS and oxidative stress during ECLS [174]. During application of CPB, hyperoxia occurs routinely by the iatrogenic high amounts of oxygen and supra-physiological partial pressure of oxygen (PaO_2) in an effort to prevent hypoxia. The hyperoxia may not only be non-beneficial in solving the possible oxygen delivery problems in this setting, it can cause oxidative stress, that triggers/exacerbates inflammatory responses induced during ECC [208].

This phenomenon can be more severe in patients with pre-existing hypoxemia due to different conditions, which have been placed under ECLS. Caputo *et al.* reported that controlled reoxygenation early during application of CPB in pediatric patients with cyanotic diseases undergoing open heart surgery (normoxic PaO_2: 50 mmHg versus hyperoxic PaO_2: 150-180 mmHg) can attenuate oxidative stress and tissue damage [209]. The same group has also shown that in single-ventricle patients undergoing cardiac surgery with CPB, hyperoxic condition (PaO_2: 150-200 mmHg) when compared with normoxic, was associated with significantly lower plasma IL-6, IL-8, cTn-I, 8-isoprostane (a marker of oxidative stress), and protein S100 (a damage-associated molecular pattern). Although in double-ventricle patients, they were not able to detect any difference in the plasma pro-inflammatory cytokines between two oxygenation policies, the normoxic CPB group showed lower 8-isoprostane and protein S100. Their results suggested that normoxic CPB has protective effects against the incidence of inflammation and oxidative stress, particularly in single-ventricle patients who suffer more severe degrees of cyanosis before the surgical correction [210].

During ECMO, hyperoxia is more of a prominent condition in VV-ECMO where the hyperoxic blood returns to the venous system, directly perfusing the damaged pulmonary vessels [138, 211]. In an experimental study of ECMO in a rat model, Fujii *et al.* reported that $PaO_2 \geq 300$ mmHg compared to PaO_2 :100-299 mmHg during 120 minutes of ECMO was associated with significantly higher plasma TNF-a and IL-6, lower anti-inflammatory IL-10, and higher edema formation in the lungs (while edema was negligible in low PaO_2 groups) [212]. Also, Trittenwein *et al.* detected a significant positive correlation between the pulmonary tissue MDA and PaO_2 during experimental ECMO in a rabbit model [211]. Retrospective evaluation of patients placed on VA-ECMO post-cardiac surgery by Sznycer-Taub *et al.* suggested that Pao2 >193 mmHg during the firs 48 hour of ECMO may be associated with higher risk of 30-day post-operative mortality and some adverse outcomes such as the need for renal dialysis [213]. Similarly, Cashen *et al.* has also suggested that hyperoxia ($PaO_2 \geq 200$ mmHg) during the first 48 hours of ECMO application is associated with a higher mortality [214]. Hyperoxia potentially may occur where oxygen is administered in percentages higher than room air (Fraction of inspired oxygen, FIO_2>21%). Taking into account that a considerable number of the patient are hypoxic/hypoxemic condition (e.g. acute respiratory distress syndrome) before application of ECMO, the negative effect of hyperoxia during ECMO may be even worse [215]. However, values to be considered as hyperoxia have not been well defined yet as different authors have defined it in a wide range, with $100 > PaO_2 \geq 485$ mmHg [58, 216]. A better definition of hyperoxia during ECLS seems necessary for better protection of patients application of ECLS.

The optimal PaO_2 in ESHP is even more uncertain. During the 20th century's ESHP experiments performed in the more physiologically-relevant working mode, typically a bloodless Krebs-Henseleit (KH) buffer was utilized for perfusate. Such a buffer would maintain a pH of 7.40 using 95% oxygen flow that produced a $PaO_2 \geq 450$ mmHg that is much higher than physiological values (PaO_2:75-100 mmHg). Later it was observes that the addition of RBC to KH to reach a 35% hematocrit can improve the oxygen delivery of the buffer to the heart with much closer to physiological values of PaO_2 [216]. More recently, in the experimental studies using an RBC-contained perfusate (either whole blood or washed RBCs), the PaO_2 is maintained in lower values, not exceeding 300-400 mmHg, and mainly in the range of $100 \leq PaO_2 < 200$ mmHg, which is able to provide and maintain a physiological oxygen saturation [58, 98, 111]

1.7.2.4. Ischemia reperfusion injury

The pathophysiological effects of IRI (tissue and systemic) start with ischemia and depletion of the cell energy, and paradoxically worsen with reperfusion which involves the interaction of inflammation, oxidative stress, cellular sub-structural alteration and damage, and cell death. During ischemia, due to the hypoxic condition in the cell and uncoupling of the oxidative phosphorylation and glycolysis, the adenosine triphosphate (ATP)-consuming cellular pumps stop, and the pH drops. Upon reperfusion, abrupt reoxygenation and pH correction leads to accumulation of Ca^{2+} in the cells and generation of RONS through various pathways together with activation of inflammatory responses. Activation of the inflammatory responses including activation and transmigration of neutrophils into the tissues, and production of pro-inflammatory cytokines will boost massive production of the RONS, exacerbating oxidative stress and inflammation [217,

218]. With cross-clamping the aorta and application of cardioplegic solution during cardiac surgery involving CPB, the heart undergoes global ischemia. The lungs also partially get ischemic as a result of the deletion of the flow that the lungs receive through the bronchial arteries. The peripheral organs also subject to global hypoperfusion. When the coss-clamp is removed, the abrupt reperfusion of the heart and lungs lead to IRI, which has a negative effect on the outcomes [176, 219]. The application of ECMO on the other side, is not directly associated with IRI. Yet, due to the indications of ECMO (respiratory and/or cardiac failure), the organs have been already subjected to different degrees of ischemia/hypoxia and hypoperfusion, thus the hyperoxic perfusion via ECMO leads to a massive production of RONS and exacerbation of oxidative stress and inflammatory responses [174]. Although during ECLS, IRI contributes to the ECLS-related inflammation and oxidative stress, the electron spin resonance spectroscopy evaluation has suggested that production of the reactive oxygen occurs throughout the application of ECLS, going beyond the RONS production in the context of IRI [159].

In ESHP, when compared to the standard donor organ preservation method (cold storage), the duration of cold ischemia is considerably shorter. However, ischemia still occurs during the procurement of the organ, and mounting of the organ on the *ex-situ* perfusion apparatus [98]. Moreover, in the case of DCD, the longer warm ischemic times are expected, due to the legal “no-touch” period that should be followed to confirm the irreversible circulatory death prior to procurement. Thus, the *ex-situ*-perfused hearts are naturally affected by IRI during early perfusion. In fact, one of the rationales of ESHP technology as a preservation method has been evaluating the IRI-related damages to the potential donor heart, and addressing it in an *ex-situ* setting. This is of particular

importance in the donor hearts with longer ischemic times (e.g. DCD hearts) with predicted severe IRI [75, 220]. Thus, IRI in the setting of ESTP is a contributor to ROS during early perfusion, and may also further trigger inflammatory responses and tissue damage.

1.7.2.5. Absorption of antioxidants/decreased antioxidative potential

Growing evidence suggest that the bioavailability of micronutrient and trace elements necessary for the function of the antioxidative enzymes such as copper, zinc, selenium, manganese, as well as may non-enzymatic antioxidants such as uric acid, vitamins C and E decrease during ECC as a result of circuit biomaterial and compartments depositing and sequestering these elements [138,174].

1.8. Targeting inflammation and oxidative stress

1.8.1 Circuit optimization

With the elucidation of the role of the artificial surfaces, air-blood contact, and shear stress in induction of inflammation and oxidative stress in ECLS, the attentions were raised to 1) detect the highly involved components of the circuit in the induction of this phenomenon, and 2) to improve the general aspects of the circuits used during ECLS to prevent/minimize it.

Targeting of the contact system in the setting of ECLS may decrease inflammation and oxidative stress. For this purpose, the main attention has been toward modification of the circuit surfaces. The surface modification of the circuit may include the application of biomimetic surfaces (e.g. heparin, and direct thrombin inhibitors); or biopassive surfaces (phosphorylcholine, albumin, and poly- 2-methoxyethylacrylate-coated circuits). The earlier attempts using molecules such as albumin and silicone did not show any beneficial

effect. However, both clinical and experimental studies have advocated that the currently commercially available heparin-coated circuits, and third-generation heparin-polymer coated ECLS circuits improve the biocompatibility and may be associated with decreased production of thrombin and pro-inflammatory cytokines, reduced activation of leukocytes, complement, and platelets, and improved clinical outcomes compared to standard circuits, despite the higher costs [221-223].

There is a lack of scientific data on the effect of different surface modifications in the setting of ESHP. Part of this lack of data is related to the fact that the circuit used in ESHP shares the same components and artificial material with the ECLS circuit, thus similar reactions to surface modifications are predicted for ESTP. Also, the organs are typically being perfused for a few hours in the clinical setting. However, in general, the size of circuit compared to the exposed organs is considerably higher [138] in the setting of isolated organ perfusion (including ESHP) compared to ECLS systems (including CPB). This situation may also exacerbate the inflammatory and oxidative stress responses. Also, the home-made circuits used in experimental studies on ESTP, particularly in the small animal models, may not follow clinically relevant reactions to different surface modification methods and material. Studies using clinically relevant platforms and circuits seem inevitable for evaluation of the biocompatibility in ESTP setting in terms of reducing inflammation and oxidative stress.

The improved knowledge on the effects of artificial, non-endothelialized surfaces in the induction of inflammation and oxidative stress during ECC, lead to the development of minimized extracorporeal circuits (MECC) with lower surface area compared to conventional circuits. These circuits are also fully “closed”, even as a CPB circuit,

preventing air-blood interface. Application of ECC with mini-circuit show lower RBC damage and systemic inflammation and oxidative stress, although they may not be suitable for highly complicated procedures/surgeries [138, 170, 224]. Regardless of the size of the circuit, there is limited data about the contribution of each circuit components in induction of the inflammatory and oxidative yet it has been shown that using smaller sized oxygenators and connectors, application of centrifugal pumps, and additional in-line filters are associated with higher hemolysis that can induce/exacerbate oxidative stress and inflammation [174, 225].

1.8.2. Leukocyte filtration

Activated leukocytes, neutrophils and monocytes in particular play a crucial role in the development of oxidative stress and inflammation during ECC, thus eliminating this phenomenon has been believed to bear a potential in decreasing inflammatory and oxidative stress responses in this setting. In this regard, leukocyte-depleting filters have been used in surgeries requiring CPB. On the other hand, these filters can be placed in either 1) arterial line, or 2) venous line. The leukocyte depletion with continuous arterial line filters incorporated into the circuit has shown mixed results in terms of the pro-inflammatory cytokine, activation of the neutrophils and complement system, induction of biomarkers of oxidative stress, and organ dysfunction during and after CPB. It has been also suggested that placing the leukocyte filter in the venous line can also successfully deplete leukocytes and decrease the inflammatory responses as well. Moreover leukocyte filtration in venous line under lower pressure and flow may cause lower leukocyte damage and elastase release [226-230]. In critically ill neonates placed on ECMO, transfusion of leukoreduced blood via leukocyte filtration is associated with

improvement of patient outcomes [231]. In a group of infants with severe pertussis needing ECMO, leukoreduction with leukocyte filtration applied before priming and the establishment of ECMO was associated with a trend for higher survival in the patients [232]. The findings of the very limited number of studies suggest that the protective effects of leukoreduction in ECMO, may be highly related to the underlying condition (e.g. inflammatory versus non-inflammatory). Yet, the prolonged, continuous leukocyte filtration, despite the reduction of circulating leukocyte count may increase hemolysis and release of free hemoglobin, leukocyte damage and rupture and can induce/exacerbate inflammation and oxidative stress in this context [233].

In clinical ESHP, the leukoreduced donor blood is used for the perfusate as the standard of care [95]. Leukocyte reduced/depleted perfusate (either using leukocyte filters or centrifuging the blood) has been also frequently used in experimental ESHP studies to reduce the assumed inflammation and oxidative stress [94, 111, 121]. However, in our previous experimental studies we have reported a significant induction of inflammatory and oxidative stress responses during ESHP that occurred despite using an in-line leukocyte filter [141]. Recently we have also reported that a perfusate containing leukocyte-depleted blood, compared with a whole blood-based perfusate did not decrease circulating concentrations of pro-inflammatory cytokines or tissue edema. Moreover, it did not improve cardiac functional preservation during extended (12-hour) ESHP [169].

1.8.3. Pharmacological interventions

1.8.3.1. Corticosteroids

Although corticosteroids have been utilized in the setting of cardiac surgery for more than half a century, there are controversies on their clinical beneficial effects. Various studies have suggested that corticosteroid administration may reduce the complement activation, expression of endothelial adhesion molecules, cytokine release and neutrophil –related tissue injury, and can improve the integrity of myocardial and pulmonary tissue [234]. Among corticosteroids, methylprednisolone and dexamethasone are the most commonly used synthetic corticosteroids in the setting of CPB, however methylprednisolone has been the most frequently administered corticosteroid due to its strong anti-inflammatory properties, and lower induction of fluid retention [235]. A strong body of evidence suggests that perioperative administration of corticosteroids ($\geq 1\ dose$) in surgeries involving CPB can attenuate generation of pro-inflammatory cytokines, markers of tissue damage (e.g. creatine kinase myocardial band, CK-MB and cTn-I), acute phase reactants (e.g. C-reactive protein), RONS, adhesion molecules and it may increase the anti-inflammatory cytokine IL-10. On the other hand clinical studies evaluating the effect of this intervention on patients outcome have shown mixed results. While some studies reported perioperative administration of corticosteroids to be associated with improved myocardial contractile function, lower incidence of post operative atrial fibrillation (AF), decreased post-operative need for mechanical ventilation and circulatory support, and shorter duration of the hospital stay while not affecting the risk of infections [236-245], other groups did not detect any beneficial effects for steroid administration regarding improving post-operational recovery, decreasing incidence of post-operative arrhythmias,

or pain, shortening of the ICU/hospital stay, shortening of needed mechanical ventilation time, and mortality [238-241, 245]. Despite the mixed findings, meta-analysis investigations suggest that prophylactic steroid in patients undergoing cardiac surgery involving cardiopulmonary bypass significantly decreases the length of ICU and hospital stay and post-operation AF in adult or pediatric patient populations [246-248]. Thus, according to AHA/ACC, steroid administration seems to reduce the inflammatory responses to extracorporeal circulation with negligible risk, the prophylactic use of it in encouraged, and currently is widely applied in patients undergoing ECC [234]. Similarly, corticosteroids have been administered widely in patients undergoing ECMO and is believed to benefit critically ill patients through reducing the systemic inflammatory response and compensating for the cortico-adrenal insufficiency. The related clinical trials overall have supported its effect in improving secondary outcomes such as decreasing the need for mechanical ventilation and the length of ICU stay. Based on the meta-analytical investigations, corticosteroid can reduce in-hospital mortality in critically ill patient with ECMO [249]. Despite these observations, the indication of corticosteroid therapy before or during ECMO is still highly depends on the justification of its use in the underlying pathological condition (e.g. influenza- induced acute respiratory distress syndrome (ARDS) versus diffuse alveolar hemorrhage-related ARDS) [250].

Corticosteroids added to the perfusate, is the standard of care in clinical protocols of ESHP [97, 128]. Most of the attention has been focused on the beneficial effects of steroids in attenuating myocardial IRI that the donated hearts (particularly DCD hearts) are subjected to before, and during organ procurement and before starting of the ESHP [97, 134]. In general, there is very limited data on the effects of steroids on reducing the

ex-situ perfusion-related inflammation and oxidative that may be masked in ESHP prefusion of ischemic hearts. Whilst corticosteroids are added to the perfusate in clinical ESHP, the few existing experimental studies have reported little or no beneficial effects for steroids in preventing the cardiac functional decline, tissue damage, and edema during ESHP [134, 251].

1.8.3.2. Statins

During the recent few decades the anti-inflammatory and antioxidative effects of statins (e.g. Rosuvastatin) has been recognized [252, 253]. The clinical trials and earlier meta-analysis of clinical trials suggested that preoperative prescription of statin in patients undergoing cardiac surgery and CPB can attenuate the inflammatory responses and induction of markers of tissue damage induced by extracorporeal circulation, and may improve the patient outcomes [140, 253, 254]. However, a more current meta-analysis by An *et al.* showed that preoperative statin, while has a beneficial effect in decreasing the incidence of post-operative AF, does not decrease the risk of post-operative myocardial infraction or stroke in the patients undergoing cardiac surgery involving CPB [255]. The meta-analysis by Putzu *et al* suggested an increased risk of acute kidney injury and in-hospital mortality with preoperative statins, while the earlier meta-analysis by this group also failed to show any improvement in patient outcomes (e.g. mortality and incidence of post-operative AF) [140, 256, 257]. Statins have been also used in adjacent to VV-ECMO for the treatment of respiratory pathologies such as ARDS [249], but to our best knowledge the effect of statins to attenuate the inflammatory and oxidative stress occurring during ECMO has not been investigated. Similarly, ESHP has been used as a study method to evaluate the beneficial effects of statins (anti-inflammatory and

antioxidant effects) in attenuating myocardial IRI for their [258, 259] but again, their effect has not been investigated in attenuating the ECC-related inflammation and oxidative stress in the setting of ESHP.

1.8.3.3. Phosphodiesterase Inhibitors

The second messengers cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) participate in the regulation of multiple physiological cell processes. These nucleotides are degraded by 11 families of phosphodiesterases (PDEs), that are distributed diversely in different tissues. Among those, cAMP-specific PDE4 is highly expressed in the cardiovascular tissues and immune cells, and its inhibition help in maintaining the intracellular level of cAMP, decreasing vascular resistance, promoting inotropy and also ameliorating the inflammatory responses [260]. The anti-inflammatory effects of PDE inhibitors have been investigated during recent decades. The anti-inflammatory and organ-protective effects of PDE inhibitors in ECLS has been somehow controversial. It has been shown in clinical and experimental studies that PDE inhibitors can adjust myocardial energetics, and ameliorate myocardial, and systemic inflammation and myocardial IRI in CPB [261-264]. Paradoxically, in an experimental study in a rat model of VA-ECMO, administration of PDE inhibitor lead to significantly lower pulmonary tissue injury, vascular endothelial cadherin expression in vein tissue and serum compared to controls, without lowering the serum levels of the pro-inflammatory cytokines such as TNF-a and IL-6 [265]. More studies are warranted on the anti-inflammatory and antioxidant properties of PDE inhibitors alongside their beneficial effect on patient outcomes in ECLS.

Similar to most of the agents discussed here, although the ESHP has been used to evaluate the cardioprotective and therapeutic properties of different families of PDE inhibitors [266, 267], yet to our best knowledge the effects of PDE inhibitors on mitigating the inflammatory responses and/or improving the functional preservation of the heart during ESHP is unknown. More experimental and clinical studies are required to evaluate the cardioprotective effects of PDE inhibitors in the setting of ESHP.

1.8.3.4. Complement inhibitors

Experimental and clinical studies evaluating different complement inhibitors including APT070 and TP10 (C3 and C5 activation inhibitor) and Pexelizumab (a recombinant antibody binding to C5) have reported a significant reduction in active complement proteins, and better protection of the lungs and myocardium during CPB, and improvement in patient outcomes [268-270]. However, some of these studies failed to show an association between diminished complement activation and inflammation [268]. It has been shown that heparin-coated ECMO circuits, lead to attenuation in complement activation due to complement-inhibitory effects of Heparin [271].

As a technique to study the physiology and pathology of the heart, ESHP has been used to evaluate the effects of complement activation and inhibition on myocardium [272, 273], but to our best knowledge there is are no scientific data on the effect of complement inhibition during ESHP with blood derived perfusates.

1.8.3.5. Protease inhibitors

Serine proteases, generated as a part of coagulation cascades, also released from the activated leukocytes are among the important mediators of inflammatory responses as they are involved in production of the pro-inflammatory cytokine, and activation of immune

cells [274]. A bovine lung-derived serine protease inhibitor (aprotinin) which was originally evaluated to improve homeostasis in patients undergoing cardiac surgeries, has also shown anti-inflammatory effects in this process. Both clinical and experimental studies suggest that aprotinin may attenuate the CPB-related inflammation by decreasing the activation of 1) contact system (preservation of Kallikrein inhibitor activity), 2) coagulation pathway (inhibition of plasmin), 3) complement system (decreased production/activation of complement factors), and 4) modulating leukocyte activation [275-278]. It may protect the heart during CPB as evidenced by lowering the levels of plasma cTn-I and CK-MB [234]. In an experimental study of ECMO in a canine model, serine protease inhibitor (nafamostat mesylate) administered during ECMO lead to similar anticoagulant effects with heparin, and a significantly lower IL-1β in plasma during the procedure. Despite these promising results, the use of serine protease inhibitors (aprotinin in particular) has raised some concerns as an observational propensity and multivariate analysis by Mangano *et al.* reported significantly higher incidence of adverse cardiac, cerebral, and renal events in patients receiving aprotinin [234, 279] .

Serine protease inhibitors have been studies in the context of ESHP, particularly to assess its effect in attenuating the damaging effects of myocardial ischemia and IRI [280, 281]. Yet, studies addressing the possible cardioprotective effects of serine protease inhibitors through their anti-inflammatory characteristics against the ECC-related inflammation and oxidative stress during ESHP will be beneficial.

1.8.3.6. Antioxidants

Antioxidants are molecules capable of neutralizing free radicals by reversing the unpaired condition of the radical by donating or receiving electrons form it. The antioxidant-oxidant

interactions can either destroy the oxidant molecule, or change it into less reactive, and less damaging molecules compared to the original molecule [170]. Among the various antioxidative agents that have been assessed to decrease the oxidative stress in ECLS is Propofol. Propofol is an agent widely used in anesthesia that also has antioxidative properties. Clinical studies have reported that propofol administration throughout cardiac surgery with CPB leads to better myocardial protection, lower circulating markers of oxidative stress, and improved patient outcomes [282].

In an experimental CPB study in a canine model, administration of N-acetylcysteine (NAC) right before and during early CPB led to a significantly better myocardial function alongside lower concentration of 8-isoprostane in coronary sinus plasma samples [283]. In patients undergoing cardiac surgery with CPB, NAC administration during the operation has been reported to significantly lower the formation of RONS, oxidative stress biomarkers, TNF-α and CK-MB in plasma samples, and improve the respiratory function [284, 285]. Similarly, dexmedetomidine, a selective α2-adrenoreceptor agonist with anti-inflammatory and antioxidant effects, when administered before application of CPB in cardiac surgery lead to lower serum TNF-α and cTn-I concentrations [286]. However, there has been a controversy in the results of the studies evaluating the beneficial effects of antioxidants in the context of ECC. In a group of patients undergoing coronary artery bypass grafting surgery, administration of vitamin C, did not improve the pro-inflammatory cytokine profile of the patients, or myocardial function [287]. In another clinical study, while simultaneous administration of vitamin C and vitamin E, significantly decreased oxidative stress during CPB, but did not decrease the incidence of post-operative AF [288]. Other antioxidants such as coenzyme Q10 (CoQ10), a lipid-soluble antioxidant,

also has shown diverse results about its protective effects against the complications of cardiac surgery and cardiopulmonary bypass. However, the meta-analysis by Frutos *et al.* suggested that CoQ10 treatment is associated with lower incidence of ventricular arrhythmias and less need for post-operative inotropic support, but is not associated with a decreased risk of post-operative AF, or with myocardial function and length of hospital stay [289].

A meta-analysis of clinical, perioperative use of antioxidant revealed that NAC, polyunsaturated fatty acids (PUFA), and vitamin C in surgeries applying CPB is associated with significantly lower risk of post operative AF, and better patient outcomes (mortality in NAC, and length of hospital stay in PUFA) [290]. Similarly, a recent meta-analysis reported that perioperative administration of Vitamin C is associated with significantly lower incidence of post-operative arrhythmia, shorter need for mechanical ventilation and a significantly shorter length of hospital and ICU stay, however, it was not associated with an improvement in mortality [291]. Interestingly, there is a significant paucity of data about beneficial effects of antioxidants in ECMO treatment, despite the fact that the endogenous antioxidant defence system is impaired in critically ill patients that would need ECLS [174]. In general, there is a considerable heterogeneity in study designs, and the chosen antioxidant agents between the studies. More experimental and clinical studies are warranted to address the effects of antioxidant administered in ECC on systemic inflammation and oxidative stress, organ damage, and patient outcomes.

With the increasing interest for transplantation of DCD organs using the *ex-situ* organ perfusion technology, naturally IRI has become a hot topic in preserving these organs. Due to the significance of myocardial IRI in cardiac health and function, targeting it with

antioxidants has been a focus of ESHP studies. In the rat hearts that had endured a significant global warm ischemia, the post-conditioning with soluble vitamin E (Trolox) has been shown to significantly reduce the IRI, and improving their functional status during 40 minutes of ESHP [292]. In a porcine model of a very long period of cold ischemia (24 hours), soluble vitamin E added to the perfusate lead to better preservation of myocardial coronary endothelium and mitochondrial morphological characteristics, and attenuated inflammation and tissue edema in the treated hearts compared to the controls [293]. Danshensu [3 (3,4dihydroxyphenyl) 2 hydroxy propanoic acid,] treatment in the rat hearts endured 30 minutes of warm ischemia followed by 30 minutes of reperfusion was associated with a significant improvement in antioxidant profile and enzymes activity including glutathione peroxidase and nuclear factor erythroid-2-related factor 2 (Nrf2) in myocardium alongside decreased secretion of the markers of myocardial damage [294]. In a rat model of myocardial IRI, addition of glutathione alone or together with vitamin C to the perfusate at the start of ESHP attenuated the myocardial IRI in the treated hearts and at the same time reduced production of peroxynitrite [295]. While these studies are focused on attenuating the myocardial IRI, to our best knowledge there are no available studies on the cardioprotective effects of antioxidants against the ECC-related oxidative stress during ESHP. Thus, studies designed to assess the effects of antioxidants in improving cardioprotection during ESHP through attenuating ECC-related inflammation and oxidative stress are inevitable.

1.9. Conclusion

ESHP provides the opportunity to assess and recondition the donated heart while it is preserved in a beating, semi-physiologic condition. Avoiding cold ischemia, and

maintenance of myocardial aerobic metabolism in this setting may lead to superior preservation of donated heart compared to conventional CSS, and may also facilitate preservation and resuscitation of extended-criteria sub-optimal hearts, thus expand the donor pool. However, both functional and metabolic assessment of the heart is essential during ESHP to prevent high-risk transplantation. Despite all the promising, cardioprotective aspects of ESHP, the functional status of the heart declines gradually during ESHP. Although the mechanisms of functional decline are yet to be discovered but the alterations related to ECC may play an important role. Inflammation and oxidative stress are induced during ECC. While these phenomena are better studied in clinical setting (e.g. CPB and ECMO), they have been significantly overlooked in *ex-situ* organ perfusion and in particular in ESHP. More importantly, the effects of addressing them in ESHP on myocardial functional preservation and outcomes of transplantation is unknown. Clinical ESHP is still in its infancy. Thus, an elaborate identification of the ECC-related effects on myocardial tissue and preservation environment (perfusate), optimal perfusion conditions (including circuit/device optimization), and efficient metabolic support for the heart during perfusion is necessary for the aim of improving the clinical protocols of ESHP and better preserving and reconditioning of the donated hearts in this setting. In this thesis we have evaluated the heart during an extended period of ESHP (12 hours) and have studied the alteration occurring during this period in functional status of the heart, metabolism, and in general ECC-related changes, together with evaluating the different mechanical perfusion parameters that may affect the viability of the heart in this setting. The specific objectives of this thesis are as below:

1) To assess the cardioprotective effect of a semi-anatomical position of heart, and semi-physiological left ventricular load during ESHP
2) To assess the incidence of cell death, and overtime changes in energy-yielding metabolism, alongside functional preservation of heart during ESHP
3) To assess the incidence of inflammatory and tissue stress responses during ESHP
4) To assess the incidence of oxidative stress and related alterations in antioxidative potential, and metabolism of the myocardial tissue and induction of cardioprotective mechanisms during ESHP in different workloads (semi-physiological ventricular load versus empty beating heart)
5) To assess the effects of ESHP with a leukocyte-depleted perfusate on inflammation, oxidative stress, metabolism and functional preservation of the heart during ESHP.

1.10. Tables and figures

Table 1.1. New York Heart Association (NYHA) Heart Failure symptom classification system [2]

NYHA Class	Level of Impairment
I	No symptom limitation with ordinary physical activity
II	Ordinary physical activity somewhat limited by dyspnea (e.g., long-distance walking, climbing two flights of stairs)
III	Exercise limited by dyspnea with moderate workload (e.g., short-distance walking, climbing one flight of stairs)
IV	Dyspnea at rest or with very little exertion

Table 1.2. American College of Cardiology- American Heart Association classification of Chronic Heart Failure [24]

Stage	Description
A: High risk for developing heart failure	Hypertension, diabetes mellitus, CAD, family history of cardiomyopathy
B: Asymptomatic heart failure	Previous MI, LV dysfunction, valvular heart disease
C: Symptomatic heart failure	Structural heart disease, dyspnea and fatigue, impaired exercise tolerance
D: Refractory end-stage heart failure	Marked symptoms at rest despite maximal medical therapy

CAD, coronary artery disease; LV, left ventricular; MI, myocardial infarction.

Table 1.3. Factors and entities of impact on the definition and usability of 'marginal cardiac donors'

Extra-cardiac factors	Age Ischemic time Size Virology status
Heart-related variables	Left ventricular hypertrophy Valvular/congenital abnormalities Coronary artery disease
Brain-death related factors	Intracranial haemorrhage Penetrating head injury Brain malignancies Drug/substance intoxication Various
Impact of optimal donor management	Metabolic/endocrine management Hemodynamic assessment Coronary evaluation Echocardiographic evaluation
Non-heart beating donation	

Table 1.4. Modified Maastricht classification for donation after circulatory determination of death [75]

Uncontrolled	**Description**
Category 1	*Found dead* Sudden unexpected circulatory arrest without any attempt of resuscitation by a medical team Category IA: out-of-hospital, Category IB: in-hospital
Category II	*Witnessed cardiac arrest* Sudden unexpected irreversible circulatory arrest with unsuccessful resuscitation by a medical team Category IIA: out-of-hospital, Category IIB: in-hospital
Category IV	*Cardiac arrest while brain dead* Sudden circulatory arrest after brain-death diagnosis during donor management but prior to planned Organ retrieval; donation proceeds after unsuccessful resuscitation by a medical team
Controlled	**Description**
Category III	*Withdrawal of life-sustaining therapy (WLST)* Planned WLST and expected circulatory arrest
Category IV	*Cardiac arrest while brain dead* In countries where legislation does not accept brain death criteria or patient will never meet the neurologic criteria for the diagnosis of brain death, the procedure for this potential DBD can be converted to a DCD

Table 1.5. Differences between extracorporeal life support techniques and *ex-situ* heart perfusion

	ECMO	CPB	ESHP
Application of artificial materials/components	Yes	Yes	Yes
Connection to body	Connected	Connected	Non-connected
Duration	Days to weeks	Minutes to hours	Minutes to hours
Haemodilution	No	Yes	Yes
Anticoagulation	Low-dose heparin	High-dose Heparin	High-dose Heparin
Reversal of anticoagulation	No	Yes (protamine)	No
Hypothermia	No	Yes	Variable
Air-blood interface	No (closed-circuit)	Yes (there are some closed-circuit variants)	Yes
Pulsatility	Variable with mode	No	Variable (dependent on device)

ECMO, extracorporeal membrane oxygenation; CPB, cardiopulmonary bypass; ESHP, *ex-situ* heart perfusion

Figure 1.1. ACC/AHA Guideline recommendations for treating patients with chronic heart failure [35]

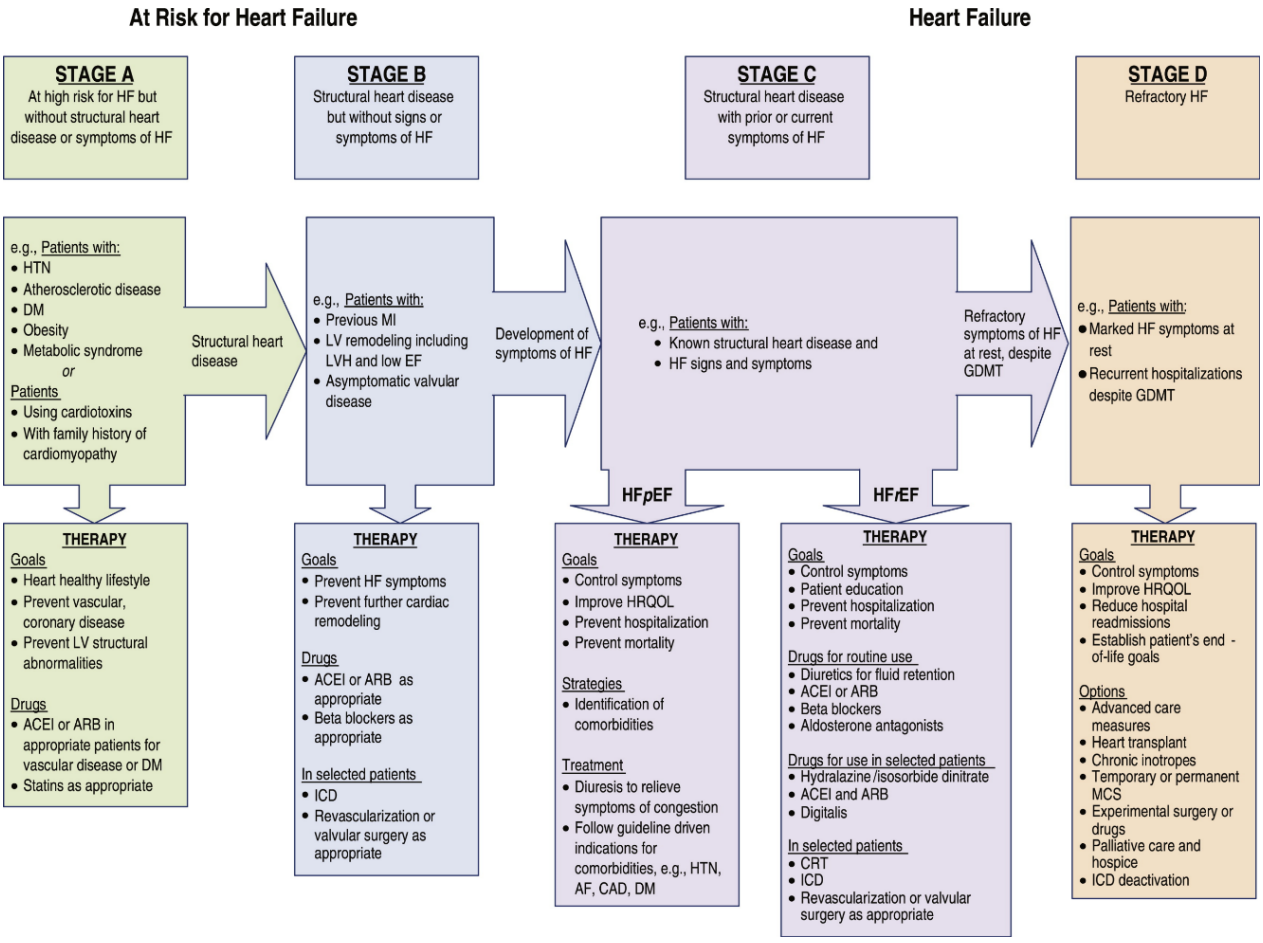
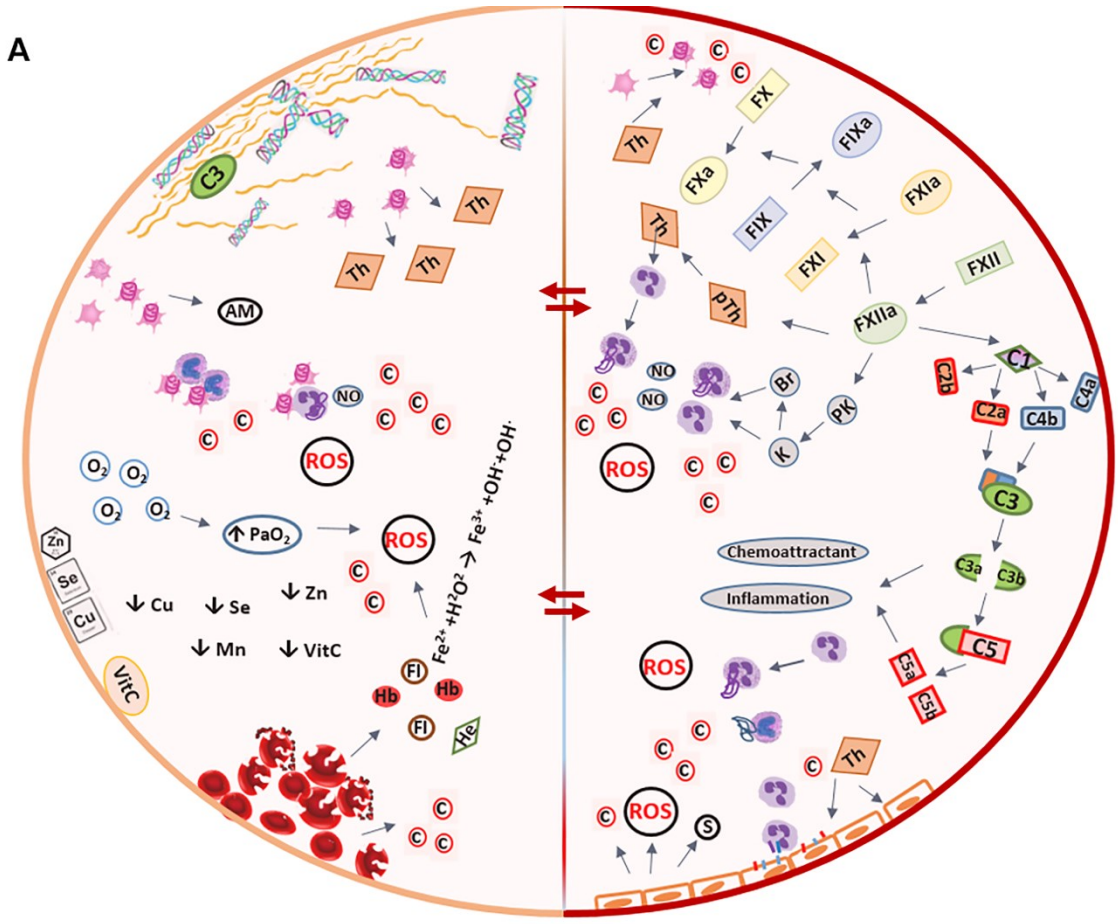


Figure 1.2. Summary of the pathophysiologic conditions occurring during extracorporeal circulation. A: Artificial biomaterial of the circuit B: Vascular system of the organs



AM, adhesion molecules; Br, bradykinin; C, complement compartment, c, cytokines; Cu, copper; Fl, free iron; FIX, factor-9; FX, factor-10; FXI, factor-11; FXII, factor-12; K, kallikrein; Mn, manganese; NO, nitric oxide; O_2, oxygen; PaO_2, partial pressure of arterial oxygen; PK, pre-kallikrein; pTh, prothrombin; ROS, reactive oxygen species; s, selectins; Se, selenium; Th, thrombin; VitC, vitamin C; Zn, zinc; , monocyte; , neutrophil; , red blood cell

1.11. References:

1. Roger VL, Weston SA, Redfield MM, Hellermann-Homan JP, Killian J, Yawn BP, Jacobsen SJ: **Trends in heart failure incidence and survival in a community-based population**. *Jama* 2004, **292**(3):344-350.
2. Murthy S, Lipman HI: **Management of end-stage heart failure**. *Prim Care* 2011, **38**(2):265-276, viii.
3. Cook C, Cole G, Asaria P, Jabbour R, Francis DP: **The annual global economic burden of heart failure**. *International journal of cardiology* 2013, **171**(3):368-376.
4. Avery CL, Loehr LR, Baggett C, Chang PP, Kucharska-Newton AM, Matsushita K, Rosamond WD, Heiss G: **The population burden of heart failure attributable to modifiable risk factors: the ARIC (Atherosclerosis Risk in Communities) study**. *J Am Coll Cardiol* 2012, **60**(17):1640-1646.
5. **Correction to: Heart Disease and Stroke Statistics-2019 Update: A Report From the American Heart Association**. *Circulation* 2020, **141**(2):e33.
6. Bui AL, Horwich TB, Fonarow GC: **Epidemiology and risk profile of heart failure**. *Nature reviews Cardiology* 2011, **8**(1):30-41.
7. Heidenreich PA, Trogdon JG, Khavjou OA, Butler J, Dracup K, Ezekowitz MD, Finkelstein EA, Hong Y, Johnston SC, Khera A *et al*: **Forecasting the future of cardiovascular disease in the United States: a policy statement from the American Heart Association**. *Circulation* 2011, **123**(8):933-944.
8. Rohde LE, Bertoldi EG, Goldraich L, Polanczyk CA: **Cost-effectiveness of heart failure therapies**. *Nature reviews Cardiology* 2013, **10**(6):338-354.
9. Virani SA, Bains M, Code J, Ducharme A, Harkness K, Howlett JG, Ross H, Sussex B, Zieroth S: **The Need for Heart Failure Advocacy in Canada**. *The Canadian journal of cardiology* 2017, **33**(11):1450-1454.
10. Jong P, Vowinckel E, Liu PP, Gong Y, Tu JV: **Prognosis and determinants of survival in patients newly hospitalized for heart failure: a population-based study**. *Arch Intern Med* 2002, **162**(15):1689-1694.
11. Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, De Simone G, Ferguson TB, Ford E, Furie K, Gillespie C *et al*: **Executive summary: heart disease and stroke statistics--2010 update: a report from the American Heart Association**. *Circulation* 2010, **121**(7):948-954.
12. Abraham WT, Smith SA: **Devices in the management of advanced, chronic heart failure**. *Nature reviews Cardiology* 2013, **10**(2):98-110.
13. Kinkhabwala MP, Mancini D: **Patient selection for cardiac transplant in 2012**. *Expert Rev Cardiovasc Ther* 2013, **11**(2):179-191.
14. Toyoda Y, Guy TS, Kashem A: **Present status and future perspectives of heart transplantation**. *Circulation journal : official journal of the Japanese Circulation Society* 2013, **77**(5):1097-1110.
15. Jencks SF, Williams MV, Coleman EA: **Rehospitalizations among patients in the Medicare fee-for-service program**. *The New England journal of medicine* 2009, **360**(14):1418-1428.
16. Kozak LJ, DeFrances CJ, Hall MJ: **National hospital discharge survey: 2004 annual summary with detailed diagnosis and procedure data**. *Vital Health Stat 13* 2006(162):1-209.
17. Roger VL: **Epidemiology of heart failure**. *Circulation research* 2013, **113**(6):646-659.
18. Haldeman GA, Croft JB, Giles WH, Rashidee A: **Hospitalization of patients with heart failure: National Hospital Discharge Survey, 1985 to 1995**. *Am Heart J* 1999, **137**(2):352-360.
19. Hunt SA, Baker DW, Chin MH, Cinquegrani MP, Feldman AM, Francis GS, Ganiats TG, Goldstein S, Gregoratos G, Jessup ML *et al*: **ACC/AHA guidelines for the evaluation and management of chronic heart failure in the adult: executive summary a report of the American College of Cardiology/American Heart Association task force on practice guidelines (committee to revise**

the 1995 guidelines for the evaluation and management of heart failure): developed in collaboration with the international society for heart and lung transplantation; endorsed by the Heart Failure Society of America. *Circulation* 2001, **104**(24):2996-3007.
20. Biermann J, Neumann T, Angermann CE, Erbel R, Maisch B, Pittrow D, Regitz-Zagrosek V, Scheffold T, Wachter R, Gelbrich G *et al*: **Economic burden of patients with various etiologies of chronic systolic heart failure analyzed by resource use and costs**. *International journal of cardiology* 2012, **156**(3):323-325.
21. Smith DH, Johnson ES, Blough DK, Thorp ML, Yang X, Petrik AF, Crispell KA: **Predicting costs of care in heart failure patients**. *BMC Health Serv Res* 2012, **12**:434.
22. Lee WC, Chavez YE, Baker T, Luce BR: **Economic burden of heart failure: a summary of recent literature**. *Heart & lung : the journal of critical care* 2004, **33**(6):362-371.
23. Iwano H, Little WC: **Heart failure: what does ejection fraction have to do with it?** *Journal of cardiology* 2013, **62**(1):1-3.
24. Pazos-López P, Peteiro-Vázquez J, Carcía-Campos A, García-Bueno L, de Torres JP, Castro-Beiras A: **The causes, consequences, and treatment of left or right heart failure**. *Vascular health and risk management* 2011, **7**:237-254.
25. Guha K, McDonagh T: **Heart failure epidemiology: European perspective**. *Current cardiology reviews* 2013, **9**(2):123-127.
26. McKee PA, Castelli WP, McNamara PM, Kannel WB: **The natural history of congestive heart failure: the Framingham study**. *The New England journal of medicine* 1971, **285**(26):1441-1446.
27. Nieminen MS, Brutsaert D, Dickstein K, Drexler H, Follath F, Harjola VP, Hochadel M, Komajda M, Lassus J, Lopez-Sendon JL *et al*: **EuroHeart Failure Survey II (EHFS II): a survey on hospitalized acute heart failure patients: description of population**. *European heart journal* 2006, **27**(22):2725-2736.
28. Davies M, Hobbs F, Davis R, Kenkre J, Roalfe AK, Hare R, Wosornu D, Lancashire RJ: **Prevalence of left-ventricular systolic dysfunction and heart failure in the Echocardiographic Heart of England Screening study: a population based study**. *Lancet (London, England)* 2001, **358**(9280):439-444.
29. McDonagh TA, Morrison CE, Lawrence A, Ford I, Tunstall-Pedoe H, McMurray JJ, Dargie HJ: **Symptomatic and asymptomatic left-ventricular systolic dysfunction in an urban population**. *Lancet (London, England)* 1997, **350**(9081):829-833.
30. Sharma K, Kass DA: **Heart failure with preserved ejection fraction: mechanisms, clinical features, and therapies**. *Circulation research* 2014, **115**(1):79-96.
31. Kelder JC, Cramer MJ, van Wijngaarden J, van Tooren R, Mosterd A, Moons KG, Lammers JW, Cowie MR, Grobbee DE, Hoes AW: **The diagnostic value of physical examination and additional testing in primary care patients with suspected heart failure**. *Circulation* 2011, **124**(25):2865-2873.
32. Mant J, Doust J, Roalfe A, Barton P, Cowie MR, Glasziou P, Mant D, McManus RJ, Holder R, Deeks J *et al*: **Systematic review and individual patient data meta-analysis of diagnosis of heart failure, with modelling of implications of different diagnostic strategies in primary care**. *Health Technol Assess* 2009, **13**(32):1-207, iii.
33. McKelvie RS, Moe GW, Ezekowitz JA, Heckman GA, Costigan J, Ducharme A, Estrella-Holder E, Giannetti N, Grzeslo A, Harkness K *et al*: **The 2012 Canadian Cardiovascular Society heart failure management guidelines update: focus on acute and chronic heart failure**. *The Canadian journal of cardiology* 2013, **29**(2):168-181.
34. Heart Failure Society Of A: **Evaluation of patients for ventricular dysfunction and heart failure**. *J Card Fail* 2006, **12**(1):e16-25.

35. Yancy CW, Jessup M, Bozkurt B, Butler J, Casey DE, Jr., Drazner MH, Fonarow GC, Geraci SA, Horwich T, Januzzi JL *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.
36. Patarroyo-Aponte M, Colvin-Adams M: **Evaluation of patients with heart failure**. *Cardiol Clin* 2014, **32**(1):47-62.
37. van Kimmenade RR, Januzzi JL, Jr.: **Emerging biomarkers in heart failure**. *Clin Chem* 2012, **58**(1):127-138.
38. Masson S, Anand I, Favero C, Barlera S, Vago T, Bertocchi F, Maggioni AP, Tavazzi L, Tognoni G, Cohn JN *et al*: **Serial measurement of cardiac troponin T using a highly sensitive assay in patients with chronic heart failure: data from 2 large randomized clinical trials**. *Circulation* 2012, **125**(2):280-288.
39. Metra M, Bettari L, Pagani F, Lazzarini V, Lombardi C, Carubelli V, Bonetti G, Bugatti S, Parrinello G, Caimi L *et al*: **Troponin T levels in patients with acute heart failure: clinical and prognostic significance of their detection and release during hospitalisation**. *Clin Res Cardiol* 2012, **101**(8):663-672.
40. Borlaug BA, Paulus WJ: **Heart failure with preserved ejection fraction: pathophysiology, diagnosis, and treatment**. *European heart journal* 2011, **32**(6):670-679.
41. McMurray JJ, Adamopoulos S, Anker SD, Auricchio A, Bohm M, Dickstein K, Falk V, Filippatos G, Fonseca C, Gomez-Sanchez MA *et al*: **ESC guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC**. *Eur J Heart Fail* 2012, **14**(8):803-869.
42. Paulus WJ, Tschope C, Sanderson JE, Rusconi C, Flachskampf FA, Rademakers FE, Marino P, Smiseth OA, De Keulenaer G, Leite-Moreira AF *et al*: **How to diagnose diastolic heart failure: a consensus statement on the diagnosis of heart failure with normal left ventricular ejection fraction by the Heart Failure and Echocardiography Associations of the European Society of Cardiology**. *European heart journal* 2007, **28**(20):2539-2550.
43. Greenberg B, Czerska B, Delgado RM, Bourge R, Zile MR, Silver M, Klapholz M, Haeusslein E, Mehra MR, Mather P *et al*: **Effects of continuous aortic flow augmentation in patients with exacerbation of heart failure inadequately responsive to medical therapy: results of the Multicenter Trial of the Orqis Medical Cancion System for the Enhanced Treatment of Heart Failure Unresponsive to Medical Therapy (MOMENTUM)**. *Circulation* 2008, **118**(12):1241-1249.
44. Liang HY, Cauduro SA, Pellikka PA, Bailey KR, Grossardt BR, Yang EH, Rihal C, Seward JB, Miller FA, Abraham TP: **Comparison of usefulness of echocardiographic Doppler variables to left ventricular end-diastolic pressure in predicting future heart failure events**. *Am J Cardiol* 2006, **97**(6):866-871.
45. Nayyar S, Magalski A, Khumri TM, Idupulapati M, Stoner CN, Kusnetzky LL, Coggins TR, Morris BA, Main ML: **Contrast administration reduces interobserver variability in determination of left ventricular ejection fraction in patients with left ventricular dysfunction and good baseline endocardial border delineation**. *Am J Cardiol* 2006, **98**(8):1110-1114.
46. Paterson I, Mielniczuk LM, O'Meara E, So A, White JA: **Imaging heart failure: current and future applications**. *The Canadian journal of cardiology* 2013, **29**(3):317-328.
47. Butler J, Khadim G, Paul KM, Davis SF, Kronenberg MW, Chomsky DB, Pierson RN, 3rd, Wilson JR: **Selection of patients for heart transplantation in the current era of heart failure therapy**. *J Am Coll Cardiol* 2004, **43**(5):787-793.
48. Chase P, Arena R, Guazzi M, Myers J, Peberdy MA, Bensimhon D: **Prognostic usefulness of the functional aerobic reserve in patients with heart failure**. *Am Heart J* 2010, **160**(5):922-927.

49. Aronson D, Krum H: **Novel therapies in acute and chronic heart failure**. *Pharmacol Ther* 2012, **135**(1):1-17.
50. Cowie MR: **Improving the management of chronic heart failure**. *Practitioner* 2010, **254**(1734):29-32, 23.
51. Atherton JJ: **Chronic heart failure: we are fighting the battle, but are we winning the war?** *Scientifica (Cairo)* 2012, **2012**:279731.
52. Scott C, Thompson JP: **Drugs acting on the heart: heart failure and coronary insufficiency**. *Anaesthesia And Intensive Care Medicine* 2012, **13**(8):378-382.
53. Guibert EE, Petrenko AY, Balaban CL, Somov AY, Rodriguez JV, Fuller BJ: **Organ preservation: current concepts and new strategies for the next decade**. *Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie* 2011, **38**(2):125-142.
54. 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**. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* 2008, **34**(2):318-325.
55. Hornby K, Ross H, Keshavjee S, Rao V, Shemie SD: **Non-utilization of hearts and lungs after consent for donation: a Canadian multicentre study**. *Canadian journal of anaesthesia = Journal canadien d'anesthesie* 2006, **53**(8):831-837.
56. Klein AS, Messersmith EE, Ratner LE, Kochik R, Baliga PK, Ojo AO: **Organ donation and utilization in the United States, 1999-2008**. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2010, **10**(4 Pt 2):973-986.
57. Ross LF, Frader J: **Are we ready to expand donation after cardiac death to the newborn population?** *The Journal of pediatrics* 2011, **158**(1):6-8.
58. White CW, Ali A, Hasanally D, Xiang B, Li Y, Mundt P, Lytwyn M, Colah S, Klein J, Ravandi A *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 : the official publication of the International Society for Heart Transplantation* 2013, **32**(7):734-743.
59. Aupperle H, Garbade J, Ullmann C, Schneider K, Krautz C, Dhein S, Gummert JF, Schoon HA: **Comparing the ultrastructural effects of two different cardiac preparation- and perfusion-techniques in a porcine model of extracorporal long-term preservation**. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* 2007, **31**(2):214-221.
60. Hatami S, Freed DH: **Machine perfusion of donor heart: state of the art**. *Current Transplantation Reports* 2019, **6**(3):242-250.
61. Monteagudo Vela M, Garcia Saez D, Simon AR: **Current approaches in retrieval and heart preservation**. *Annals of cardiothoracic surgery* 2018, **7**(1):67-74.
62. Goila AK, Pawar M: **The diagnosis of brain death**. *Indian J Crit Care Med* 2009, **13**(1):7-11.
63. Dhanwate AD: **Brainstem death: A comprehensive review in Indian perspective**. *Indian J Crit Care Med* 2014, **18**(9):596-605.
64. McKeown DW, Bonser RS, Kellum JA: **Management of the heartbeating brain-dead organ donor**. *Br J Anaesth* 2012, **108 Suppl 1**:i96-107.
65. Chen EP, Bittner HB, Kendall SW, Van Trigt P: **Hormonal and hemodynamic changes in a validated animal model of brain death**. *Critical care medicine* 1996, **24**(8):1352-1359.

66. Marasco S, Kras A, Schulberg E, Vale M, Chan P, Lee GA, Bailey M: **Donor brain death time and impact on outcomes in heart transplantation**. *Transplantation proceedings* 2013, **45**(1):33-37.
67. Cohen O, De La Zerda DJ, Beygui R, Hekmat D, Laks H: **Donor brain death mechanisms and outcomes after heart transplantation**. *Transplantation proceedings* 2007, **39**(10):2964-2969.
68. de Jonge N, Kirkels JH, Klopping C, Lahpor JR, Caliskan K, Maat AP, Brugemann J, Erasmus ME, Klautz RJ, Verwey HF *et al*: **Guidelines for heart transplantation**. *Neth Heart J* 2008, **16**(3):79-87.
69. Edwards N, Chen J, Mazzeo P, Chen J, Edwards N: **Donor Selection and Management of the High-Risk Donor**. In: *Cardiac Transplantation.* edn.: Humana Press; 2004: 19-36.
70. Wittwer T, Wahlers T: **Marginal donor grafts in heart transplantation: lessons learned from 25 years of experience**. *Transplant international : official journal of the European Society for Organ Transplantation* 2008, **21**(2):113-125.
71. Longnus SL, Mathys V, Dornbierer M, Dick F, Carrel TP, Tevaearai HT: **Heart transplantation with donation after circulatory determination of death**. *Nature reviews Cardiology* 2014, **11**(6):354-363.
72. Steinbrook R: **Organ donation after cardiac death**. *The New England journal of medicine* 2007, **357**(3):209-213.
73. Detry O, Le Dinh H, Noterdaeme T, De Roover A, Honore P, Squifflet JP, Meurisse M: **Categories of donation after cardiocirculatory death**. *Transplantation proceedings* 2012, **44**(5):1189-1195.
74. Manara AR, Murphy PG, O'Callaghan G: **Donation after circulatory death**. *Br J Anaesth* 2012, **108 Suppl 1**:i108-121.
75. Choong JW, Ou R, Lim YW, Rosenfeldt FL: **Cold Crystalloid Perfusion Provides Cardiac Preservation Superior to Cold Storage for Donation After Circulatory Death**. *Transplantation* 2016, **100**(3):546-553.
76. Collins MJ, Ozeki T, Zhuo J, Gu J, Gullapalli R, Pierson RN, Griffith BP, Fedak PW, Poston RS: **Use of diffusion tensor imaging to predict myocardial viability after warm global ischemia: possible avenue for use of non-beating donor hearts**. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* 2007, **26**(4):376-383.
77. Ozeki T, Kwon MH, Gu J, Collins MJ, Brassil JM, Miller MB, Jr., Gullapalli RP, Zhuo J, Pierson RN, 3rd, Griffith BP *et al*: **Heart preservation using continuous ex vivo perfusion improves viability and functional recovery**. *Circulation journal : official journal of the Japanese Circulation Society* 2007, **71**(1):153-159.
78. Amberger A, Schneeberger S, Hernegger G, Brandacher G, Obrist P, Lackner P, Margreiter R, Mark W: **Gene expression profiling of prolonged cold ischemia and reperfusion in murine heart transplants**. *Transplantation* 2002, **74**(10):1441-1449.
79. Buckberg GD, Brazier JR, Nelson RL, Goldstein SM, McConnell DH, Cooper N: **Studies of the effects of hypothermia on regional myocardial blood flow and metabolism during cardiopulmonary bypass. I. The adequately perfused beating, fibrillating, and arrested heart**. *The Journal of thoracic and cardiovascular surgery* 1977, **73**(1):87-94.
80. Hassanein WH, Zellos L, Tyrrell TA, Healey NA, Crittenden MD, Birjiniuk V, Khuri SF: **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.
81. Poston RS, Gu J, Prastein D, Gage F, Hoffman JW, Kwon M, Azimzadeh A, Pierson RN, 3rd, Griffith BP: **Optimizing donor heart outcome after prolonged storage with endothelial function analysis and continuous perfusion**. *The Annals of thoracic surgery* 2004, **78**(4):1362-1370;; discussion 1362-1370.
82. Deng M, Soltesz E, Hsich E, Naka Y, Mancini D, Esmailian F, Kobashigawa J, Camacho M, Baran D, Madsen J *et al*: **Ex-vivo perfusion of human donor hearts reduces cold ischemia time**. *The JournalofHeartandLungTransplantation* 2013, **32**(4s):410.

83. Schneeberger S, Amberger A, Mandl J, Hautz T, Renz O, Obrist P, Meusburger H, Brandacher G, Mark W, Strobl D *et al*: **Cold ischemia contributes to the development of chronic rejection and mitochondrial injury after cardiac transplantation**. *Transplant international : official journal of the European Society for Organ Transplantation* 2010, **23**(12):1282-1292.
84. Hansen TN, Haworth RA, Southard JH: **Warm and cold ischemia result in different mechanisms of injury to the coronary vasculature during reperfusion of rat hearts**. *Transplantation proceedings* 2000, **32**(1):15-18.
85. Halazun KJ, Al-Mukhtar A, Aldouri A, Willis S, Ahmad N: **Warm ischemia in transplantation: search for a consensus definition**. *Transplantation proceedings* 2007, **39**(5):1329-1331.
86. Iyer A, Gao L, Doyle A, Rao P, Jayewardene D, Wan B, Kumarasinghe G, Jabbour A, Hicks M, Jansz PC *et al*: **Increasing the tolerance of DCD hearts to warm ischemia by pharmacological postconditioning**. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2014, **14**(8):1744-1752.
87. Liao R, Podesser BK, Lim CC: **The continuing evolution of the Langendorff and ejecting murine heart: new advances in cardiac phenotyping**. *American journal of physiology Heart and circulatory physiology* 2012, **303**(2):H156-167.
88. Barnard CN: **The operation. A human cardiac transplant: an interim report of a successful operation performed at Groote Schuur Hospital, Cape Town**. *South African medical journal = Suid-Afrikaanse tydskrif vir geneeskunde* 1967, **41**(48):1271-1274.
89. Rivard AL, Gallegos R, Ogden IM, Bianco RW: **Perfusion preservation of the donor heart: basic science to pre-clinical**. *The journal of extra-corporeal technology* 2009, **41**(3):140-148.
90. Wicomb WN, Cooper DK, Novitzky D, Barnard CN: **Cardiac transplantation following storage of the donor heart by a portable hypothermic perfusion system**. *The Annals of thoracic surgery* 1984, **37**(3):243-248.
91. Minasian SM, Galagudza MM, Dmitriev YV, Karpov AA, Vlasov TD: **Preservation of the donor heart: from basic science to clinical studies**. *Interactive cardiovascular and thoracic surgery* 2015, **20**(4):510-519.
92. Dhital KK, Chew HC, Macdonald PS: **Donation after circulatory death heart transplantation**. *Current opinion in organ transplantation* 2017, **22**(3):189-197.
93. White CW, Ambrose E, Muller A, Li Y, Le H, Hiebert B, Arora R, Lee TW, Dixon I, Tian G *et al*: **Assessment of donor heart viability during ex vivo heart perfusion**. *Canadian journal of physiology and pharmacology* 2015, **93**(10):893-901.
94. White CW, Messer SJ, Large SR, Conway J, Kim DH, Kutsogiannis DJ, Nagendran J, Freed DH: **Transplantation of hearts donated after circulatory death**. *Frontiers in cardiovascular medicine* 2018, **5**:8.
95. Messer S, Ardehali A, Tsui S: **Normothermic donor heart perfusion: current clinical experience and the future**. *Transplant international : official journal of the European Society for Organ Transplantation* 2015, **28**(6):634-642.
96. Latchana N, Peck JR, Whitson B, Black SM: **Preservation solutions for cardiac and pulmonary donor grafts: a review of the current literature**. *Journal of thoracic disease* 2014, **6**(8):1143-1149.
97. Ardehali A, Esmailian F, Deng M, Soltesz E, Hsich E, Naka Y, Mancini D, Camacho M, Zucker M, Leprince P *et al*: **Ex-vivo perfusion of donor hearts for human heart transplantation (PROCEED II): a prospective, open-label, multicentre, randomised non-inferiority trial**. *Lancet (London, England)* 2015, **385**(9987):2577-2584.
98. Hatami S, White CW, Ondrus M, Qi X, Buchko M, Himmat S, Lin L, Cameron K, Nobes D, Chung HJ *et al*: **Normothermic ex situ heart perfusion in working mode: assessment of cardiac function and metabolism**. *Journal of visualized experiments : JoVE* 2019(143).

99. Taylor MJ, Baicu SC: **Current state of hypothermic machine perfusion preservation of organs: The clinical perspective**. *Cryobiology* 2010, **60**(3 Suppl):S20-35.
100. Rosenbaum DH, Peltz M, DiMaio JM, Meyer DM, Wait MA, Merritt ME, Ring WS, Jessen ME: **Perfusion preservation versus static preservation for cardiac transplantation: effects on myocardial function and metabolism**. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* 2008, **27**(1):93-99.
101. Steen S, Paskevicius A, Liao Q, Sjoberg T: **Safe orthotopic transplantation of hearts harvested 24 hours after brain death and preserved for 24 hours**. *Scandinavian cardiovascular journal : SCJ* 2016, **50**(3):193-200.
102. Michel SG, La Muraglia GM, 2nd, Madariaga ML, Titus JS, Selig MK, Farkash EA, Allan JS, Anderson LM, Madsen JC: **Preservation of donor hearts using hypothermic oxygenated perfusion**. *Annals of transplantation : quarterly of the Polish Transplantation Society* 2014, **19**:409-416.
103. Fitton TP, Barreiro CJ, Bonde PN, Wei C, Gage F, Rodriguez R, Conte JV: **Attenuation of DNA damage in canine hearts preserved by continuous hypothermic perfusion**. *The Annals of thoracic surgery* 2005, **80**(5):1812-1820.
104. Rosenfeldt F, Ou R, Woodard J, Esmore D, Marasco S: **Twelve-hour reanimation of a human heart following donation after circulatory death**. *Heart, lung & circulation* 2014, **23**(1):88-90.
105. Van Caenegem O, Beauloye C, Bertrand L, Horman S, Lepropre S, Sparavier G, Vercruysse J, Bethuyne N, Poncelet AJ, Gianello P *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 : official journal of the European Association for Cardio-thoracic Surgery* 2016, **49**(5):1348-1353.
106. Van Raemdonck D, Rega F, Rex S, Neyrinck A: **Machine perfusion of thoracic organs**. *Journal of thoracic disease* 2018, **10**(Suppl 8):S910-s923.
107. Hatami S, White C, Shan S, Haromy A, Qi X, Ondrus M, Kinnear A, Himmat S, Michelakis E, Nagendran J *et al*: **Myocardial functional decline during prolonged ex situ heart perfusion**. *The Annals of thoracic surgery* 2019, **108**:499-507.
108. Garbade J, Krautz C, Aupperle H, Ullmann C, Lehmann S, Kempfert J, Borger MA, Dhein S, Gummert JF, Mohr FW: **Functional, metabolic, and morphological aspects of continuous, normothermic heart preservation: effects of different preparation and perfusion techniques**. *Tissue engineering Part C, Methods* 2009, **15**(2):275-283.
109. Garcia Saez D, Zych B, Sabashnikov A, Bowles CT, De Robertis F, Mohite PN, Popov AF, Maunz O, Patil NP, Weymann A *et al*: **Evaluation of the organ care system in heart transplantation with an adverse donor/recipient profile**. *The Annals of thoracic surgery* 2014, **98**(6):2099-2105; discussion 2105-2096.
110. Iyer A, Gao L, Doyle A, Rao P, Cropper JR, Soto C, Dinale A, Kumarasinghe G, Jabbour A, Hicks M *et al*: **Normothermic ex vivo perfusion provides superior organ preservation and enables viability assessment of hearts from DCD donors**. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2015, **15**(2):371-380.
111. White CW, Hasanally D, Mundt P, Li Y, Klein J, Xiang B, Müller A, Ambrose E, Ravandi A, Arora RC *et al*: **A wholeblood–based perfusate provides superior preservation of myocardial function during ex vivo heart perfusion**. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* 2014, **S1053-2498**(14):01356-01354.

112. Brant SM, Cobert ML, West LM, Shelton JM, Jessen ME, Peltz M: **Characterizing cardiac donation after circulatory death: implications for perfusion preservation**. *The Annals of thoracic surgery* 2014, **98**(6):2107-2113; discussion 2113-2104.
113. Rosenfeldt F, Ou R, Salamonsen R, Marasco S, Zimmet A, Byrne J, Cosic F, Saxena P, Esmore D: **A novel combination technique of cold crystalloid perfusion but not cold storage facilitates transplantation of canine hearts donated after circulatory death**. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* 2016, **35**(11):1358-1364.
114. Schraufnagel DP, Steffen RJ, Vargo PR, Attia T, Elgharably H, Hasan SM, Bribriesco A, Wierup P: **Devices for ex vivo heart and lung perfusion**. *Expert review of medical devices* 2018, **15**(3):183-191.
115. Lucchinetti E, Lou PH, Hatami S, Qi X, Clanachan AS, Freed DH, Zaugg M: **Enhanced myocardial protection in cardiac donation after circulatory death using Intralipid((R)) postconditioning in a porcine model**. *Canadian journal of anaesthesia = Journal canadien d'anesthesie* 2019.
116. White C, Ambrose E, Muller A, Hatami S, Li Y, Le H, Thliveris J, Arora R, Lee T, Dixon I *et al*: **Impact of reperfusion calcium and pH on the resuscitation of hearts donated after circulatory death**. *The Annals of thoracic surgery* 2017, **103**(1):122-130.
117. Page A, Messer S, Large SR: **Heart transplantation from donation after circulatory determined death**. *Annals of cardiothoracic surgery* 2018, **7**(1):75-81.
118. Michel SG, La Muraglia GM, 2nd, Madariaga ML, Titus JS, Selig MK, Farkash EA, Allan JS, Anderson LM, Madsen JC: **Twelve-hour hypothermic machine perfusion for donor heart preservation leads to improved ultrastructural characteristics compared to conventional cold storage**. *Annals of transplantation : quarterly of the Polish Transplantation Society* 2015, **20**:461-468.
119. Tolboom H, Olejnickova V, Reser D, Rosser B, Wilhelm MJ, Gassmann M, Bogdanova A, Falk V: **Moderate hypothermia during ex vivo machine perfusion promotes recovery of hearts donated after cardiocirculatory deathdagger**. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* 2016, **49**(1):25-31.
120. Bishawi M, Roan J-N, Milano CA, Daneshmand MA, Schroder JN, Chiang Y, Lee FH, Brown ZD, Nevo A, Watson MJ *et al*: **A normothermic ex vivo organ perfusion delivery method for cardiac transplantation gene therapy**. *Scientific reports* 2019, **9**(1):8029.
121. Church JT, Alghanem F, Deatrick KB, Trahanas JM, Phillips JP, Hee Song M, Perkins EM, Bartlett RH, Rojas-Pena A, Bocks ML *et al*: **Normothermic ex vivo heart perfusion: effects of live animal blood and plasma cross circulation**. *ASAIO journal (American Society for Artificial Internal Organs : 1992)* 2017, **63**(6):766-773.
122. Trahanas JM, Witer LJ, Alghanem F, Bryner BS, Iyengar A, Hirschl JR, Hoenerhoff MJ, Potkay JA, Bartlett RH, Rojas-Pena A *et al*: **Achieving 12 hour normothermic ex situ heart perfusion: an experience of 40 porcine hearts**. *ASAIO journal (American Society for Artificial Internal Organs : 1992)* 2016, **62**(4):470-476.
123. Messer S, Page A, Axell R, Berman M, Hernandez-Sanchez J, Colah S, Parizkova B, Valchanov K, Dunning J, Pavlushkov E *et al*: **Outcome after heart transplantation from donation after circulatory-determined death donors**. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* 2017, **36**(12):1311-1318.
124. Taylor MJ: **Hypothermic blood substitution: special considerations for protection of cells during ex vivo and in vivo preservation**. *Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie* 2007, **34**:226-244.

125. Giannelli S, Jr., McKenna JP, Bordiuk JM, Miller LD, Jerome CR: **Prevention of increased hemoglobin-oxygen affinity in open-heart operations with inosine-phosphate-pyruvate solution**. *The Annals of thoracic surgery* 1976, **21**(5):386-396.
126. Vercaemst L: **Hemolysis in cardiac surgery patients undergoing cardiopulmonary bypass: a review in search of a treatment algorithm**. *The journal of extra-corporeal technology* 2008, **40**(4):257-267.
127. Schroder JN, D'Alessandro D, Esmailian F, Boeve T, Tang P, Liao K, Wang I, Anyanwu A, Shah A, Mudy K *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.
128. Dhital KK, Iyer A, Connellan M, Chew HC, Gao L, Doyle A, Hicks M, Kumarasinghe G, Soto C, Dinale A *et al*: **Adult heart transplantation with distant procurement and ex-vivo preservation of donor hearts after circulatory death: a case series**. *Lancet (London, England)* 2015, **385**(9987):2585-2591.
129. Hamed A, Tsui S, Huber J, Lin R, . Poggio E, Ardehali4 A: **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 : the official publication of the International Society for Heart Transplantation* 2009, **28**(2):S71.
130. Stamp NL, Shah A, Vincent V, Wright B, Wood C, Pavey W, Cokis C, Chih S, Dembo L, Larbalestier R: **Successful heart transplant after ten hours out-of-body time using the TransMedics Organ Care System**. *Heart, lung & circulation* 2015, **24**(6):611-613.
131. Page A, Messer S, Axell R, Naruka V, Colah S, Fakelman S, Ellis C, Abu-Omar Y, Ali A, Berman M *et al*: **Does the assessment of DCD donor hearts on the Organ Care System using lactate need redefining?** *The Journal of Heart and Lung Transplantation* 2017, **36**(4):S16-S17.
132. Freed DH, White CW: **Donor heart preservation: straight up, or on the rocks?** *Lancet (London, England)* 2015, **385**(9987):2552-2554.
133. National Institude for Health and Care Excellence. In: *OCS Heart system for heart transplant.* London; 2016.
134. Sandha JK, White CW, Muller A, Avery E, Thliveris J, Dixon IMC, Arora RC, Tian G, Hryshko LV, Nagendran J *et al*: **Steroids limit myocardial edema during ex vivo perfusion of hearts donated after circulatory death**. *The Annals of thoracic surgery* 2018, **105**(6):1763-1770.
135. Ferdinandy P, Panas D, Schulz R: **Peroxynitrite contributes to spontaneous loss of cardiac efficiency in isolated working rat hearts**. *The American journal of physiology* 1999, **276**(6):H1861-1867.
136. Cobert ML, Peltz M, West LM, Merritt ME, Jessen ME: **Glucose is an ineffective substrate for preservation of machine perfused donor hearts**. *The Journal of surgical research* 2012, **173**(2):198-205.
137. Jahania MS, Sanchez JA, Narayan P, Lasley RD, Mentzer RM, Jr.: **Heart preservation for transplantation: principles and strategies**. *The Annals of thoracic surgery* 1999, **68**(5):1983-1987.
138. McDonald CI, Fraser JF, Coombes JS, Fung YL: **Oxidative stress during extracorporeal circulation**. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* 2014, **46**(6):937-943.
139. Millar JE, Fanning JP, McDonald CI, McAuley DF, Fraser JF: **The inflammatory response to extracorporeal membrane oxygenation (ECMO): a review of the pathophysiology**. *Critical care (London, England)* 2016, **20**(1):387.

140. Al-Fares A, Pettenuzzo T, Del Sorbo L: **Extracorporeal life support and systemic inflammation**. 2019, **7**(Suppl 1):46.
141. Hatami S, White C, Qi X, Buchko M, Ondrus M, Kinnear A, Himmat S, Sergi C, Nagendran J, Freed D: **Immunity and stress responses are induced during ex-situ heart perfusion**. *Circ Heart Fail* 2020, **13**(6):e006552.
142. Chen Q, Yu W, Shi J, Shen J, Hu Y, Gong J, Li J, Li N: **The effect of extracorporeal membrane oxygenation therapy on systemic oxidative stress injury in a porcine model**. *Artificial organs* 2014, **38**(5):426-431.
143. Mc IRB, Timpa JG, Kurundkar AR, Holt DW, Kelly DR, Hartman YE, Neel ML, Karnatak RK, Schelonka RL, Anantharamaiah GM *et al*: **Plasma concentrations of inflammatory cytokines rise rapidly during ECMO-related SIRS due to the release of preformed stores in the intestine**. *Laboratory investigation; a journal of technical methods and pathology* 2010, **90**(1):128-139.
144. Graulich J, Walzog B, Marcinkowski M, Bauer K, Kössel H, Fuhrmann G, Bührer C, Gaehtgens P, Versmold HT: **Leukocyte and endothelial activation in a laboratory model of extracorporeal membrane oxygenation (ECMO)**. *Pediatric Research* 2000, **48**(5):679-684.
145. Golej J, Winter P, Schoffmann G, Kahlbacher H, Stoll E, Boigner H, Trittenwein G: **Impact of extracorporeal membrane oxygenation modality on cytokine release during rescue from infant hypoxia**. *Shock (Augusta, Ga)* 2003, **20**(2):110-115.
146. Passmore MR, Fung YL, Simonova G, Foley SR, Dunster KR, Diab SD, Tung JP, Minchinton RM, McDonald CI, Anstey CM *et al*: **Inflammation and lung injury in an ovine model of extracorporeal membrane oxygenation support**. *American journal of physiology Lung cellular and molecular physiology* 2016, **311**(6):L1202-l1212.
147. Wang S, Krawiec C, Patel S, Kunselman AR, Song J, Lei F, Baer LD, Undar A: **Laboratory evaluation of hemolysis and systemic inflammatory response in neonatal nonpulsatile and pulsatile extracorporeal life support systems**. *Artificial organs* 2015, **39**(9):774-781.
148. Thangappan K, Cavarocchi NC, Baram M, Thoma B, Hirose H: **Systemic inflammatory response syndrome (SIRS) after extracorporeal membrane oxygenation (ECMO): Incidence, risks and survivals**. *Heart & lung : the journal of critical care* 2016, **45**(5):449-453.
149. Hirthler M, Simoni J, Dickson M: **Elevated levels of endotoxin, oxygen-derived free radicals, and cytokines during extracorporeal membrane oxygenation**. *Journal of pediatric surgery* 1992, **27**(9):1199-1202.
150. Fortenberry JD, Bhardwaj V, Niemer P, Cornish JD, Wright JA, Bland L: **Neutrophil and cytokine activation with neonatal extracorporeal membrane oxygenation**. *The Journal of pediatrics* 1996, **128**(5 Pt 1):670-678.
151. Vitkova V, Panek M, Janec P, Sibikova M, Vobruba V, Haluzik M, Zivny J, Janota J: **Endothelial Microvesicles and Soluble Markers of Endothelial Injury in Critically Ill Newborns**. 2018, **2018**:1975056.
152. Vallhonrat H, Swinford RD, Ingelfinger JR, Williams WW, Ryan DP, Tolkoff-Rubin N, Cosimi AB, Pascual M: **Rapid activation of the alternative pathway of complement by extracorporeal membrane oxygenation**. *ASAIO journal (American Society for Artificial Internal Organs : 1992)* 1999, **45**(1):113-114.
153. Ohata T, Sawa Y, Kadoba K, Taniguchi K, Ichikawa H, Masai T, Shimazaki Y, Matsuda H: **Normothermia has beneficial effects in cardiopulmonary bypass attenuating inflammatory reactions**. *ASAIO journal (American Society for Artificial Internal Organs : 1992)* 1995, **41**(3):M288-291.
154. Halter J, Steinberg J, Fink G, Lutz C, Picone A, Maybury R, Fedors N, DiRocco J, Lee HM, Nieman G: **Evidence of systemic cytokine release in patients undergoing cardiopulmonary bypass**. *The journal of extra-corporeal technology* 2005, **37**(3):272-277.

155. Ito H, Hamano K, Gohra H, Katoh T, Fujimura Y, Tsuboi H, Esato K: **Relationship between respiratory distress and cytokine response after cardiopulmonary bypass**. *Surgery today* 1997, **27**(3):220-225.
156. Stassano P, Di Tommaso L, Monaco M, Iesu S, Brando G, Buonpane S, Ambrosio G, Di Benedetto G, Pepino P: **Myocardial revascularization by left ventricular assisted beating heart is associated with reduced systemic inflammatory response**. *The Annals of thoracic surgery* 2009, **87**(1):46-52.
157. Chen YF, Tsai WC, Lin CC, Tsai LY, Lee CS, Huang CH, Pan PC, Chen ML: **Effect of leukocyte depletion on endothelial cell activation and transendothelial migration of leukocytes during cardiopulmonary bypass**. *The Annals of thoracic surgery* 2004, **78**(2):634-642; discussion 642-633.
158. Liu JH, Shen JM, Li L, Chang YT: **[Effects of fentanyl on cytokines and MDA during cardiopulmonary bypass in patients undergoing valve replacement]**. *Zhong nan da xue xue bao Yi xue ban = Journal of Central South University Medical sciences* 2005, **30**(1):80-83.
159. Clermont G, Vergely C, Jazayeri S, Lahet JJ, Goudeau JJ, Lecour S, David M, Rochette L, Girard C: **Systemic free radical activation is a major event involved in myocardial oxidative stress related to cardiopulmonary bypass**. *Anesthesiology* 2002, **96**(1):80-87.
160. Chenoweth DE, Cooper SW, Hugli TE, Stewart RW, Blackstone EH, Kirklin JW: **Complement activation during cardiopulmonary bypass: evidence for generation of C3a and C5a anaphylatoxins**. *The New England journal of medicine* 1981, **304**(9):497-503.
161. Steinberg JB, Kapelanski DP, Olson JD, Weiler JM: **Cytokine and complement levels in patients undergoing cardiopulmonary bypass**. *The Journal of thoracic and cardiovascular surgery* 1993, **106**(6):1008-1016.
162. Deng MC, Wiedner M, Erren M, Mollhoff T, Assmann G, Scheld HH: **Arterial and venous cytokine response to cardiopulmonary bypass for low risk CABG and relation to hemodynamics**. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* 1995, **9**(1):22-29.
163. McBride WT, Armstrong MA, Gilliland H, McMurray TJ: **The balance of pro and anti-inflammatory cytokines in plasma and bronchoalveolar lavage (BAL) at paediatric cardiac surgery**. *Cytokine* 1996, **8**(9):724-729.
164. Haeffner-Cavaillon N, Roussellier N, Ponzio O, Carreno MP, Laude M, Carpentier A, Kazatchkine MD: **Induction of interleukin-1 production in patients undergoing cardiopulmonary bypass**. *The Journal of thoracic and cardiovascular surgery* 1989, **98**(6):1100-1106.
165. Hosgood SA, Moore T, Kleverlaan T, Adams T, Nicholson ML: **Haemoadsorption reduces the inflammatory response and improves blood flow during ex vivo renal perfusion in an experimental model**. *Journal of Translational Medicine* 2017, **15**(1):216.
166. Iskender I, Cosgun T, Arni S, Trinkwitz M, Fehlings S, Yamada Y, Cesarovic N, Yu K, Frauenfelder T, Jungraithmayr W *et al*: **Cytokine filtration modulates pulmonary metabolism and edema formation during ex vivo lung perfusion**. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* 2017.
167. Noda K, Tane S, Haam SJ, D'Cunha J, Hayanga AJ, Luketich JD, Shigemura N: **Targeting circulating leukocytes and pyroptosis during ex vivo lung perfusion improves lung preservation**. *Transplantation* 2017, **101**(12):2841-2849.
168. Seewald M, Coles JA, Jr., Sigg DC, Iaizzo PA: **Featured Article: Pharmacological postconditioning with delta opioid attenuates myocardial reperfusion injury in isolated porcine hearts**. *Experimental biology and medicine (Maywood, NJ)* 2017, **242**(9):986-995.
169. Hatami S, Qi X, White C, Buchko M, Bozso S, Himmat S, Nagendran J, Freed D: **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.
170. Zakkar M, Guida G, Suleiman MS, Angelini GD: **Cardiopulmonary bypass and oxidative stress**. *Oxidative medicine and cellular longevity* 2015, **2015**:189863.
171. Pathak M, Kaira BG, Slater A, Emsley J: **Cell receptor and cofactor interactions of the contact activation system and factor XI**. *Frontiers in medicine* 2018, **5**:66.
172. Doyle AJ, Hunt BJ: **Current understanding of how extracorporeal membrane oxygenators activate haemostasis and other blood components**. *Frontiers in medicine* 2018, **5**:352.
173. Datzmann T, Trager K: **Extracorporeal membrane oxygenation and cytokine adsorption**. *Journal of thoracic disease* 2018, **10**(Suppl 5):S653-s660.
174. Raffaeli G, Ghirardello S, Passera S, Mosca F, Cavallaro G: **Oxidative stress and neonatal respiratory extracorporeal membrane oxygenation**. *Frontiers in physiology* 2018, **9**(1739).
175. Ranucci M, Baryshnikova E: **Inflammation and coagulation following minimally invasive extracorporeal circulation technologies**. *Journal of thoracic disease* 2019, **11**(Suppl 10):S1480-s1488.
176. Warren OJ, Smith AJ, Alexiou C, Rogers PL, Jawad N, Vincent C, Darzi AW, Athanasiou T: **The inflammatory response to cardiopulmonary bypass: part 1--mechanisms of pathogenesis**. *Journal of cardiothoracic and vascular anesthesia* 2009, **23**(2):223-231.
177. Graulich J, Sonntag J, Marcinkowski M, Bauer K, Kossel H, Buhrer C, Obladen M, Versmold HT: **Complement activation by in vivo neonatal and in vitro extracorporeal membrane oxygenation**. *Mediators of inflammation* 2002, **11**(2):69-73.
178. Gemmell CH, Ramirez SM, Yeo EL, Sefton MV: **Platelet activation in whole blood by artificial surfaces: identification of platelet-derived microparticles and activated platelet binding to leukocytes as material-induced activation events**. *The Journal of laboratory and clinical medicine* 1995, **125**(2):276-287.
179. Gorbet MB, Sefton MV: **Biomaterial-associated thrombosis: roles of coagulation factors, complement, platelets and leukocytes**. *Biomaterials* 2004, **25**(26):5681-5703.
180. Weerasinghe A, Taylor KM: **The platelet in cardiopulmonary bypass**. *The Annals of thoracic surgery* 1998, **66**(6):2145-2152.
181. Rinder CS, Gaal D, Student LA, Smith BR: **Platelet-leukocyte activation and modulation of adhesion receptors in pediatric patients with congenital heart disease undergoing cardiopulmonary bypass**. *The Journal of thoracic and cardiovascular surgery* 1994, **107**(1):280-288.
182. Passaroni AC, Felicio ML, Campos N, Silva MAM, Yoshida WB: **Hemolysis and inflammatory response to extracorporeal circulation during On-Pump CABG: comparison between roller and centrifugal pump systems**. *Brazilian journal of cardiovascular surgery* 2018, **33**(1):64-71.
183. Keyser A, Hilker MK, Diez C, Philipp A, Foltan M, Schmid C: **Prospective randomized clinical study of arterial pumps used for routine on pump coronary bypass grafting**. *Artificial organs* 2011, **35**(5):534-542.
184. Van Avondt K, Nur E, Zeerleder S: **Mechanisms of haemolysis-induced kidney injury**. *Nature reviews Nephrology* 2019, **15**(11):671-692.
185. Ricci Z: **Hemolysis during pediatric cardiac surgery: an old issue with renewed concerns**. *Journal of Laboratory and Precision Medicine* 2018, **3**(2).
186. Vermeulen Windsant IC, Hanssen SJ, Buurman WA, Jacobs MJ: **Cardiovascular surgery and organ damage: time to reconsider the role of hemolysis**. *The Journal of thoracic and cardiovascular surgery* 2011, **142**(1):1-11.

187. Mariani AW, Pego-Fernandes PM, Abdalla LG, Jatene FB: **Ex vivo lung reconditioning: a new era for lung transplantation**. *Jornal brasileiro de pneumologia : publicacao oficial da Sociedade Brasileira de Pneumologia e Tisilogia* 2012, **38**(6):776-785.
188. Wu ZJ, Gartner M, Litwak KN, Griffith BP: **Progress toward an ambulatory pump-lung**. *The Journal of thoracic and cardiovascular surgery* 2005, **130**(4):973-978.
189. Cypel M, Yeung JC, Hirayama S, Rubacha M, Fischer S, Anraku M, Sato M, Harwood S, Pierre A, Waddell TK *et al*: **Technique for prolonged normothermic ex vivo lung perfusion**. *The Journal of Heart and Lung Transplantation* 2008, **27**(12):1319-1325.
190. Kappler B, Ledezma CA, van Tuijl S, Meijborg V, Boukens BJ, Ergin B, Tan PJ, Stijnen M, Ince C, Díaz-Zuccarini V *et al*: **Investigating the physiology of normothermic ex vivo heart perfusion in an isolated slaughterhouse porcine model used for device testing and training**. *BMC Cardiovascular Disorders* 2019, **19**(1):254.
191. Karsten E, Breen E, Herbert BR: **Red blood cells are dynamic reservoirs of cytokines**. *Scientific reports* 2018, **8**(1):3101.
192. Tang J, Tao K, Zhou J, Zhang C, Gong L, Luo N, Du L: **Long-term leukocyte filtration should be avoided during extracorporeal circulation**. *Mediators of inflammation* 2013, **2013**:612848.
193. Alexiou C, Sheppard S, Tang A, Rengarajan A, Smith D, Haw M, Gibbs R: **Leukocytes-depleting filters preferentially remove activated leukocytes and reduce the expression of surface adhesion molecules during the simulated extracorporeal circulation of human blood**. *ASAIO journal (American Society for Artificial Internal Organs : 1992)* 2006, **52**(4):438-444.
194. Warren O, Alexiou C, Massey R, Leff D, Purkayastha S, Kinross J, Darzi A, Athanasiou T: **The effects of various leukocyte filtration strategies in cardiac surgery**. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* 2007, **31**(4):665-676.
195. Aboelnazar NS, Himmat S, Hatami S, White CW, Burhani MS, Dromparis P, Matsumura N, Tian G, Dyck JRB, Mengel M *et al*: **Negative pressure ventilation decreases inflammation and lung edema during normothermic ex-vivo lung perfusion**. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* 2018, **37**(4):520-530.
196. Brown RA, Leung E, Kankaanranta H, Moilanen E, Page CP: **Effects of heparin and related drugs on neutrophil function**. *Pulmonary pharmacology & therapeutics* 2012, **25**(2):185-192.
197. Farneti PA, Sbrana S, Spiller D, Cerillo AG, Santarelli F, Di Dario D, Del Sarto PA, Glauber M: **Reduction of blood coagulation and monocyte-platelet interaction following the use of a minimal extracorporeal circulation system (Synergy) in coronary artery bypass grafting (CABG)**. *Perfusion* 2008, **23**(1):49-56.
198. Sbrana S, Parri MS, De Filippis R, Gianetti J, Clerico A: **Monitoring of monocyte functional state after extracorporeal circulation: a flow cytometry study**. *Cytometry Part B, Clinical cytometry* 2004, **58**(1):17-24.
199. Rinder CS, Rinder HM, Smith MJ, Fitch JC, Tracey JB, Chandler WL, Rollins SA, Smith BR: **Antithrombin reduces monocyte and neutrophil CD11b up regulation in addition to blocking platelet activation during extracorporeal circulation**. *Transfusion* 2006, **46**(7):1130-1137.
200. Rinder CS, Rinder HM, Johnson K, Smith M, Lee DL, Tracey J, Polack G, Higgins P, Yeh CG, Smith BR: **Role of C3 cleavage in monocyte activation during extracorporeal circulation**. *Circulation* 1999, **100**(5):553-558.
201. Mossanen JC, Jansen TU, Pracht J, Liepelt A, Buendgens L, Stoppe C, Goetzenich A, Simon TP, Autschbach R, Marx G *et al*: **Elevated circulating CD14(++)CD16(+) intermediate monocytes are independently associated with extracardiac complications after cardiac surgery**. *Scientific reports* 2020, **10**(1):947.

202. Philippidis P, Athanasiou T, Nadra I, Ashrafian H, Haskard DO, Landis RC, Taylor KM: **Anti-inflammatory haemoglobin scavenging monocytes are induced following coronary artery bypass surgery**. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* 2010, **37**(6):1360-1366.
203. Perttila J, Salo M, Pirttikangas CO, Jalonen J, Vainio O: **Effects of cardiopulmonary bypass on lymphocytes and their subset counts with or without use of autotransfusion devices**. *Journal of cardiothoracic and vascular anesthesia* 1994, **8**(5):532-535.
204. Franke A, Lante W, Kurig E, Zoller LG, Weinhold C, Markewitz A: **Hyporesponsiveness of T cell subsets after cardiac surgery: a product of altered cell function or merely a result of absolute cell count changes in peripheral blood?** *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* 2006, **30**(1):64-71.
205. Panagiotopoulos I, Palatianos G, Michalopoulos A, Chatzigeorgiou A, Prapas S, Kamper EF: **Alterations in biomarkers of endothelial function following on-pump coronary artery revascularization**. *Journal of clinical laboratory analysis* 2010, **24**(6):389-398.
206. Giacinto O, Satriano U, Nenna A, Spadaccio C, Lusini M, Mastroianni C, Nappi F, Chello M: **Inflammatory response and endothelial dysfunction following cardiopulmonary bypass: pathophysiology and pharmacological targets**. *Recent patents on inflammation & allergy drug discovery* 2019, **13**(2):158-173.
207. Jacob-Ferreira AL, Schulz R: **Activation of intracellular matrix metalloproteinase-2 by reactive oxygen-nitrogen species: Consequences and therapeutic strategies in the heart**. *Archives of biochemistry and biophysics* 2013, **540**(1-2):82-93.
208. Spoelstra-de Man AM, Smit B, Oudemans-van Straaten HM, Smulders YM: **Cardiovascular effects of hyperoxia during and after cardiac surgery**. *Anaesthesia* 2015, **70**(11):1307-1319.
209. Caputo M, Mokhtari A, Rogers CA, Panayiotou N, Chen Q, Ghorbel MT, Angelini GD, Parry AJ: **The effects of normoxic versus hyperoxic cardiopulmonary bypass on oxidative stress and inflammatory response in cyanotic pediatric patients undergoing open cardiac surgery: a randomized controlled trial**. *The Journal of thoracic and cardiovascular surgery* 2009, **138**(1):206-214.
210. Caputo M, Mokhtari A, Miceli A, Ghorbel MT, Angelini GD, Parry AJ, Suleiman SM: **Controlled reoxygenation during cardiopulmonary bypass decreases markers of organ damage, inflammation, and oxidative stress in single-ventricle patients undergoing pediatric heart surgery**. *The Journal of thoracic and cardiovascular surgery* 2014, **148**(3):792-801.e798; discussion 800-791.
211. Trittenwein G, Rotta AT, Gunnarsson B, Steinhorn DM: **Lipid peroxidation during initiation of extracorporeal membrane oxygenation after hypoxia in endotoxemic rabbits**. *Perfusion* 1999, **14**(1):49-57.
212. Fujii Y, Tatsumi E, Nakamura F, Oite T: **PaO 2 greater than 300 mmHg promotes an inflammatory response during extracorporeal circulation in a rat extracorporeal membrane oxygenation model**. *Journal of thoracic disease* 2020, **12**(3):749-757.
213. Sznycer-Taub NR, Lowery R, Yu S, Owens ST, Hirsch-Romano JC, Owens GE: **Hyperoxia Is Associated With Poor Outcomes in Pediatric Cardiac Patients Supported on Venoarterial Extracorporeal Membrane Oxygenation**. *Pediatric critical care medicine : a journal of the Society of Critical Care Medicine and the World Federation of Pediatric Intensive and Critical Care Societies* 2016, **17**(4):350-358.
214. Cashen K, Reeder R, Dalton HJ, Berg RA, Shanley TP, Newth CJL, Pollack MM, Wessel D, Carcillo J, Harrison R *et al*: **Hyperoxia and hypocapnia during pediatric extracorporeal membrane oxygenation: associations with complications, mortality, and functional status among**

survivors. *Pediatric critical care medicine : a journal of the Society of Critical Care Medicine and the World Federation of Pediatric Intensive and Critical Care Societies* 2018, **19**(3):245-253.
215. Hayes RA, Shekar K, Fraser JF: **Is hyperoxaemia helping or hurting patients during extracorporeal membrane oxygenation? Review of a complex problem**. *Perfusion* 2013, **28**(3):184-193.
216. Kuzmiak-Glancy S, Jaimes R, 3rd, Wengrowski AM, Kay MW: **Oxygen demand of perfused heart preparations: how electromechanical function and inadequate oxygenation affect physiology and optical measurements**. *Experimental physiology* 2015, **100**(6):603-616.
217. Sanada S, Komuro I, Kitakaze M: **Pathophysiology of myocardial reperfusion injury: preconditioning, postconditioning, and translational aspects of protective measures**. *American journal of physiology Heart and circulatory physiology* 2011, **301**(5):H1723-1741.
218. Soares ROS, Losada DM, Jordani MC, Evora P, Castro ESO: **Ischemia/reperfusion injury revisited: an overview of the latest pharmacological strategies**. *International journal of molecular sciences* 2019, **20**(20).
219. Salameh A, Dhein S: **Strategies for pharmacological organoprotection during extracorporeal circulation targeting ischemia-reperfusion injury**. *Frontiers in pharmacology* 2015, **6**:296.
220. Charles EJ, Hunter Mehaffey J, Huerter ME, Sharma AK, Stoler MH, Roeser ME, Walters DM, Tribble CG, Kron IL, Laubach VE: **Ex vivo assessment of porcine donation after circulatory death lungs that undergo increasing warm ischemia times**. *Transplantation direct* 2018, **4**(12):e405.
221. Mollnes TE, Videm V, Gotze O, Harboe M, Oppermann M: **Formation of C5a during cardiopulmonary bypass: inhibition by precoating with heparin**. *The Annals of thoracic surgery* 1991, **52**(1):92-97.
222. Mahmood S, Bilal H, Zaman M, Tang A: **Is a fully heparin-bonded cardiopulmonary bypass circuit superior to a standard cardiopulmonary bypass circuit?** *Interactive cardiovascular and thoracic surgery* 2012, **14**(4):406-414.
223. Ontaneda A, Annich GM: **Novel surfaces in extracorporeal membrane oxygenation circuits**. *Frontiers in medicine* 2018, **5**:321.
224. Vohra HA, Whistance R, Modi A, Ohri SK: **The inflammatory response to miniaturised extracorporeal circulation: a review of the literature**. *Mediators of inflammation* 2009, **2009**:707042.
225. Williams DC, Turi JL, Hornik CP, Bonadonna DK, Williford WL, Walczak RJ, Watt KM, Cheifetz IM: **Circuit oxygenator contributes to extracorporeal membrane oxygenation-induced hemolysis**. *ASAIO journal (American Society for Artificial Internal Organs : 1992)* 2015, **61**(2):190-195.
226. Day JR, Taylor KM: **The systemic inflammatory response syndrome and cardiopulmonary bypass**. *International journal of surgery (London, England)* 2005, **3**(2):129-140.
227. de Amorim CG, Malbouisson LM, da Silva FC, Jr., Fiorelli AI, Murakami CK, Carmona MJ: **Leukocyte depletion during CPB: effects on inflammation and lung function**. *Inflammation* 2014, **37**(1):196-204.
228. Karaiskos TE, Palatianos GM, Triantafillou CD, Kantidakis GH, Astras GM, Papadakis EG, Vassili MI: **Clinical effectiveness of leukocyte filtration during cardiopulmonary bypass in patients with chronic obstructive pulmonary disease**. *The Annals of thoracic surgery* 2004, **78**(4):1339-1344.
229. Boodram S, Evans E: **Use of leukocyte-depleting filters during cardiac surgery with cardiopulmonary bypass: a review**. *The journal of extra-corporeal technology* 2008, **40**(1):27-42.
230. Dvorak L, Pirk J, Cerny S, Kovar J: **The role of leukocyte depleting filters in heart transplantation: early outcomes in prospective, randomized clinical trial**. *European Journal of Cardio-Thoracic Surgery* 2006, **30**(4):621-627.

231. Jackson HT, Oyetunji TA, Thomas A, Oyetunji AO, Hamrick M, Nadler EP, Wong E, Qureshi FG: **The impact of leukoreduced red blood cell transfusion on mortality of neonates undergoing extracorporeal membrane oxygenation**. *The Journal of surgical research* 2014, **192**(1):6-11.
232. Canter MO, Daniels J, Bridges BC: **Adjunctive therapies during extracorporeal membrane oxygenation to enhance multiple organ support in critically ill children**. In: *Front Pediatr.* vol. 6; 2018: 78.
233. Tang J, Tao K, Zhou J, Zhang C, Gong L, Luo N, Du L: **Long-term leukocyte filtration should be avoided during extracorporeal circulation**. *Mediators of inflammation* 2013, **2013**:612848.
234. Warren OJ, Watret AL, de Wit KL, Alexiou C, Vincent C, Darzi AW, Athanasiou T: **The inflammatory response to cardiopulmonary bypass: part 2--anti-inflammatory therapeutic strategies**. *Journal of cardiothoracic and vascular anesthesia* 2009, **23**(3):384-393.
235. Chaney MA: **Corticosteroids and cardiopulmonary bypass : a review of clinical investigations**. *Chest* 2002, **121**(3):921-931.
236. Anic D, Gasparovic H, Ivancan V, Batinic D: **Effects of corticosteroids on inflammatory response following cardiopulmonary bypass**. *Croatian medical journal* 2004, **45**(2):158-161.
237. Celik JB, Gormus N, Okesli S, Gormus ZI, Solak H: **Methylprednisolone prevents inflammatory reaction occurring during cardiopulmonary bypass: effects on TNF-alpha, IL-6, IL-8, IL-10**. *Perfusion* 2004, **19**(3):185-191.
238. Giomarelli P, Scolletta S, Borrelli E, Biagioli B: **Myocardial and lung injury after cardiopulmonary bypass: role of interleukin (IL)-10**. *The Annals of thoracic surgery* 2003, **76**(1):117-123.
239. Demir T, Ergenoglu MU, Demir HB, Tanrikulu N, Sahin M, Gok E, Korkut K, Demirsoy E: **Pretreatment with methylprednisolone improves myocardial protection during on-pump coronary artery bypass surgery**. *The heart surgery forum* 2015, **18**(4):E171-177.
240. Liakopoulos OJ, Schmitto JD, Kazmaier S, Brauer A, Quintel M, Schoendube FA, Dorge H: **Cardiopulmonary and systemic effects of methylprednisolone in patients undergoing cardiac surgery**. *The Annals of thoracic surgery* 2007, **84**(1):110-118; discussion 118-119.
241. Fillinger MP, Rassias AJ, Guyre PM, Sanders JH, Beach M, Pahl J, Watson RB, Whalen PK, Yeo KT, Yeager MP: **Glucocorticoid effects on the inflammatory and clinical responses to cardiac surgery**. *Journal of cardiothoracic and vascular anesthesia* 2002, **16**(2):163-169.
242. Halonen J, Halonen P, Jarvinen O, Taskinen P, Auvinen T, Tarkka M, Hippelainen M, Juvonen T, Hartikainen J, Hakala T: **Corticosteroids for the prevention of atrial fibrillation after cardiac surgery: a randomized controlled trial**. *Jama* 2007, **297**(14):1562-1567.
243. Kilger E, Weis F, Briegel J, Frey L, Goetz AE, Reuter D, Nagy A, Schuetz A, Lamm P, Knoll A *et al*: **Stress doses of hydrocortisone reduce severe systemic inflammatory response syndrome and improve early outcome in a risk group of patients after cardiac surgery**. *Critical care medicine* 2003, **31**(4):1068-1074.
244. Bourbon A, Vionnet M, Leprince P, Vaissier E, Copeland J, McDonagh P, Debre P, Gandjbakhch I: **The effect of methylprednisolone treatment on the cardiopulmonary bypass-induced systemic inflammatory response**. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* 2004, **26**(5):932-938.
245. Toft P, Christiansen K, Tonnesen E, Nielsen CH, Lillevang S: **Effect of methylprednisolone on the oxidative burst activity, adhesion molecules and clinical outcome following open heart surgery**. *Scandinavian cardiovascular journal : SCJ* 1997, **31**(5):283-288.
246. Ho KM, Tan JA: **Benefits and risks of corticosteroid prophylaxis in adult cardiac surgery: a dose-response meta-analysis**. *Circulation* 2009, **119**(14):1853-1866.
247. Whitlock RP, Chan S, Devereaux PJ, Sun J, Rubens FD, Thorlund K, Teoh KH: **Clinical benefit of steroid use in patients undergoing cardiopulmonary bypass: a meta-analysis of randomized trials**. *European heart journal* 2008, **29**(21):2592-2600.

248. Robertson-Malt S, Afrane B, El Barbary M: **Prophylactic steroids for pediatric open heart surgery**. *The Cochrane database of systematic reviews* 2007(4):Cd005550.
249. Pappalardo F, Montisci A: **Adjunctive therapies during veno-venous extracorporeal membrane oxygenation**. *Journal of thoracic disease* 2018, **10**(Suppl 5):S683-s691.
250. Puri N, Baram M, N C: **Extracorporeal membrane oxygenation (ECMO), an issue of critical care clinics**. In. Edited by Pfaff K, vol. 33: Elsevier Health Sciences; 2017.
251. Hayes M, Langley M, Langley M, Schneider B, Drake D, Rojas-Peña A, Owens GE, Bartlett R, Haft JW: **Steroid use in ex-vivo normothermic heart perfusion**. In: *American Society for Artificial Internal Organs.* San Francisco, CA, US; 2019.
252. Mahalwar R, Khanna D: **Pleiotropic antioxidant potential of rosuvastatin in preventing cardiovascular disorders**. *European journal of pharmacology* 2013, **711**(1-3):57-62.
253. Morgan C, Zappitelli M, Gill P: **Statin prophylaxis and inflammatory mediators following cardiopulmonary bypass: a systematic review**. *Critical care (London, England)* 2009, **13**(5):R165.
254. Chee YR, Watson RW, McCarthy J, Chughtai JZ, Nolke L, Healy DG: **High dose statin prophylaxis in cardiopulmonary bypass related surgery: clinical utility**. *Journal of cardiothoracic surgery* 2017, **12**(1):20.
255. An J, Shi F, Liu S, Ma J, Ma Q: **Preoperative statins as modifiers of cardiac and inflammatory outcomes following coronary artery bypass graft surgery: a meta-analysis**. *Interactive cardiovascular and thoracic surgery* 2017, **25**(6):958-965.
256. Putzu A, Capelli B, Belletti A, Cassina T, Ferrari E, Gallo M, Casso G, Landoni G: **Perioperative statin therapy in cardiac surgery: a meta-analysis of randomized controlled trials**. *Critical Care* 2016, **20**(1):395.
257. Putzu A, de Carvalho e Silva CMPD, de Almeida JP, Belletti A, Cassina T, Landoni G, Hajjar LA: **Perioperative statin therapy in cardiac and non-cardiac surgery: a systematic review and meta-analysis of randomized controlled trials**. *Annals of Intensive Care* 2018, **8**(1):95.
258. Velez DE, Mestre-Cordero VE, Hermann R, Perego J, Harriet S, Fernandez-Pazos MLM, Mourglia J, Marina-Prendes MG: **Rosuvastatin protects isolated hearts against ischemia-reperfusion injury: role of Akt-GSK-3beta, metabolic environment, and mitochondrial permeability transition pore**. *Journal of physiology and biochemistry* 2020, **76**(1):85-98.
259. Liu CW, Yang F, Cheng SZ, Liu Y, Wan LH, Cong HL: **Rosuvastatin postconditioning protects isolated hearts against ischemia-reperfusion injury: The role of radical oxygen species, PI3K-Akt-GSK-3beta pathway, and mitochondrial permeability transition pore**. *Cardiovascular therapeutics* 2017, **35**(1):3-9.
260. Li H, Zuo J, Tang W: **Phosphodiesterase-4 Inhibitors for the Treatment of Inflammatory Diseases**. *Frontiers in pharmacology* 2018, **9**:1048.
261. Hamamoto M, Suga M, Takahashi Y, Sato Y, Inamori S, Yagihara T, Nakatani T, Kitamura S: **Suppressive effect of phosphodiesterase type 4 inhibition on systemic inflammatory responses after cardiopulmonary bypass**. *Journal of artificial organs : the official journal of the Japanese Society for Artificial Organs* 2006, **9**(3):144-148.
262. Ustunsoy H, Sivrikoz MC, Tarakcioglu M, Bakir K, Guldur E, Celkan MA: **The effects of pentoxifylline on the myocardial inflammation and ischemia-reperfusion injury during cardiopulmonary bypass**. *Journal of cardiac surgery* 2006, **21**(1):57-61.
263. Yamaura K, Akiyoshi K, Irita K, Taniyama T, Takahashi S: **Effects of olprinone, a new phosphodiesterase inhibitor, on gastric intramucosal acidosis and systemic inflammatory responses following hypothermic cardiopulmonary bypass**. *Acta anaesthesiologica Scandinavica* 2001, **45**(4):427-434.

264. Dabbagh A, Rajaei S, Bahadori Monfared A, Keramatinia AA, Omidi K: **Cardiopulmonary bypass, inflammation and how to defy it: focus on pharmacological interventions**. *Iranian journal of pharmaceutical research : IJPR* 2012, **11**(3):705-714.
265. Wollborn J, Siemering S, Steiger C, Buerkle H, Goebel U, Schick MA: **Phosphodiesterase-4 inhibition reduces ECLS-induced vascular permeability and improves microcirculation in a rodent model of extracorporeal resuscitation**. *American journal of physiology Heart and circulatory physiology* 2019, **316**(3):H751-h761.
266. Reffelmann T, Kloner RA: **Phosphodiesterase 5 inhibitors: are they cardioprotective?** *Cardiovascular research* 2009, **83**(2):204-212.
267. Osadchii OE, Woodiwiss AJ, Norton GR: **Contractile responses to selective phosphodiesterase inhibitors following chronic beta-adrenoreceptor activation**. *Pflugers Archiv : European journal of physiology* 2006, **452**(2):155-163.
268. De Silva RJ, Vuylsteke A, Fritchley SJ, Trull AK, Dunning JJ, Wallwork J: **APT070 inhibits complement activation during in vitro cardiopulmonary bypass**. *European Journal of Cardio-Thoracic Surgery* 2006, **30**(1):72-76.
269. Li JS, Jaggers J, Anderson PAW: **The use of TP10, soluble complement receptor 1, in cardiopulmonary bypass**. *Expert Rev Cardiovasc Ther* 2006, **4**(5):649-654.
270. Verrier ED, Shernan SK, Taylor KM, Van de Werf F, Newman MF, Chen JC, Carrier M, Haverich A, Malloy KJ, Adams PX *et al*: **Terminal complement blockade with pexelizumab during coronary artery bypass graft surgery requiring cardiopulmonary bypass: a randomized trial**. *Jama* 2004, **291**(19):2319-2327.
271. Kopp R, Mottaghy K, Kirschfink M: **Mechanism of complement activation during extracorporeal blood-biomaterial interaction: effects of heparin coated and uncoated surfaces**. *ASAIO journal (American Society for Artificial Internal Organs : 1992)* 2002, **48**(6):598-605.
272. Homeister JW, Satoh P, Lucchesi BR: **Effects of complement activation in the isolated heart. Role of the terminal complement components**. *Circulation research* 1992, **71**(2):303-319.
273. Gralinski MR, Wiater BC, Assenmacher AN, Lucchesi BR: **Selective inhibition of the alternative complement pathway by sCR1[desLHR-A] protects the rabbit isolated heart from human complement-mediated damage**. *Immunopharmacology* 1996, **34**(2-3):79-88.
274. Soualmia F, El Amri C: **Serine protease inhibitors to treat inflammation: a patent review (2011-2016)**. *Expert opinion on therapeutic patents* 2018, **28**(2):93-110.
275. Hill GE, Alonso A, Spurzem JR, Stammers AH, Robbins RA: **Aprotinin and methylprednisolone equally blunt cardiopulmonary bypass–induced inflammation in humans**. *The Journal of thoracic and cardiovascular surgery* 1995, **110**(6):1658-1662.
276. Mojcik CF, Levy JH: **Aprotinin and the systemic inflammatory response after cardiopulmonary bypass**. *The Annals of thoracic surgery* 2001, **71**(2):745-754.
277. Putnam JB, Royston D, Chambers AF, Dunbar S, Lemmer JH, Norman P, Travis E, Vaporciyan AA, Yang S, Zacharski LR: **Evaluating the role of serine protease inhibition in the management of tumor micrometastases**. *Oncology (Williston Park, NY)* 2003, **17**(10 Suppl 10):9-30; quiz 31-32.
278. Hsia TY, McQuinn TC, Mukherjee R, Deardorff RL, Squires JE, Stroud RE, Crawford FA, Bradley SM, Reeves ST, Spinale FG: **Effects of aprotinin or tranexamic acid on proteolytic/cytokine profiles in infants after cardiac surgery**. *The Annals of thoracic surgery* 2010, **89**(6):1843-1852; discussion 1852.
279. Mangano DT, Rieves RD, Weiss KD: **Judging the safety of aprotinin**. *The New England journal of medicine* 2006, **355**(21):2261-2262.
280. Prompunt E, Sanit J, Barrère-Lemaire S, Nargeot J, Noordali H, Madhani M, Kumphune S: **The cardioprotective effects of secretory leukocyte protease inhibitor against myocardial ischemia/reperfusion injury**. *Experimental and therapeutic medicine* 2018, **15**(6):5231-5242.

281. Shibata T, Yamamoto F, Suehiro S, Kinoshita H: **Effects of protease inhibitors on postischemic recovery of the heart**. *Cardiovascular Drugs and Therapy* 1997, **11**(4):547-556.
282. Xia Z, Huang Z, Ansley DM: **Large-dose propofol during cardiopulmonary bypass decreases biochemical markers of myocardial injury in coronary surgery patients: a comparison with isoflurane**. *Anesthesia and analgesia* 2006, **103**(3):527-532.
283. Fischer UM, Cox CS, Jr., Allen SJ, Stewart RH, Mehlhorn U, Laine GA: **The antioxidant N-acetylcysteine preserves myocardial function and diminishes oxidative stress after cardioplegic arrest**. *The Journal of thoracic and cardiovascular surgery* 2003, **126**(5):1483-1488.
284. Orhan G, Yapici N, Yuksel M, Sargin M, Şenay Ş, Yalçin AS, Aykaç Z, Aka SA: **Effects of N-acetylcysteine on myocardial ischemia–reperfusion injury in bypass surgery**. *Heart and Vessels* 2006, **21**(1):42-47.
285. Eren N, Cakir O, Oruc A, Kaya Z, Erdinc L: **Effects of N-acetylcysteine on pulmonary function in patients undergoing coronary artery bypass surgery with cardiopulmonary bypass**. *Perfusion* 2003, **18**(6):345-350.
286. Zhou H, Zhou D, Lu J, Wu C, Zhu Z: **Effects of pre–cardiopulmonary bypass administration of dexmedetomidine on cardiac injuries and the inflammatory response in valve replacement surgery with a sevoflurane postconditioning protocol: a pilot study**. *Journal of Cardiovascular Pharmacology* 2019, **74**(2):91-97.
287. Jouybar R, Kabgani H, Kamalipour H, Shahbazi S, Allahyari E, Rasouli M, Akhlaghi S, Shafa M, Ghazinpoor M, Moeinvaziri MT *et al*: **The perioperative effect of ascorbic acid on the inflammatory response in coronary artery bypass graft surgery; a randomized control trial oronary artery bypass graft surgery**. *International Cardiovascular Research Journal* 2012, **6**(1):-.
288. Stanger O, Aigner I, Schimetta W, Wonisch W: **Antioxidant supplementation attenuates oxidative stress in patients undergoing coronary artery bypass graft surgery**. *The Tohoku journal of experimental medicine* 2014, **232**(2):145-154.
289. de Frutos F, Gea A, Hernandez-Estefania R, Rabago G: **Prophylactic treatment with coenzyme Q10 in patients undergoing cardiac surgery: could an antioxidant reduce complications? A systematic review and meta-analysis**. *Interactive cardiovascular and thoracic surgery* 2015, **20**(2):254-259.
290. Ali- Hasan- Al- Saegh S, Mirhosseini SJ, Tahernejad M, Mahdavi P, Shahidzadeh A, Karimi-Bondarabadi AA, Dehghan AM, Rahimizadeh E, Haddad F, Ghodratipour Z *et al*: **Impact of antioxidant supplementations on cardio-renal protection in cardiac surgery: an updated and comprehensive meta-analysis and systematic review**. *Cardiovascular therapeutics* 2016, **34**(5):360-370.
291. Hill A, Clasen KC, Wendt S, Majoros Á G, Stoppe C, Adhikari NKJ: **Effects of Vitamin C on Organ Function in Cardiac Surgery Patients: A Systematic Review and Meta-Analysis**. 2019, **11**(9).
292. Sagach VF, Scrosati M, Fielding J, Rossoni G, Galli C, Visioli F: **The water-soluble vitamin E analogue Trolox protects against ischaemia/reperfusion damage in vitro and ex vivo. A comparison with vitamin E**. *Pharmacological research* 2002, **45**(6):435-439.
293. Alvarez-Ayuso L, Gómez-Heras SG, Jorge E, Guardiola JM, Torralba A, Granado F, Millán I, Roda JR, Calero P, Fernández-García H *et al*: **Vitamin E action on oxidative state, endothelial function and morphology in long-term myocardial preservation**. *Histology and histopathology* 2010, **25**(5):577-587.
294. Yu J, Wang L, Akinyi M, Li Y, Duan Z, Zhu Y, Fan G: **Danshensu protects isolated heart against ischemia reperfusion injury through activation of Akt/ERK1/2/Nrf2 signaling**. *International journal of clinical and experimental medicine* 2015, **8**(9):14793-14804.

295. Gao F, Yao CL, Gao E, Mo QZ, Yan WL, McLaughlin R, Lopez BL, Christopher TA, Ma XL: **Enhancement of glutathione cardioprotection by ascorbic acid in myocardial reperfusion injury**. *The Journal of pharmacology and experimental therapeutics* 2002, **301**(2):543-550.

Chapter 2: Advanced protocol for normothermic *ex-situ* heart perfusion: semi-physiological and semi-anatomical dynamic preservation and evaluation of the donor heart

Sanaz Hatami[1], Christopher W. White[1], Martin Ondrus[1], Xiao Qi[1], Sayed Himmat, Jayan Nagendran[1,4], Darren H. Freed[1,2,3,4]

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

[2]Department of Physiology, Faculty of Medicine, University of Alberta, Edmonton, Alberta, Canada

[3]Department of Biomedical Engineering, Faculty of Medicine, University of Alberta, Edmonton, Alberta, Canada

[4]Canadian National Transplant Research Program

This work has been published as: **Hatami S**, White CW, Ondrus M, Qi X, Buchko M, Himmat S, Lin L, Cameron K, Nobes D, Chung HJ *et al*: **Normothermic *Ex-situ* Heart Perfusion in Working Mode: Assessment of Cardiac Function and Metabolism**. *Journal of visualized experiments : JoVE* 2019(143).

2.1. Abstract

The current standard method for organ preservation (cold storage, CS), exposes the heart to a period of cold ischemia that limits the safe preservation time and increases the risk of adverse post-transplantation outcomes. Moreover, the static nature of CS does not allow for organ evaluation or intervention during the preservation interval. Normothermic *ex-situ* heart perfusion (ESHP) is a novel method for preservation of the donated heart that minimizes cold ischemia by providing oxygenated, nutrient-rich perfusate to the heart. ESHP has been shown to be non-inferior to CS in the preservation of standard-criteria donor hearts and has also facilitated the clinical transplantation of the hearts donated after the circulatory determination of death. Currently, the only available clinical ESHP device perfuses the heart in an unloaded, non-working state, limiting assessments of myocardial performance. Conversely, ESHP in working mode provides the opportunity for comprehensive evaluation of cardiac performance by assessment of functional and metabolic parameters under physiologic conditions. Moreover, earlier experimental studies have suggested that ESHP in working mode may result in improved functional preservation. Here, we describe the protocol for *ex-situ* perfusion of the heart in a large mammal (porcine) model, which is reproducible for different animal models and heart sizes. The software program in this ESHP apparatus allows for real-time and automated control of the pump speed to maintain desired aortic and left atrial pressure and evaluates a variety of functional and electrophysiological parameters with minimal need for supervision/manipulation.

2.2. Introduction

2.2.1. Clinical relevance

While most aspects of cardiac transplantation have evolved significantly since the first heart transplant in 1967, cold storage (CS) remains the standard for donor heart preservation [1]. CS exposes the organ to a period of cold ischemia that limits the safe preservation interval (4-6 hours) and increases the risk of primary graft dysfunction [2-4]. Due to the static nature of CS, assessments of function or therapeutic interventions are not possible in the time between the organ procurement and transplantation. This is a particular limitation in extended criteria donors including hearts donated after circulatory of death (DCD), creating an obstacle for overcoming the considerable gap between demand and the current donor pool [5, 6]. To address this limitation, *ex-situ* heart perfusion has been proposed as a novel, semi-physiologic method of preserving donated hearts, minimizing exposure to cold ischemia by providing oxygenated, nutrient-rich perfusate to the heart during preservation time [1, 7, 8].

2.2.2. *Ex-situ* heart perfusion

One of the most frequently used methods for *ex-situ* examination of the isolated heart is Langendorff perfusion. In this method, introduced by Oskar Langendorff in 1895, the blood flows into the coronary arteries and out the coronary sinus of the isolated heart, with the heart in an empty and beating state [9, 10]. Clinical ESHP in a Langendorff mode with the Transmedics Organ Care System apparatus (OCS) has been shown to be non-inferior to CS in the preservation of standard-criteria donor hearts, [1] and has facilitated the clinical transplantation of DCD hearts [11]. However, there are concerns about the ability of the device to evaluate organ viability, as a number of donor hearts primarily

thought to be transplantable, were discarded after perfusion on the OCS [3]. The OCS supports the heart in the Langendorff (non-working) mode, and thus possesses a limited capacity for evaluation of the pumping function of the heart [3, 12]. A growing body of evidence suggests that functional parameters offer a better way to assess organ viability, suggesting that assessments of cardiac function may become a reliable tool for the evaluation and selection of hearts for transplantation during ESHP [3, 12-14]. Furthermore, our studies on *ex-situ* perfused porcine hearts suggest that ESHP in working mode provides enhanced functional preservation of the heart during the perfusion interval [15, 16].

An ESHP apparatus capable of preserving the heart in a working mode must possess a level of automation to safely and precisely maintain preload, afterload and flow rates. Also, such a system should possess the flexibility to facilitate comprehensive assessments of cardiac function to be undertaken. The ESHP apparatus used here is equipped with custom software that 1) provides and maintains desired aortic (Ao) and left atrial (LA) pressure /flow, 2) provides real-time analysis of functional parameters and visual evaluation of pressure waveforms with minimal need for supervision. Pressure data is acquired with standard fluid-filled pressure transducers, and flow data is acquired with transit-time doppler flow probes. These signals are digitized with a bridge and analog input, respectively. The heart is positioned horizontally with a slight elevation to the great vessels on a soft silicone membrane. The cannulation attachments pass through the membrane, incorporating a compliance chamber for dampening ventricular ejection. The goal of this manuscript is to provide researchers in the field of cardiac transplantation with

a protocol for *ex-situ* perfusion and evaluation of the heart, under normothermic, semi-physiologic conditions in working mode, in a large mammal (Yorkshire pig) model.

2.3. Protocol

All the procedures in this manuscript were performed in compliance with the guidelines of the Canadian Council on Animal Care and the guide for the care and use of laboratory animals. The protocols were approved by the institutional animal care committee of the University of Alberta.

2.3.1. Animals

This protocol has been applied in female juvenile Yorkshire pigs between 35-50 kg.

2.3.2. Biohazard

All individuals involved in ESHP procedures had received proper biosafety training.

2.3.3. Pre-surgical preparations

- Place the organ chamber properly on the apparatus cart and install the silicon support membrane inside the organ chamber. The Ao, pulmonary artery (PA) and LA connection points can be seen in Figure 2.1.

- Install the ESHP tubing network (represented in Figure 2.2A&B), oxygenator and filter. Attach the heat exchanger water lines and the sweep gas tubing to the oxygenator.

- Place the flow probes for measuring coronary sinus/PA and LA flow on the corresponding tubing.

- Connect the Ao and LA pressure transducers to the representative lines on the circuit.

- Ensure that all the tubing connections are firmly attached, and all the stopcocks and luer locks are properly closed on the unattached sites.

- Prime the circuit with 750 ml of modified Krebs-Henseleit buffer (NaCl, 85; KCl, 4.6; $NaHCO_3$, 25; KH_2PO, 1.2; $MgSO_4$, 1.2; glucose, 11; and $CaCl_2$, 1.25 mmol/L containing 8% albumin). De-air the Ao and LA pumps by positioning the pump outlet above the inlet so that the air leaves the pump chamber (Figure 2.3). The solution typically does not need to be oxygenized before the start of the perfusion.

- Initiate the software after the Ao and LA pumps are de-aired and the circuit is primed.

2.3.4. ESHP software initialization and adjustments

Note: The ESHP apparatus used here is equipped with a custom software program to allow control of pump speed in order to achieve and maintain desired LA and Ao pressures. The software also analyzes functional parameters and provides a visual evaluation of pressure waveforms (Figure 2.4).

- To start the ESHP program, click on the program shortcut on the monitor.

- In the “setting” page, click “initialize”. The initializing message will appear on the board (Figure 2.5).

- On the same page, zero the flow sensors clicking the “zero LA flow” and “zero PA flow”. The message will appear on the board.

- Adjust the height of the pressure transducers to the height of the silicon support**.** To zero the pressure transducers, open the Ao and LA pressure transducers (and any other

transducers you have set to check the pressure) to the atmosphere, then click “zero all pressures” button. The message will appear on the board.

- In the “main” page, increase the Ao pump speed gradually up to the point where flow from the Ao cannula appears in the organ chamber. In the present system, this is achieved with 900-1000 revolutions per minute (RPM).

- Add 750 mL of blood to the perfusate solution to bring the total perfusate volume to 1.5 L (as described in “Surgery, harvesting blood, and heart procurement” section) and then increase the LA pump PRM (800-900 RPM) so that no air remains in the LA cannula or the LA tubing beneath the silicone support membrane.

- After initializing the controlling software and de-airing of the ESHP apparatus, donor heart procurement may proceed.

2.3.5. Preparations and anesthesia

- Administer 20 mg/kg of ketamine intramuscularly for premedication.

- Transfer the pig to the surgical suite and place the pig on the operating table with tabletop heating to maintain normothermia.

- Titrate oxygen flow rate for mask induction according to animal weight and the anesthetic system. For the closed-circle anesthetic circuits the oxygen flow should be 20 - 40 mL/kg.

- Turn on isoflurane to 4 - 5%; after one or two minutes this may be reduced to 3%.

- Evaluate the depth of anesthesia. The pig is in the surgical plane if there is no withdrawal reflex when a pinch is applied between the hooves or along the coronary band (pedal reflex).

- After confirmation of the appropriate depth of anesthesia, proceed to intubation.

- Place the pulse oximeter probe on the tongue (preferred) or ear. The oxygen saturation measured by pulse oximetry should remain above 90%.

- Shave patches of hair off on the left and right elbow regions, and left stifle. Wash off skin oils with soap and water, rinse with rubbing alcohol and dry completely. Place the ECG contacts. Avoid lead wire interference with the surgical site. Connect the leads to the correct locations.

- To maintain the anesthesia, adjust oxygen flow (20 - 40 mL/kg) and inhalant gas rate (1 - 3%). The heart rate should be 80 - 130 beats/min. Respiration rate should be 12 - 30 breaths/min.

- Shave, wash and aseptically prepare the incision site.

2.3.6. Blood collection and heart procurement

- Evaluate the anesthesia level every minimum every 5 minutes to confirm the surgical plane (no pedal reflex and no blink reflex, no response to painful stimuli).

- Perform a median sternotomy.

- Identify jugulum and xiphoid as landmarks.

- Using electrocautery, develop the midline between the landmarks by dividing the subcutaneous tissue and the fascia between the fibers of the pectoralis major muscle.

- Mark the midline along the sternal bone with the cautery. Perform sternal osteotomy with an electric or air-powered saw. To prevent creating injuries to the underlying structures (e.g. pericardium and brachiocephalic vein, and innominate artery), proceed gradually with the saw.

- Retract the sternum gradually, using a sternal retractor. To avoid excessive tension and vascular injury, do not place the retractor too far cranially.

- Free the sternopericardial ligaments from the posterior surface of the sternum using cautery.

- Open the pericardium with a Metzenbaum Scissor and fix the pericardial edges to the sternum using 1-0 silk suture.

- Extend the midline incision cranially by 2-3 cm and expose the right common carotid artery and internal jugular vein.

- Obtain proximal and distal control of the vessels by encircling the vessels with silk ties (2-0).

- Tie the cranial encircling ties on each vessel.

- Open the anterior 1/3 of each vessel with an 11-blade and then insert a 5-6 F sheath into each vessel. Tie the caudal encircling tie around each vessel to secure the respective sheathes.

- Monitor the arterial and central venous pressures by connecting each sheath to a pressure transducer.

- Deliver 1000 U/kg heparin intravenously.

- Place a 3-0 polypropylene purse-string suture around the right atrial appendage and secure it with a snare.

- Inside the purse-string suture, create a 1 cm incision on the appendage using an 11 blade. Insert a two-stage venous cannula (28/36 FR) inside the incision and position the distal tip in the IVC. Secure the cannula by tieing snare to the venous cannula. Control the outlet of the cannula with a tubing clamp.

- From the two-stage venous cannula placed in the right atrium, collect 750 mL of whole blood from the pig gradually over a period of 15 min, into an autoclaved glass container, and simultaneously replace the volume with 1 L of an isotonic crystalloid solution such as Plasmalyte A.

- Add the blood to the perfusion circuit (which has been previously primed with 750 mL Krebs-Henseleit buffer containing 8% albumin) to reach a final volume of 1.5 L of perfusate. The perfusate is a 1:1 combination of Krebs-Henseleit containing 8% albumin solution and whole blood from the donor animal [17].

- Place a cardioplegia needle (14-16 F) in the ascending Ao and secure it with a snare.

- Connect the cardioplegia cannula to the cardioplegia bag, to add 100 mL of blood to 400 mL of cardioplegia (St. Thomas Hospital Solution No. 2) to reach a final volume of 500 mL blood cardioplegia.

- Euthanize the pig by exsanguination. If you are intended to add more blood to the perfusate after starting of the perfusion (according to the aims of the study), collect the blood and add 10-30 U/mL of heparin to it and store it in a glass container or a plastic bag at 4 ˚C for short durations (hours). For longer storage, follow the institutional guidelines.

- Cross-clamp the ascending Ao with an Ao clamp and deliver the cardioplegic solution into the Ao root.

- After delivery of the cardioplegic solution is completed, remove the cross-clamp and perform the cardiectomy.

- For ease of attaching the Ao and PA to their representative cannula, partially dissect the ascending Ao from the PA using a Metzenbaum scissors.

- Transect the superior and inferior vena cava, leaving roughly 1 cm of length on each.

- Separate the heart from the posterior mediastinum by transecting the pulmonary veins.

- Excise the heart ensuring all of the Ao arch vessels are procured along with a segment of descending Ao. Preserve up to the PA bifurcation.

- Weigh the empty heart. The amount of weight gain over the *ex-situ* preservation interval can be used as a metric for organ edema.

2.3.7. Placement of the heart onto the ESHP apparatus and initiation of perfusion

- Trim excess tissue around the LA with a Metzenbaum scissor, and cut between the pulmonary veins to create a common orifice.

- Place a purse-string suture around the LA orifice using a 3-0 polypropylene suture.

- Place the LA cannula into the LA orifice and secure it with a snare (Figure 2.6).

- Suture and close the inferior vena cava with a 3-0 polypropylene suture. Leave the superior vena cava open at the beginning of the perfusion to ensure the right ventricle (RV) remains decompressed until the perfusate warmed and an organized rhythm is achieved.

- Gently squeeze the ventricles to de-air the heart. Place the LA cannula over the magnet embedded in the silicon membrane. Ensure the magnet in the silicone and the corresponding metal ring in the LA cannula are properly engaged.

- Attach the Ao to the Ao cannula embedded in the silicone membrane. Secure the Ao around the cannula with a silk tie. Trim the Ao to achieve a proper lie without tension or kinking.

- Increase the Ao pump speed to 1600 RPM. The remaining air in Ao root will be ejected through the innominate and subclavian branches.

- Connect the Ao purge line to the innominate artery. Secure the connection with a silk tie.

- Snare the left subclavian artery orifice with a silk tie. Secure the closure with a snare and snap. Through the orifice of the subclavian artery, place an introducer sheath (5f). Ensure that the length of the catheter and its orientation is properly adjusted so that it does not interfere with Ao valve function.

- Connect the Ao pressure transducer to the introducer sheath side port.

- Read the Ao pressure on the monitor. Adjust the Ao pump speed to reach a mean pressure of 30 mm Hg. At this point (Time 0) the perfusion has started in the non-working mode (Langendorff mode) and appearance of a dark deoxygenated perfusate in the PA line is a reflector of reestablishment of coronary flow. Set a timer to follow duration of the perfusion if needed.

- Turn on the heat exchanger and set the temperature to 38 ˚C. The perfusate will warm up to 37-38 ˚C in approximately 10 minutes. For normothermic perfusion of a porcine heart, keep the temperature at 38 ˚C throughout the perfusion.

- Maintain the perfusion in non-working mode for the first hour of the perfusion. Adjust the LA pump speed to maintain the LA pressure at 0 mmHg.

- Once the perfusate temperature is > 34 ˚C, evaluate the heart rhythm and pace or defibrillate as required (5-20 joules). Ensure the heart is completely decompressed before attempting cardioversion.

- Check the dissolved gas status using a blood gas analyzer. Adjust the gas mixture to maintain a pH: 7.35-7.45, arterial partial pressure of carbon dioxide (P_aCO_2): 35-45 mmHg, arterial partial pressure of oxygen (P_aO_2): of 100-150 mmHg, and oxygen saturation (sO_2) ≥ 95 %.

- Once the heart is normothermic and in a stable rhythm, ligate the superior vena cava.

- Attach temporary pacemaker leads to the right atrial wall and pace the heart in an AAI mode at 100 beats/min.

- Attach the epicardial electrocardiography electrodes to the surface of the heart.

- Switch to working mode after 1 hour of perfusion in Langendorff mode. For this purpose, enter the desire LA pressure (typically 6-8 mmHg) on the left side of the main page, in the "desired LAP" section of the software, and click on the button to initiate the feedback loop. The activated working mode will appear as a green button, and the LA pump speed will automatically increase and decrease to reach and maintain the desired LA pressure.

- As the heart begins to work, coronary vascular resistance will drop resulting in a low diastolic pressure. Adjust the Ao pump speed to maintain the Ao diastolic pressure of 40 mmHg as afterload during perfusion in the working mode.

2.3.8. Metabolic support during ESHP

Note: Organ perfusion solutions, including Krebs-Henseleit buffer solution, typically contain glucose as the primary energy substrate.

- Check the glucose level (e.g. with blood gas analysis) at regular intervals during the perfusion. In accordance with the consumption rates, using a standard infusion pump replace glucose by continuous arterial infusion and/or bolus doses, to maintain an arterial concentration of 6-8 mmol/L of glucose throughout the perfusion.

- Using a separate infusion pump, deliver 2 U/h of insulin to the perfusate throughout the perfusion. You may change the rate of insulin infusion according to the aims of the study.

- For β-adrenoceptor stimulation of the heart, deliver 0.08 mcg/min of epinephrine to the perfusate using a standard infusion pump, and continue throughout the perfusion. Alternatively, an infusion of 4 mcg/min of dobutamine may be used.

2.3.9. Anti-microbial / anti-inflammatory agents:

- Add a broad-spectrum antibiotic (e.g. 3.375 grams of piperacillin-tazobactam) to the perfusate at the start of perfusion.

- You may also add anti-inflammatory agents (e.g. 500 mg of methylprednisolone) to the perfusate, in accordance with the aims of the study.

2.3.10. Assessment of function

Note: The ESHP controlling software automatically calculates and records steady-state hemodynamic and functional indices every ten seconds.

- Assessment of steady state systolic and diastolic function

- For assessment and recording of the steady state data, through the introducer sheath placed earlier in the subclavian artery, place a fluid-filled pigtail catheter into the left ventricle (LV) while in working mode.

- Flush the pigtail catheter with saline and place the guide wire inside it.

- Gently insert the catheter into the Sheath cannula previously placed in the subclavian artery. As soon as it passes through the Ao valve, remove the guidewire slowly and connect the pigtail catheter to the LV pressure line.

- Follow the LV pressure wave on the monitor. The diastolic portion of the pressure wave will reach zero when the catheter has properly placed inside of the LV. Of note, this step is only possible in working mode since the Ao valve must be opening normally for the pigtail catheter to be able to enter the chamber. Once the pigtail catheter is placed in the LV and connected to the LV pressure transducer, the LV maximum and minimum rate of pressure change (dPdT min and dPdT max) will be recorded automatically.

- Determine the myocardial performance by indexing the measured flow on the LA line, for heart mass ($mL \cdot min^{-1} \cdot g^{-1}$), at a given constant LA pressure (8 mmHg), and an Ao diastolic pressure of 40 mm Hg, and a heart rate of 100 $beats \cdot min^{-1}$. The LA pressure equals the cardiac output, assuming there is no Ao insufficiency. Examine the Ao pressure waveform to ensure there is no Ao insufficiency.

- Assessment of preload recruitable stroke work (PRSW)

Note: PRSW is the linear relationship between end-diastolic volume and LV stroke work (LVSW) and represents an index for the evaluation of ventricular function, independent of preload, afterload, and size of the ventricle [18, 19]. PRSW can be measured with this system in a non-invasive fashion as described below [13].

- Remove the pigtail catheter from the LV, since the catheter may induce arrhythmias during PRSW analysis that will negatively affect the accuracy of the results.

- On the main page, in the “Capture PVL” section, adjust the desired rate of drop in LA pump speed during the analysis (typically 100-200 RPM) and desired time during which the analysis will take place (typically 10-12 sec) (Figure 2.4).

- After performing the adjustments mentioned above, click on “Record PVL”. The software will automatically exit working mode and gradually reduce LA pump RPM while simultaneously recording LVSW and LA pressure. At the conclusion of data collection, the software will perform linear regression on the newly acquired dataset to yield PRSW. After the ESHP software has completed the analysis, a message will appear on the main page, showing the correlation coefficient of the analysis. Press “OK” if the coefficient (R-value) is desirable (typically > 0.95). The PRSW analysis results will be recorded.

- After performing the analysis, to return to perfusion in the working mode, click on “Press To Start Working Mode” otherwise the software will continue in Langendorff (non-working) mode. The gray button will turn to green indicating a return to working mode. If repeated PRSW analysis is needed, before each new attempt ensure that the LA pressure/flow values return to the previous steady state values.

2.3.11. Metabolic assessment of the *ex-situ* perfused heart

- Assess the metabolic state of the heart and the perfusate during ESHP, using the information obtained from the blood gas analysis of the perfusate samples collected from both Ao (arterial), and PA (venous) lines every 1-2 hours.

- Perform blood gas analysis (every 1-2 hours) to monitor the gas and ionic state of the perfusate. Adjust the gas composition (O_2 and CO_2) and sweep speed to maintain a pH of 7.35-7.45, paO_2 of 100-150 mmHg, and $paCO_2$ of 35-45 mmHg. Adjust and maintain the perfusate ionic concentration of potassium and calcium in the physiologic range during the perfusion (e.g. by addition of calcium chloride if needed).

- Use the information obtained from the blood gas analysis and coronary blood flow to calculate metabolic parameters. For example, myocardial oxygen consumption (MVO_2), and LV mechanical efficiency (ME) can be calculated as follows:

MVO_2 (mL O2 · min^{-1} · 100 g^{-1}) will be determined by multiplying the coronary blood flow (CBF) by the arterial-venous difference in oxygen content ($CaO_2 – CvO_2$).

MVO_2 = [CaO_2 - CvO_2 (mL O2 · 100 mL^{-1})] × CBF (ml . min^{-1} . 100 g heart mass), where;

Arterial oxygen content (CaO_2) = [1.34 (mL O_2 . g Hb^{-1}) × Hb concentration (g · 100 mL^{-1}) × oxygen saturation (%)] + [0.00289 (mL O_2 · mm Hg^{-1} · 100 mL^{-1}) × PaO_2 (mm Hg)]

Venous oxygen content (CvO_2) = [1.34 (mL O_2 · g Hb^{-1}) × Hb concentration (g · 100mL^{-1}) × oxygen saturation (%)] + [0.00289 (mL O_2 · mm Hg^{-1} · 100 mL^{-1}) × PvO_2 (mm Hg)]

LV mechanical efficiency (ME) will be calculated as follows:

LVSW (J . beat^{-1}) / MVO_2 (J . beat^{-1}) where

Stroke work = {mean arterial pressure (mmHg) - LA pressure (mmHg)} × {LA flow (ml . min^{-1})/ heart rate (beats . min^{-1})} × 0.0001334 (J . mL^{-1} . mmHg^{-1}), and

MVO_2 (J . beat^{-1}) = {MVO_2 (ml . min^{-1})/heart rate (beats . min^{-1})} × 20 (joules . mL^{-1})

2.3.12. Removing the heart from ESHP apparatus (end of perfusion)

- Exit the working mode. Bring the LA pump RPM to zero.

- Decrease the Ao pump RPM to zero.

- Remove the pigtail and sheaths.

- Quickly remove all the attachments to the heart.

- Weigh the empty heart to determine the degree of myocardial edema formation.

- Quickly take tissue samples of proper size from the left and right ventricles and place them in optimum cutting temperature (OCT) gel, formalin and/or snap freeze them in liquid nitrogen. Store the samples for future investigations (OCT and snap frozen samples in -80˚freezer, formalin-stored samples in a properly sealed container at room temperature).

- Close the program, all the recorded data will be saved.

- Discard the remaining tissue, blood, bioactive materials and used ESHP apparatus components according to institutional protocols.

- Clean the ESHP cart using a sanitizing hard surface cleaner (e.g. 70% ethanol) thoroughly.

2.4. Representative results

At the starting of the perfusion (in non-working mode), the heart will normally resume a sinus rhythm when the temperature of the system and perfusate approaches normothermia. When entering working mode, as the LA pressures are approaching the desired values, ejection on the Ao pressure tracing should be observed and the LA flow (a reflection of cardiac output) should increase gradually. In a Yorkshire pig model (35-50 kg) and a starting heart weight of 180-220 grams, the initial LA flow will be ~2000 mL/min and this will typically approach ~2750 mL/min during the first hour of perfusion in working mode. Figure 2.7 displays trends in Ao pressure (A) as well as LA and pulmonary arterial flow (B) over 12 hours of perfusion.

During ESHP in the physiologic working mode, various metabolic assessments of the heart are also possible. Blood gas analysis/metabolic assessments performed on the perfusate samples obtained during ESHP provide extensive information on the metabolic status of the heart over time (Table 2.1 & 2.2) and (Figure 2.8A&B) [20]. In addition to blood gas analysis, perfusate samples can be collected and assessed for different biomarkers such as brain natriuretic peptide and troponin-I; however,

It should be noted that ESHP occurs in a closed system, with no exchange of perfusate solution. In the absence of the organs that naturally metabolize/clear these factors (e.g. kidneys), the accumulation of biomarkers over time in the perfusate solution is typically observed (Figure 2.9).

Functional assessment of the heart using this platform may include both load-dependent parameters [including myocardial performance (cardiac index, CI), LVSW, maximum and minimum rates of pressure change (dp/dt max and min)], and load-independent parameters (PRSW) (Table 2.3). Figure 2.10 demonstrates the evaluation of LV PRSW during a computer-controlled linear reduction in the LA pressure. [13] In our experience with ESHP of >200 porcine hearts and >10 human hearts, the use of an automated ESHP software program has been in association with the development of standard operating procedures resulting in minimal inter- and intra-operator variability in the functional parameters. The ESHP apparatus and software system used here have been designed to maintain the desired pressures and collect the functional parameters with minimal need for manual adjustments, and we have observed an interclass correlation coefficient (ICC) ≥ 0.9 for all of the assessed parameters (e.g. LVSW, and dp/dt max and min) that accounts for excellent inter-rater, intra-rater and test-retest reliability. In this system, the electrocardiographic monitoring of the heart during perfusion can also take place using two electrodes as described in the protocol, providing information on the heart rate and rhythm during perfusion (Figure 2.4).

The assessment of the heart during ESHP may be extended to different imaging modalities. Echocardiography during ESHP can provide additional information on myocardial function (e.g. ventricular ejection fraction) and anatomical parameters (Figures 2.11 and 2.12). Moreover, and assessment of the coronary vasculature is possible with angiographic imaging [21].

Performing a linear regression analysis identifies which parameters best correlated with myocardial performance (cardiac index: $mL \cdot min^{-1} \cdot g^{-1}$) during ESHP. We previously showed that despite the significant variation in the ability of the measured functional parameters to predict myocardial performance, overall, functional parameters exhibit a high correlation with cardiac output. The best functional predictors included systolic stroke work [coefficient of determination (R^2) = 0.759], for systolic function, and minimum dP/dt, (R^2 = 0.738) for diastolic function. Interestingly, metabolic parameters alone show a very limited ability to predict myocardial performance (oxygen consumption: R^2 = 0.28; coronary vascular resistance: R^2 = 0.20; lactate concentration: R^2 = 0.02) [13]. Perfusion of the heart in a normothermic working mode offers the opportunity to obtain comprehensive metabolic and functional assessments of the heart during organ preservation. A clinical ESHP device with the ability to support the donor heart in working mode will provide the healthcare team with the opportunity to made decisions about organ viability based on objective data before transplantation.

2.5. Discussion

2.5.1. Critical steps of the protocol

Successful perfusion is defined according to the aims of the study; however, this should include uninterrupted ESHP for the desired amount of time and complete collection of the data on cardiac function during the perfusion. For this purpose, a few critical steps in the protocol must be followed.

The heart is an organ with high oxygen and energy demands, and minimizing the ischemic time before cannulation and perfusion is an important principle that must be followed. The

process of procurement, mounting the heart on the ESHP apparatus, and initiating perfusion should not exceed 20-30 minutes.

For efficient perfusion and reliable functional assessment, the process of mounting the heart on the apparatus bears critical importance. Proper anatomical alignment of the great vessels plays an important role in this regard. The heart should be procured with an adequate length of PA and Ao arch branches so that these vessels are not stretched when attached to the representative cannulae**.** From the start of the perfusion, efficient coronary perfusion plays a pivotal role in the protection of the heart during *ex-situ* perfusion. After starting of the perfusion in non-working mode, the Ao pressure should be monitored and adjusted on at least 30 mmHg to support the coronary perfusion efficiently. The appearance of a dark deoxygenated perfusate in the PA line is a reflector of the reestablishment of coronary flow. After switching to the working mode, the Ao pressure should be adjusted to 40 mmHg to provide adequate coronary perfusion pressure for the working heart.

Deairing the heart chambers and Ao is essential for successful ESHP. At the time of attaching the LA cannula, squeezing the chambers will help in deairing the heart. Any air remaining the LV that is ejected should recirculate through the purge line in the innominate artery, which minimizes the risk of coronary air embolism. However, if substantial air remains in the left heart at the time of switching to the working mode, coronary air embolism is possible leading to a significant decline in myocardial function.

2.5.2. Advantages of ESHP in the working mode

The goal of the presented approach is to provide a reproducible and reliable platform for experimental ESHP studies in large mammal models. Such a system provides the opportunity for perfusion in a physiologic working mode, and for extensive evaluation of the perfused heart. This provides an opportunity to evaluate cardioprotective protocols aimed at resuscitating dysfunctional donor organs. This system facilitates simple and reproducible assessments of cardiac functional parameters alongside metabolic parameters during ESHP, providing objective data that can be used to identify viable organs for transplantation. Such a comprehensive assessment is of particular importance when evaluating extended criteria donated hearts and hearts donated after circulatory death. Moreover, according to our observations in the setting of experimental ESHP, hearts perfused in a working mode display superior preservation of systolic and diastolic function over time compared to hearts preserved in a Langendorff mode, and may help extend the safe preservation time.

2.5.3. Limitations of ESHP in the working mode

ESHP in a working mode, is an efficient method to preserve the donated heart and assess its viability, yet it is an artificial setting, lacking many of physiologic aspect of the body (e.g. real-time hormonal and nutritional balance/support, and free radical scavenging systems). The heart is an organ with sophisticated energy/metabolic demands. Thus, providing consistent, efficient metabolic support to the heart perfused is critically important. We have observed a decline in the function of the *ex-situ* perfused heart, particularly during extended perfusion times [22]. Such a decline may be reflective of

metabolic inefficiencies affecting the function of working mode-perfused heart. More studies are warranted to characterize the optimal metabolic support for the heart during ESHP. An additional challenge is the complexity of working mode heart perfusion. Despite the enhanced simplicity of ESHP in this system, working mode perfusion should be performed by well-trained personnel.

2.5.4. Future directions

The ESHP apparatus with the capacity to perform a comprehensive functional and metabolic assessment of the hearts in a large mammal model, offers a great potential to develop translational therapeutic protocols to improve the dysfunctional/suboptimal donated hearts. ESHP may serve as a platform to administer therapeutic interventions targeting a wide range of conditions (e.g. ischemia reperfusion injury), and evaluate their effects on the metabolic and functional parameters of the perfused heart [12]. Moreover, working mode ESHP may facilitate extension of the safe preservation interval, which may help to overcome geographic limitations of organ donation and facilitate better allocation of donated hearts.

2.6. Disclosure

DHF holds patents on *ex-situ* organ perfusion technology and methods. DHF and JN are founders and major shareholders of Tevosol, Inc.

2.7. Acknowledgment

This work was supported by grants from the Canadian National Transplant Research Program. SH is the recipient of a Faculty of Medicine and Dentistry Motyl Graduate Studentship in Cardiac Sciences. DHF is a recipient of a Collaborative Research Projects

(CHRP) grant in aid from the National Sciences and Engineering Research Council and Canadian Institutes of Health Research. We acknowledge Dr. Lily Lin for her assistance with acquisition of echocardiographic images of the *ex-situ* perfused heart.

2.8. References

1. Ardehali A, Esmailian F, Deng M, Soltesz E, Hsich E, Naka Y, Mancini D, Camacho M, Zucker M, Leprince P *et al*: **Ex-vivo perfusion of donor hearts for human heart transplantation (PROCEED II): a prospective, open-label, multicentre, randomised non-inferiority trial**. *Lancet (London, England)* 2015, **385**(9987):2577-2584.
2. 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**. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* 2008, **34**(2):318-325.
3. Freed DH, White CW: **Donor heart preservation: straight up, or on the rocks?** *Lancet (London, England)* 2015, **385**(9987):2552-2554.
4. Guibert EE, Petrenko AY, Balaban CL, Somov AY, Rodriguez JV, Fuller BJ: **Organ preservation: current concepts and new strategies for the next decade**. *Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie* 2011, **38**(2):125-142.
5. Collins MJ, Ozeki T, Zhuo J, Gu J, Gullapalli R, Pierson RN, Griffith BP, Fedak PW, Poston RS: **Use of diffusion tensor imaging to predict myocardial viability after warm global ischemia: possible avenue for use of non-beating donor hearts**. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* 2007, **26**(4):376-383.
6. White CW, Ali A, Hasanally D, Xiang B, Li Y, Mundt P, Lytwyn M, Colah S, Klein J, Ravandi A *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 : the official publication of the International Society for Heart Transplantation* 2013, **32**(7):734-743.
7. Iyer A, Gao L, Doyle A, Rao P, Cropper JR, Soto C, Dinale A, Kumarasinghe G, Jabbour A, Hicks M *et al*: **Normothermic ex vivo perfusion provides superior organ preservation and enables viability assessment of hearts from DCD donors**. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2015, **15**(2):371-380.
8. Peltz M, He TT, Adams GAt, Koshy S, Burgess SC, Chao RY, Meyer DM, Jessen ME: **Perfusion preservation maintains myocardial ATP levels and reduces apoptosis in an ex vivo rat heart transplantation model**. *Surgery* 2005, **138**(4):795-805.
9. Liao R, Podesser BK, Lim CC: **The continuing evolution of the Langendorff and ejecting murine heart: new advances in cardiac phenotyping**. *American journal of physiology Heart and circulatory physiology* 2012, **303**(2):H156-167.
10. Rivard L, Gallegos R, Ogden I, Bianco R: **Perfusion preservation of the donor heart: basic science to pre-clinical**. *J Extra Corpor Technol* 2009, **41**(3):140-148.
11. Dhital KK, Iyer A, Connellan M, Chew HC, Gao L, Doyle A, Hicks M, Kumarasinghe G, Soto C, Dinale A *et al*: **Adult heart transplantation with distant procurement and ex-vivo preservation of donor hearts after circulatory death: a case series**. *Lancet (London, England)* 2015, **385**(9987):2585-2591.
12. Messer S, Ardehali A, Tsui S: **Normothermic donor heart perfusion: current clinical experience and the future**. *Transplant international : official journal of the European Society for Organ Transplantation* 2015, **28**(6):634-642.

13. White CW, Ambrose E, Muller A, Li Y, Le H, Hiebert B, Arora R, Lee TW, Dixon I, Tian G *et al*: **Assessment of donor heart viability during ex vivo heart perfusion**. *Canadian Journal of Physiology and Pharmacology* 2015, **93**(10):893-901.
14. Messer SJ, Axell RG, Colah S, White PA, Ryan M, Page AA, Parizkova B, Valchanov K, White CW, Freed DH *et al*: **Functional assessment and transplantation of the donor heart after circulatory death**. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* 2016, **35**(12):1443-1452.
15. Hatami S, Qi X, White CW, Burhani M, Aboelnazar N, Himmat S, Wu A, Matsumura N, Nagendran J, Freed DH: **Endoplasmic reticulum stress in ex vivo heart prfusion: a comparison between working vs non-working modes**. *Canadian Journal of cardiology* 2017, **33**(10).
16. White CW, Shan S, Hatami S, Gurtu V, Kinnard A, Matsumura N, Aboelnazar N, Dyck JR, Lopaschuk GD, Michelakis E *et al*: **Ex vivo perfusion in a loaded state improves the preservation of donor heart function**. *Canadian Journal of cardiology* 2015, **31**(10):s202.
17. White CW, Hasanally D, Mundt P, Li Y, Klein J, Xiang B, Müller A, Ambrose E, Ravandi A, Arora RC *et al*: **A wholeblood–based perfusate provides superior preservation of myocardial function during ex vivo heart perfusion**. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* 2014, **S1053-2498**(14):01356-01354.
18. Lips DJ, van der Nagel T, Steendijk P, Palmen M, Janssen BJ, van Dantzig JM, de Windt LJ, Doevendans PA: **Left ventricular pressure-volume measurements in mice: comparison of closed-chest versus open-chest approach**. *Basic research in cardiology* 2004, **99**(5):351-359.
19. Morita S: **Is there a crystal ball for predicting the outcome of cardiomyopathy surgery? Preload recruitable stroke work, may be a possible candidate**. *Journal of cardiology* 2018, **71**(4):325-326.
20. Hatami S, White C, Shan S, Haromy A, Ondrus M, Kinnear A, Qi X, Nagendran J, Freed H: **Functional decline of the ex vivoperfused heart is not due to cell death**. In: *Canadian Sosciety for Transplantation, Halifax.* 2017.
21. Anthony C, Michel J, Christofi M, Wilson SH, Granger E, Cropper J, Dhital K, Macdonald P: **Ex vivo coronary angiographic evaluation of a beating donor heart**. *Circulation* 2014, **130**(25):e341-343.
22. Sandha JK, White CW, Muller A, Avery E, Thliveris J, Dixon IMC, Arora RC, Tian G, Hryshko LV, Nagendran J *et al*: **Steroids limit myocardial edema during ex vivo perfusion of hearts donated after circulatory death**. *The Annals of thoracic surgery* 2018.

2.9. Tables and figures

T**able 2.1.**: A case of the blood gas analysis performed during the *ex-situ* heart perfusion.

	Aortic (arterial) parameters			PA (venous) parameters		
	T1	**T5**	**T11**	**T1**	**T5**	**T11**
Blood Gas values						
pH	7.28	7.44	7.33	7.25	4.42	7.30
pO_2 (mmHg)	123.00	149.00	141.00	44.00	55.40	57.80
pCO_2 (mmHg)	38.00	33.90	42.50	43.00	37.10	46.10
Oximetry Values						
Hb (g/dL)	4.20	4.10	3.90	4.20	4.10	3.90
sO_2 (%)	100.00	100.00	100.00	64.00	95.50	92.00
Electrolyte Values						
K^+ (mmol/L)	4.20	4.60	5.20	4.20	4.60	5.20
Na^+ (mmol/L)	142.00	144.00	149.00	142.00	144.00	149.00
Ca^{2+} (mmol/L)	1.02	1.20	1.40	1.02	1.20	1.40
Cl^- (mmol/L)	107.00	109.00	114.00	107.00	109.00	114.00
Osm (mmol/kg)	291.30	292.50	302.40	291.90	292.90	302.40
Metabolite values						
Glucose (mmol/L)	7.00	5.30	5.10	7.00	5.20	5.00
Lactate (mmol/L)	3.00	2.30	2.00	3.10	2.40	1.90
Acid Base status						
$Hco3^-$ (mmol/L)	17.60	23.10	21.90	18.50	23.70	22.40

Ca^{2+}, calcium ion; Cl^-, chloride ion; Hb, hemoglobin; HCO_3^-, bicarbonate ion; K^+, potassium ion; Na^+, sodium ion; Osm, osmolarity; $paCO_2$, arterial partial pressure of carbon dioxide; paO_2, arterial partial pressure of oxygen; sO_2, oxygen saturation; T1, 1-

hour of ex-situ perfusion (early perfusion); T5, 5-hours of ex-situ perfusion (mid-perfusion); T11, 11-hours of ex-situ perfusion (late perfusion)

Table 2.2. A number of metabolic parameters calculated using the blood gas analysis data.

Metabolic Parameters	**T1**	**T5**	**T11**
MVO_2 L/min	7.02	5.24	3.74
Venous Lactate mmol/L	2.8	2.1	1.9
Arterio-Venous lactate difference mmol/L	0	0.1	0
Glucose Utilization g/hr	1.34	1.16	1.3

MVO2, myocardial oxygen consumption; T1, 1-hour of ex-situ perfusion (early perfusion); T5, 5-hours of ex-situ perfusion (mid-perfusion); T11, 11-hours of ex-situ perfusion (late perfusion

Table 2.3. A case of Left ventricular functional parameters assessed during *ex-situ* heart perfusion.

Functional Parameters	T1	T5	T11
CI (mL/min/g)	10.26	9.66	7.50
SW (mmHg*mL)	2253	1965	1323
dP/dT max (mmHg/s)	1781	1783	1482
Sys p (mmHg)	128	121	91
ME (%)	6.69	16.85	21.68
PRSW	399	348.38	248.63
dP/dT min (mmHg/s)	-1444	-2350	-844

CI, cardiac index; dP/dT max, maximum rate of pressure change; dP/dT min, minimum rate of pressure change; ME, mechanical efficiency; PRSW, preload recruitable stroke work; SW, stroke work; Sys p, systolic pressure*;* T1, 1-hour of ex-situ perfusion (early perfusion); T5, 5-hours of ex-situ perfusion (mid-perfusion); T11, 11-hours of ex-situ perfusion (late perfusion)

Figure 2.1.: The silicone support membrane for the heart

with A: integrated aortic cannula, B: Left atrial cannula, C: pulmonary artery cannula.

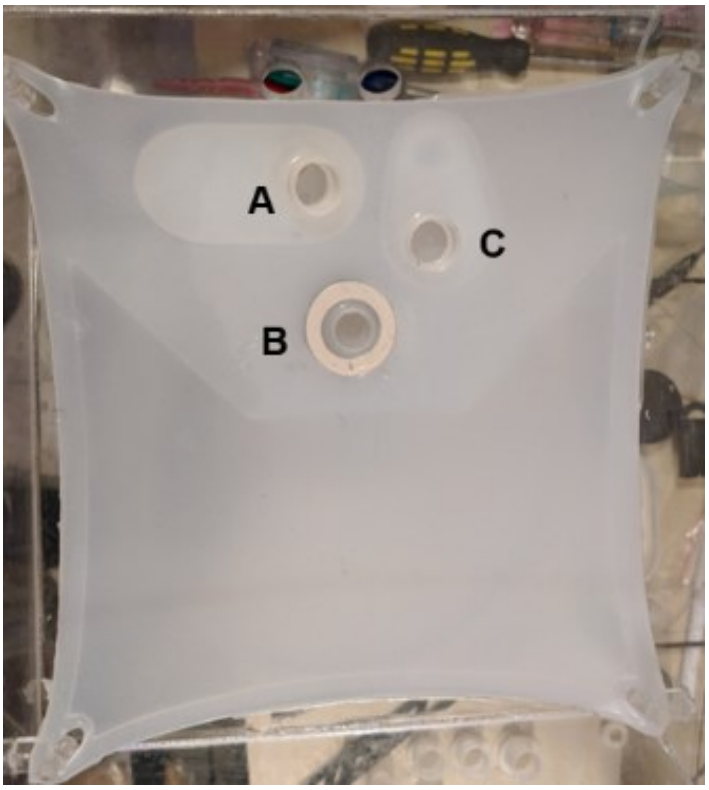
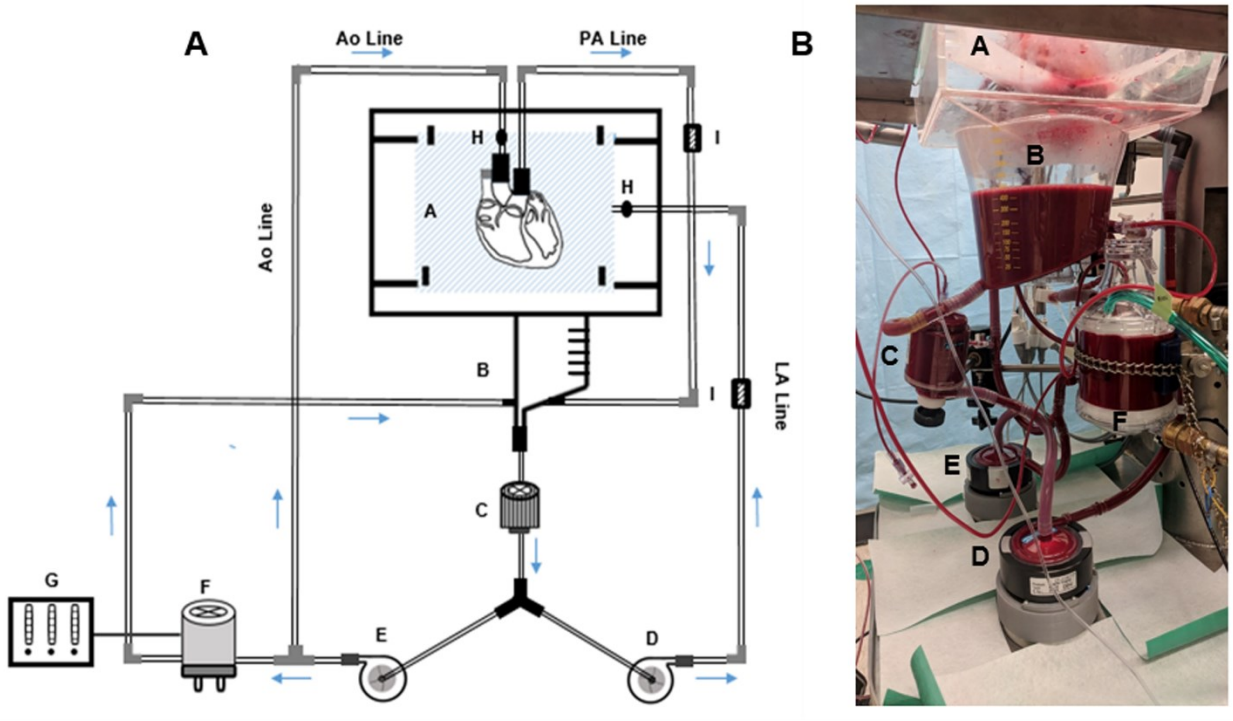


Figure 2.2.: The ESHP circuit. (A) Schematic figure of the ESHP circuit. (B) ESHP apparatus used in our setting



A: organ chamber and silicone support membrane, B: reservoir, C: arterial line filter, D: left atrial pump,

E: aortic pump, F: membrane oxygenator and heat exchanger, G: gas mixer, H: tube flow sensor, I: pressure sensor, J: stopcock/ luer lock

Figure 2.3.: De-airing the pumps by positioning the pump outlet to a higher level

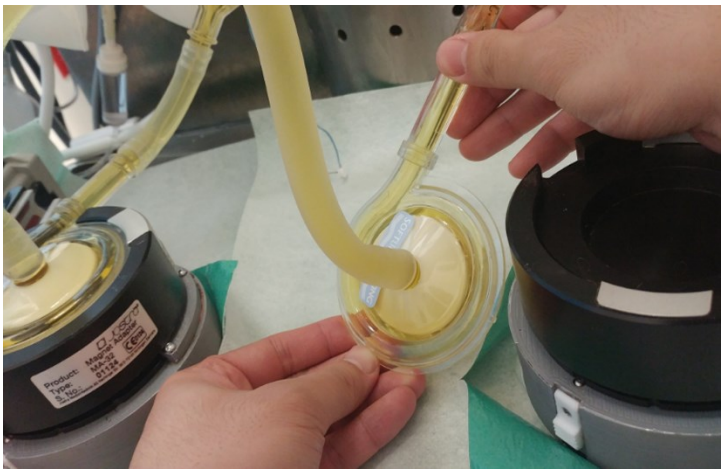


Figure 2.4.: Screen shot from the running ESHP software program showing cardiac functional parameters.

Figure 2.5. Screen shot from the initialized ESHP software program.

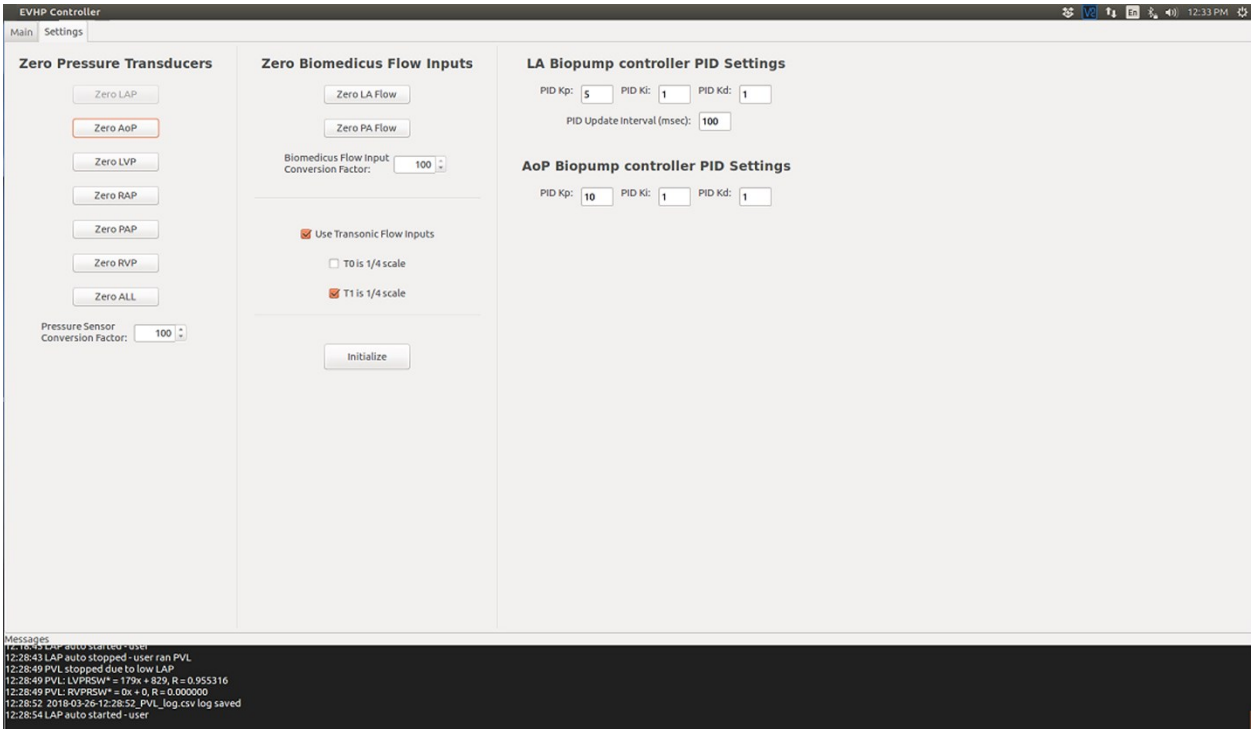


Figure 2.6. The magnetic left atrial cannula secured to the posterior aspect of the left atrium.

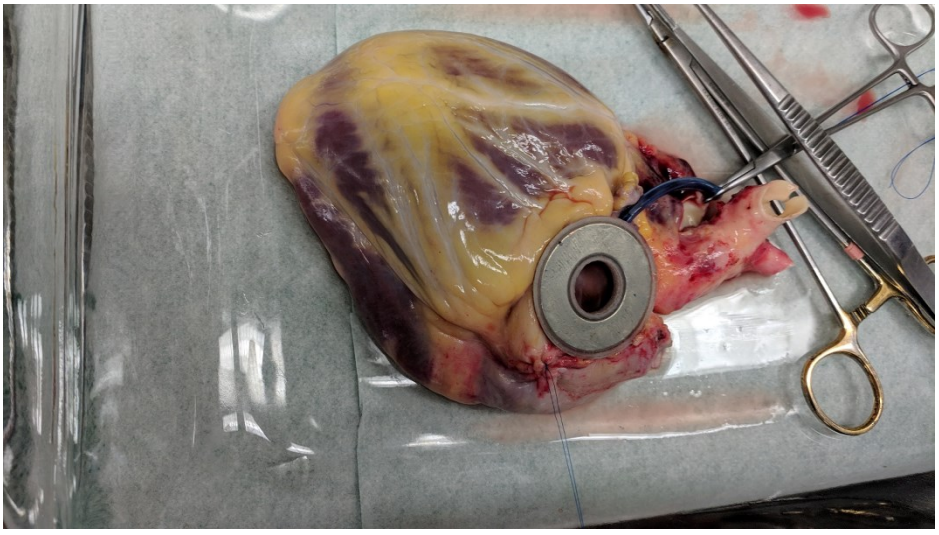


Figure 2.7. Monitoring pressures and flows during the perfusion. A: Trends in the aortic pressure during 12 hours of ESHP, B: Trends in the left atrial and pulmonary artery flows during 12 hours of ESHP

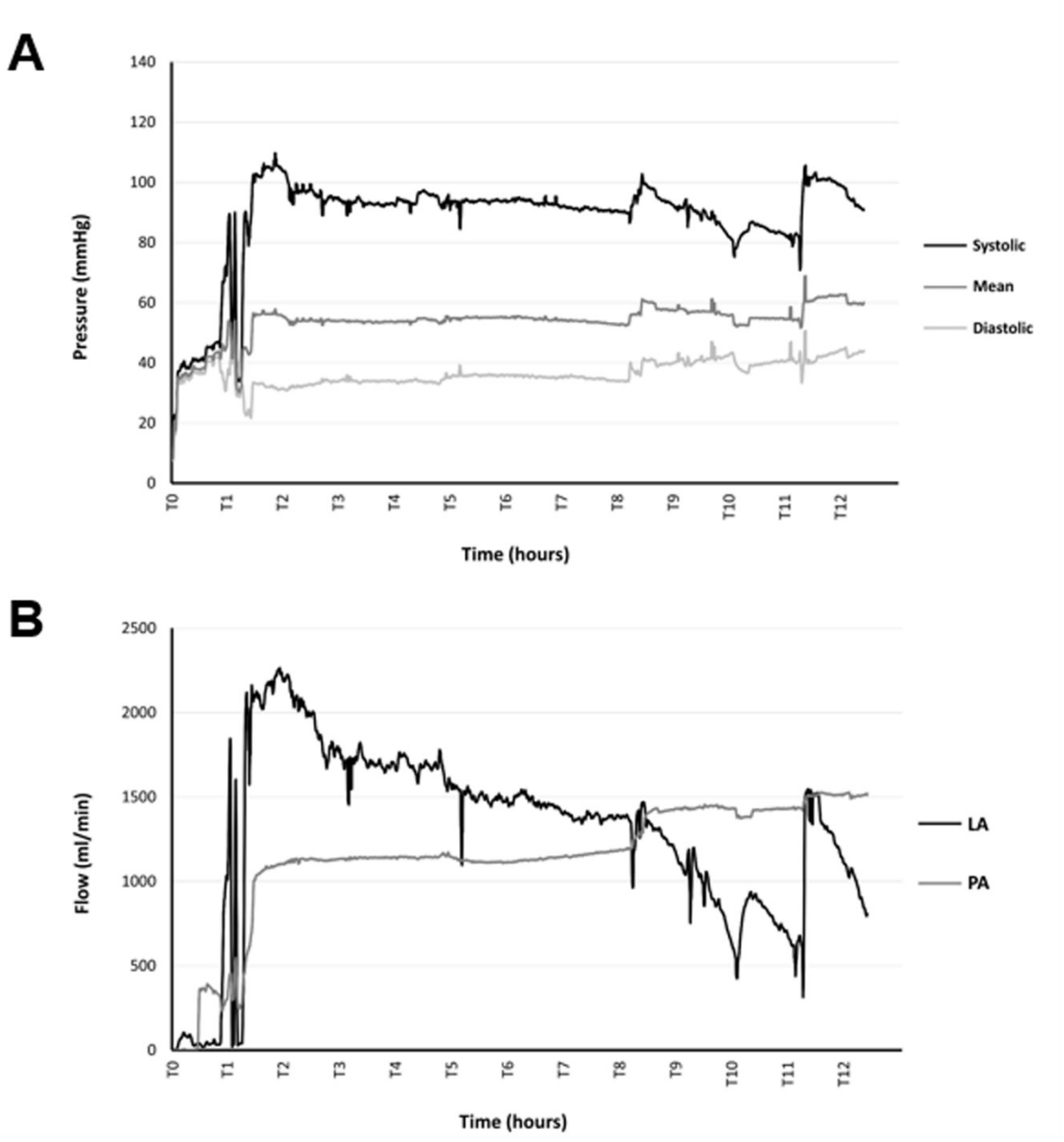


Figure 2.8. Overtime trend. A: myocardial oxygen consumption, B: venous lactate concentration during 12 hours of ESHP

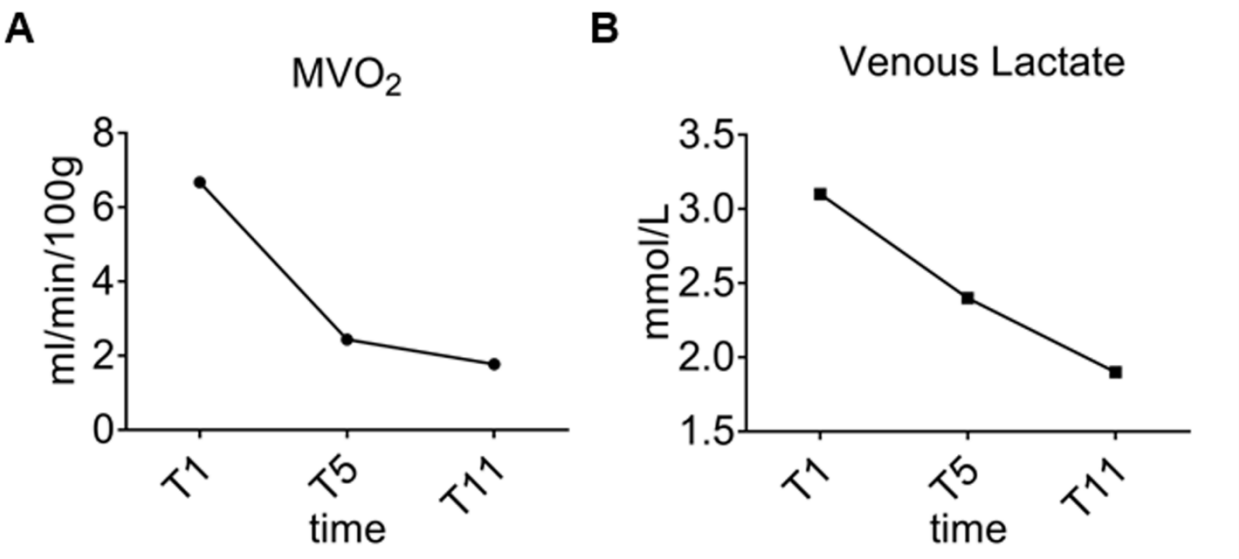


Figure 2.9. Overtime trend in perfusate concentration of cardiac troponin-I during 12 hours of ESHP.

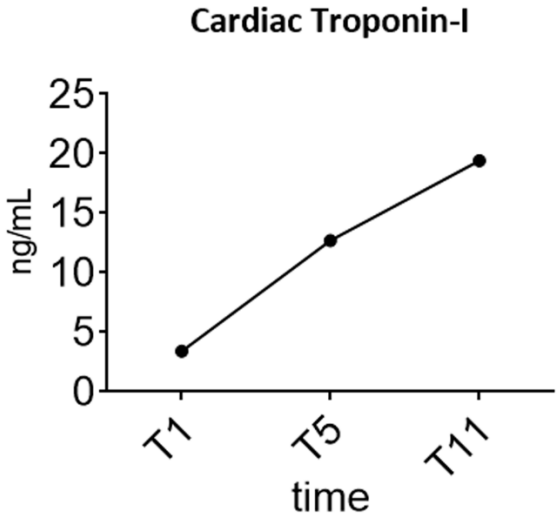


Figure 2.10. Assessment of preload recruitable stroke work a poorly-functioning heart (grey) versus a well-functioning heart (black).

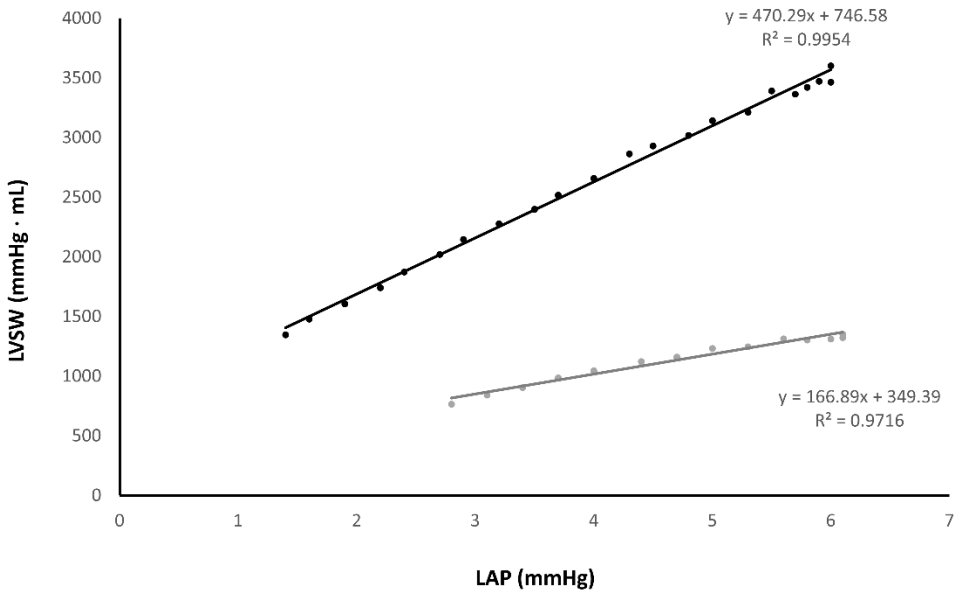


Figure 2.11. Representative 2d echocardiographic images.

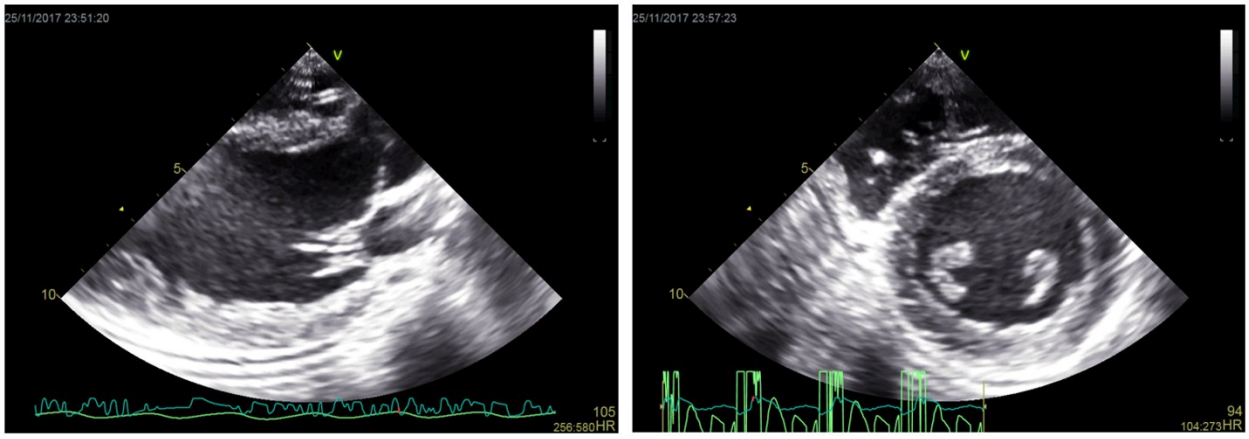
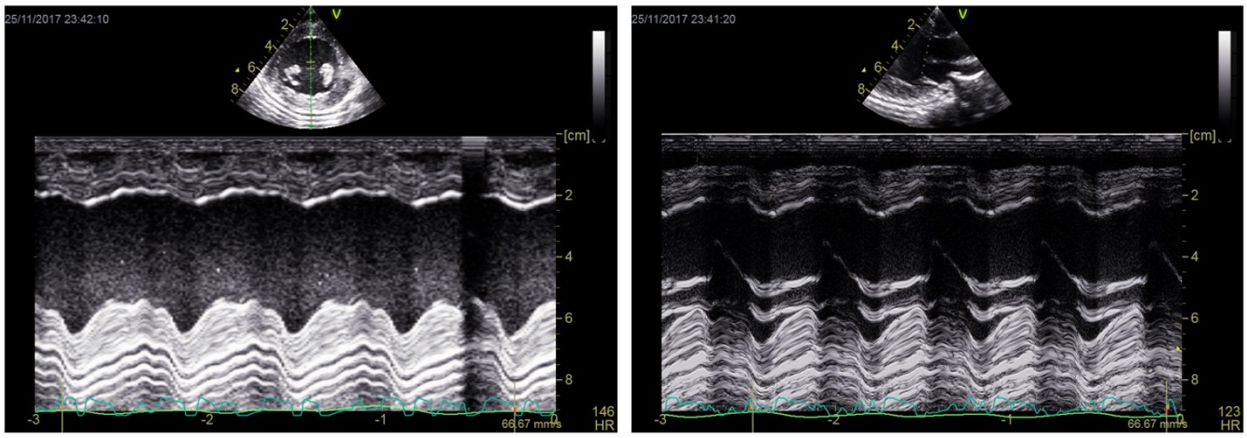


Figure 2.12. Representative M-mode echocardiographic images.



Chapter 3: The Position of the Heart During Normothermic *ex-situ* Heart Perfusion is an Important Factor in Preservation and Recovery of Myocardial Function

Sanaz Hatami, MD,[1,5] Xiao Qi, MD,[1,5], Christopher W. White MD PhD[1], Sabin J. Bozso, MD[1,5], Sayed Himmat, MD[1,5], Consolato Sergi, MD, PhD, MPH [4], Jayan Nagendran, MD, PhD,[1,5,6] Hyun-Joong Chung PhD[7], David S. Nobes PhD[8], Darren H. Freed, MD, PhD,[1,2,3,5,6]

Affiliations:

Departments of Surgery[1], Physiology[2], and Biomedical Engineering[3], Laboratory Medicine and Pathology[4], Faculty of Medicine

Alberta Transplant Institute[5], Edmonton, AB, Canada

Canadian Donation and Transplantation Research Program[6], Canada

Departments of Chemical and Materials Engineering[7] and Mechanical Engineering[8], Faculty of Engineering

University of Alberta, Edmonton, AB, Canada

This work is submitted to “The Journal For Heart and Lung Transplantation”: Hatami **S**, Qi X, White CW, Bozso, M. Himmat S, Nagendran J. Freed, DH:

Positioning of the Heart During Normothermic *Ex-situ* Heart Perfusion Is an Important Factor in Preservation and Recovery of Myocardial Function.

3.1. Abstract:

Background: *Ex-situ* heart perfusion (ESHP) is being investigated as a method for the continuous preservation of the myocardium in a semi-physiologic state for subsequent transplantation. Most methods of ESHP position the isolated heart in a hanging state, representing a considerable departure from the *in-vivo* anatomical positioning of the heart. This setting may negatively affect the functional preservation of the heart. Also, as a variable, it may affect the results of studies on ESHP. **Methods:** In the present study, cardiac functional and metabolic parameters were assessed in healthy pig hearts, perfused for 12 hours, in either a hanging (H), or supported (S) position, either in non-working mode (NWM), or working mode (WM). **Results:** The cardiac function was best preserved in the hearts perfused in a supported position in WM (median 11-hour cardiac index / 1-hour cardiac index%: WM-S= 94.77% versus NWM-S= 62.80%, WM-H= 36.18%, NWM-H= 9.75%, $p<0.001$). Delivery of pyruvate bolus, while leading to significant improvement of function in supported-perfused groups, only partially reversed myocardial dysfunction in the hanging-perfused heart groups. **Conclusion:** The hearts perfused *ex-situ* in a semi-anatomical supported position, and in physiologic WM had better functional preservation and recovery compared to the hearts perfused in a hanging, non-supported position. Optimizing the positional support for the *ex-situ*-perfused hearts may improve the quality of myocardial preservation during ESHP.

3.2. Background:

During the recent few decades, normothermic *ex-situ* heart perfusion (ESHP) has been proposed as a promising technology for potentially superior preservation of donor hearts in comparison to static cold storage (SCS), [1, 2]. The technology of ESHP is branched from the setting of isolated heart perfusion, which has been widely used to study the heart for more than a century [2]. The *ex-situ* perfusion technology was investigated during the early years of heart transplantation for the preservation of the donated heart. However, it was soon replaced with SCS due to its simplicity and generally acceptable results [2]. Myocardial preservation in ESHP is based on continuous perfusion of myocardial tissue; thus, it supports the metabolism of the heart during preservation time [1, 2]. There has been renewed interest in ESHP for the preservation of donor hearts with the aims of 1) longer safe preservation periods, 2) possibility of viability assessment during preservation time, 3) potential improvement of the donated heart, and 4) expansion of the donor pool [2]. However, despite the promising results of experimental and clinical studies on ESHP, it has been observed that myocardial function declines gradually during perfusion, limiting the potential advantages of this technology [3, 4]. *Ex-situ* perfusion of the heart has been performed using a wide variety of devices and experimental variables. These variables include animal species, perfusion temperature, pump perfusion versus gravity-feed perfusion, perfusion with or without submerging the heart in perfusate, single-mode or multi-mode operating apparatuses, consisting of one pump or two pumps, and varying perfusates [5-13].

For designing cardioprotective ESHP protocols, most of the attention has been focused on improving the composition of the solutions involved in the process of organ

procurement and ESHP, with the aim of minimizing the ischemia-reperfusion injury, providing efficient metabolic support for the heart, and preventing excess edema [5, 14-16]. Little attention has been given to the effects of the physical positioning of the heart during ESHP. In the majority of experimental ESHP studies, the heart has been suspended from the aorta (hanging), a position considerably different from the *in-vivo* position in the mediastinum wherein the myocardial mass is supported by the pericardium and the diaphragm which balances the hydrostatic, inertial, and gravitational forces, prevent excessive dilatation and wall stress, and modulate the weight borne by the heart [17]. Thus, in the present translational study in a pig model, we sought to compare the effects of *ex-situ* perfusion of the heart in hanging supported semi-anatomical position, in either working mode (WM), or non-working mode (NWM) on myocardial functional and metabolic preservation.

3.3. Material and methods

3.3.1. Animals

Twenty-four female domestic pigs (41-55 kg) were used as heart and blood donors. The institutional animal care committee of the University of Alberta approved the experimental protocol as compliant with the animal research ethics and Canadian Council on Animal Care Guidelines and federal and provincial regulations/legislation.

3.3.2. Procurement and preparation of the heart

The hearts were procured and prepared, as described in detail before [3, 16]. Briefly, an intramuscular dose of ketamine (20 mg/kg) and atropine (0.05 mg/kg) was injected as premedication. Orotracheal intubation was established, and general anesthesia was maintained with isoflurane (1-2%). The heart was exposed via a standard median

sternotomy. After intravenous delivery of heparin (1000 U/kg bolus) and Ringer's lactate solution (1000 mL), 850 ml of whole blood was retrieved through a two-stage venous cannula placed into the right atrium and was used to prime the ESHP circuit. The pigs were then euthanized by exsanguination. After a cross-clamp was placed across the ascending aorta, the hearts were arrested with 500 ml of reverse 4:1 blood cardioplegia (modified Del Nido) and were excised, weighed, immediately mounted on the custom ESHP apparatus and perfused for 12 hours.

3.3.3. *Ex-situ* heart perfusion, apparatus, perfusate, and data collection

The hanging heart perfusion circuit was constructed using a membrane oxygenator (D 905, LivaNova, LDN, UK), a venous reservoir, two centrifugal pumps (ROTAFLOW, MAQUET, CA, US), and an arterial line filter (LivaNova, LDN, UK). After cannulation of the LA, aorta (Ao), and pulmonary artery (PA), the heart was perfused in a hanging position in the open area of the reservoir, hanging from the Ao, La, PA cannula connections [3], (Figure 3.1A&B).

The supporting heart perfusion circuit was constructed using two centrifugal pumps (ROTAFLOW, MAQUET, CA, US), an arterial line filter (LivaNova, LDN, UK), a membrane oxygenator (D 905, LivaNova, LDN, UK), and a custom-built organ chamber containing a silicone support membrane with integrated Ao, La, and Pa cannulas. The hearts were perfused in a semi-horizontal position on the supporting membrane in a slightly downward angle (~ 20°), [7], (Figures 3.1C&D).

The ESHP apparatuses used here (in both hanging- and supported-perfusion groups) were equipped with a custom software program allowing consistent control of pump speed to achieve and maintain desired LA and Ao pressures, analyzing functional

parameters, and providing a visual evaluation of pressure waveforms [7]. Perfusion was initiated in a non-working (Langendorff) mode (NWM, LA pressure = 0 mmHg). The physiologic working mode (WM) was achieved by increasing the LAP to 6 mmHg.

Whole blood from the donor animal was mixed with modified Krebs-Henseleit buffer containing 8% albumin (1:1) to achieve 1700 mL of circulating perfusate [3, 16]. The perfusate was infused with glucose (0.5-1gram/hour), insulin (2 units/hour), and dobutamine (4 micrograms/minute) during ESHP. A pH of 7.35-7.45, partial pressure of arterial oxygen (paO_2) of 100-150 mmHg, and partial pressure of arterial carbon dioxide ($paCO_2$) of 35-45 mmHg was maintained during the perfusion. The coronary sinus perfusate flow and La flow were measured using a *tubing flow module and* two flow meters (TS410, Transonic, NY, US) [7].

At T0, (start of perfusion), T5, and T11 perfusate samples were collected from the aortic root and were stored in -80℃ for further analysis. Blood gas analysis was performed regularly, including at T1, T5, and T11 on the perfusate samples collected from Ao root, and venous line (PA), (ABL 800 blood gas analyzer, Radiometer, ON, CA).

3.3.4. Experimental groups

ESHP was initiated in the non-working mode (NWM) in all groups. The heart and the perfusate were warmed from room temperature to 38 °C gradually over 60-minutes. The hearts were paced with atrial wires at 100 beats/minute during the perfusion.

3.3.4.1. Hanging, working mode perfusion group (WM-H) (n = 6)

The hearts were perfused in a suspended position (Figure 3.1A&B). After the initial perfusion in NWM, the hearts were transitioned into the working mode (WM) by increasing

left atrial perfusate flow to achieve a left atrial pressure (LAP) of 6 mmHg and were maintained in WM over the remainder of the 12-hour perfusion.

3.3.4.2. Hanging heart non-working mode perfusion group (NWM-H) (n = 6)

The hearts were perfused in a suspended position (Figure 3.1 A&B) and were maintained in the unloaded, NWM over the 12-hour preservation period except for the brief transitions into WM (LAP=6 mmHg) at T1 (1-hour of *ex-situ* perfusion, early perfusion) T5 (5-hours of *ex-situ* perfusion, mid-perfusion), and T11 (11-hours of *ex-situ* perfusion, late perfusion) to assess myocardial function and metabolism.

3.3.4.3. Supported heart, working mode perfusion group (WM-S) (n = 6)

The hearts were perfused in a semi-horizontal position with the myocardial mass supported by a silicone membrane (Figure 3.1C&D). After the initial NWM perfusion, the hearts were transitioned into WM by increasing LAP to 6 mmHg. WM perfusion was maintained over the remainder of the 12-hour perfusion.

3.3.4.4. Supported heart non-working mode perfusion group (NWM-S) (n = 6)

The hearts were perfused in a semi-horizontal position with the myocardial mass supported by a silicone membrane (Figure 3.1C&D) The hearts were perfused in an unloaded, NWM state over the 12-hour preservation period except for the brief transitions into WM (LAP=6 mmHg) at T1, T5, and T11 to assess myocardial function and metabolism.

3.3.5. Assessment of myocardial functional parameters

Myocardial function was evaluated at T1, T5, and T11 in a physiologic working mode (LAP=6 mmHg) in all the perfusion groups. The cardiac index (CI) ($mL.minute^{-1}.gram^{-1}$),

left ventricular (LV) maximum and minimum rates of pressure change (max dP/dt and min dP/dt), and LV stroke work (LVSW) were calculated as described before [3]. To assess the preservation of myocardial function in experimental groups, the relative values of the parameters at T5 and T11 (compared to baseline values assessed at T1) were calculated and compared between the experimental groups. Since unlike the hanging-perfused hearts, the LV filling is not facilitated by the force of gravity, the functional potential of these hearts was also assessed during a gradual increase of the LAP from 6 mmHg to 12 mmHg.

3.3.6. Assessment of myocardial metabolism

Myocardial oxygen consumption (MVO_2) was measured as described previously [3].

Venoarterial lactate difference was calculated using the arterial and venous perfusate lactate concentrations measured with blood gas analysis (ABL 800 blood gas analyzer, Radiometer, ON, CA).

3.3.7. Functional recovery with energy substrate supplementation

After the late perfusion assessment (at T11), we assessed the potential recovery/improvement of the myocardial function with administration of exogenous pyruvate (the end product of glycolysis pathway, energy substrate) in the perfusate to achieve a pyruvate concentration of 5 mmol/L (Fisher Scientific, AB, CA) [3]. Myocardial function was assessed after the addition of the pyruvate, upon the effect reaching a plateau.

3.3.8. Myocardial injury

3.3.8.1. Myocardial edema

The weight gained by the heart over the 12-hour ESHP (edema formation) was measured and reported as a percentage of initial heart weight as described previously [3, 4].

3.3.8.2. Troponin-I

The perfusate concentration of cardiac troponin-I (adjusted with heart weight) was determined at T1, T5, and T11 using a porcine ELISA Kit (Life Diagnostics, US).

3.3.8.3. Histological changes

Histologic sections (6 μm thick) of the OCT-embedded myocardial tissue samples collected from the anterolateral wall of the LV in *ex-situ* perfused, and in-vivo hearts were stained with hematoxylin and eosin (H & E). All the histological assessments were performed by a single investigator (CS) who was blinded to the groups. Each slide was covered, and no labeling was associated with the slide at the time of the evaluation. The cardiomyocyte characteristics (nuclear number, size, area) was assessed using an operator-interactive, semi-automated method for quantification of the fiber data as previously reported [18-22]. This method is at least equivalent to Cavalieri stereology [23, 24] with minimal differences but harbors more accuracy than the Cavalieri stereology. The parameters measured in the H & E-stained sections of LV were the variation of area, perimeter, and width of the nuclei as well as the variation of their angle, circularity, the Feret diameter (as the longest distance between any two points along the selection boundary), skewness, and kurtosis.

3.3.9. Statistical analysis

The normally- distributed data (assessed with the Shapiro-Wilk test and evaluation of the histogram plot of the data distributions) were reported as the mean ± the standard error of the mean. The non-normally- distributed continuous data were transformed to normality using the “two-step transformation to normality” as described before [25] and were reported as median [Interquartile Range (IQR)]. The data were compared between the groups using the independent samples t-test, or the analysis of variance (one way-ANOVA), followed by the Fisher’s Least Significant Difference (LSD) or Games-Howell post hoc tests where appropriate. The Welch correction was applied when the assumption of equal variances was not met. The overtime trend of changes within each group was assessed using a linear regression model. A 2-sided p-value < 0.05 was considered statistically significant. The analysis was performed with the IBM SPSS statistics software (Version 21.0).

3.4. Results

There was no statistically meaningful differences in heart weight, defibrillation requirements for reanimation, and the main perfusate characteristics between experimental groups (Supplementary Table s3.1). Overall, the total ischemic time was significantly higher in supported-perfused (S) compared to hanging-perfused (H) hearts (S: 19.33±1.38, H: 5.5±0.45 min, $p<0.001$). Mounting the heart on the apparatus takes longer in the setting of supported perfusion system, due to its requirement for different adjustments for attachment of the La cannula, and more detailed de-airing of chambers in the lying position, before the reestablishment of the aortic perfusion. There was no

significant difference in ischemic time between WM and NWM in each perfusion position (WM-S: 19.66±1.76, NWM-S: 19.00±2.29 min, p=0.995 and WM-H: 5.83±0.87, NWM-H: 5.16±0.30 min, p=0.886).

3.4.1. Myocardial functional parameters

The cardiac index (CI), left ventricular stroke work (SW), ventricular maximum rate of pressure change (dp/dt maximum), and minimum rate of pressure change (dp/dt minimum) values were all significantly higher in hanging-perfused hearts at baseline (T1, 1 hour after starting of the perfusion, early perfusion) compared to supported-perfused hearts regardless of the perfusion mode. However, over the 12-hour perfusion CI did not change significantly in the WM-S (p=0.506), while it significantly declined in NWM-S (p=0.028), WM-H (p=0.002), and NWM-H (p<0.001). The LVSW did not change in the WM-S (p=0.831) but significantly decreased in NWM-S (p=0.008), and both WM-H (p<0.001), and NWM-H (p<0.001), (Table 3.1), (Figure 3.2A&B).

While ventricular dp/dt maximum improved over perfusion time in WM-S (p=0.026), it did not change in NWM-S (p=0.937). However, it significantly declined in both WM-H (p<0.001) and NWM-H (p<0.001). The ventricular dp/dt minimum, did not change in the WM-S group (p=0.366), but it significantly declined in NWM-S (p=0.003), in WM-H (p<0.001), and in NWM-H (p<0.001), (Table 3.1), (Figure 3.2C&D).

Gradual increasing of the left atrial pressure (LAP) from 6 mmHg to 12 mmHg in the supported-perfused hearts, lead to a significant improvement in CI in WM-S at all the assessment time points, (T1: p<0.001, T5 (5 hours after starting of the perfusion, mid-perfusion: p=0.001, and T11 (11 hours after starting of the perfusion, late perfusion: p=0.011). In the NWM-S hearts, increasing LAP lead to improvement of CI at baseline

(T1: p=0.012), but it did not affect CI significantly at T5 (T5: p=0.062), and T11 (T11: p=0.384), (Figure 3.2E).

At both T5 (mid-perfusion) and T11 (late perfusion), the relative (preserved) CI was significantly higher in WM-S compared NWM-S (T5: p=0.009 and T11: p=0.001), WM-H (T5: p=0.009 and T11: p<0.001), and NWM-H (T5: p<0.001 and T11: p<0.001). At T5, relative SW was similar between WM-S and NWM-S (p=0.053), and it was significantly higher in WM-S compared to WM-H (p=0.002) and NWM-H (p<0.001). At T11, the relative SW was higher in WM-S compared to NWM-S (p=0.004), WM-H (p<0.001), and NWM-H (p<0.001), (Table 3.2), (Figure 3.3A&B).

The relative max dp/dt at T5 was significantly higher in WM-S compared to WM-H (p=0.002) and NWM-H (p<0.001). The relative max dp/dt at T5 was similar between the WM-S and NWM-S (p=0.994). At T11 the relative max dp/dt was significantly higher in WM-S WM-H (p<0.001), and NWM-H (p<0.001) however, it was similar between WM-S and NWM-S (p=0.058). At T5 the relative min dp/dt, while similar between WM-S and NWM-S (p= 0.077), was significantly better in WM-S compared to WM-H (p=0.030), and NWM-H (p<0.001). At T11, the relative min dp/dt was significantly higher in WM-S compared to NWM-S (p=0.027), WM-H (p<0.001), and NWM-sups (p<0.001), (Table 3.2), (Figure 3.3C&D).

3.4.2. Myocardial metabolism

The myocardial oxygen consumption (MVO_2) did not change during perfusion in WM-S (T1: 3.55±0.25, T5: 3.03±0.19, T11: 2.97±0.45 $mL.min^{-1}.100g^{-1}$, p=0.212), however, it significantly decreased in NWM-S (T1: 3.73±0.38, T5: 2.53±0.23, T11: 2.66±0.36 $mL.min^{-1}.100g^{-1}$, p=0.047), WM-H (T1: 6.80±1.02, T5: 4.77±0.20, T11: 2.93±0.52, $mL.min^{-1}.100g^{-}$

^{1}, p=0.001), and NWM-H (T1: 7.08±0.84, T5: 4.47±0.82, T11: 2.22±0.72, mL.min^{-1}.100g^{-1}, p<0.001), (Figure 3.4 A).

During the whole perfusion time, the arterial and venous lactate were <5 mmol/L in all the cases except in one case (NWM-H, T11 venous, and arterial lactate) (Figure 3.4B). The veno-arterial lactate difference did not change significantly during the perfusion in either of the perfusion groups (WM-S: p=0.074, NWM-S: p=0.799, WM-H: p=0.505 and NWM-H: p=0.331) and was not different between the groups at any time point (T1: p=0.249, T5: p=0.388, and T11: p=0.079).

3.4.3. Functional recovery with energy substrate supplementation

After T11, the addition of pyruvate to the perfusate solution led to a significant improvement of the myocardial performance compared in all the perfusion groups (WM-S p=0.041, NWM-S p=0.007, WM-H p=0.012, NWM-H p=0.026). There was no significant difference in the time to reach the peak of the effect (data not shown) and duration of the effect (WM-S: 69.50±4.26, NWM-S: 74.83±3.31, WM-H: 69.83±4.41, NWM-H: 77.83±5.21 min, p=0.477). When compared to baseline (T1) functional status, the functional recovery was partial in both of the hanging-perfused groups, and post-pyruvate absolute CI was still significantly lower than baseline (T1) values (WM-H: CI T1: 15.85 [13.13,20.99], CI post-pyruvate: 9.31 [8.51,12.70] mL.min^{-1}.g^{-1}, p= 0.016, versus NWM-H: CI T1: 16.68 [12.58,20.00] versus CI post-pyruvate: 3.95 [1.87, 7.04] mL.min^{-1}.g^{-1}, p=0.003). By contrast, in the NWM-S group, pyruvate supplementation lead to a statistically complete recovery (NWM-S: CI T1: 5.54 [4.69,6.47] mL.min^{-1}.g^{-}1 versus CI post-pyruvate: 5.28 [3.75,6.07] mL.min^{-1}.g^{-1}, p=0.172). As outlined above, the WM-S had

not developed a significant functional decline before the pyruvate supplementation either (Figure 3.5A).

When assessing the relative CI after pyruvate supplementation, it was still significantly higher in WM-S compared to NWM-S (WM-S: 112.06 [100.27,126.61]% versus NWM-S: 84.31 [74.65,92.89]%, p=0.002), WM-H (WM-S: 112.06 [100.27,126.61]% versus WM-H: 61.44 [50.16,69.03]%, p<0.001), and NWM-H (WM-S: 112.06 [100.27,126.61]% versus p<0.001). The post-pyruvate relative CI in NWM-S group was also significantly higher compared to the hanging-perfused experimental groups [WM-H (p=0.004) and NWM-H (p<0.001)], (Figure 3.5B).

3.4.4 Myocardial injury

3.4.4.1. Myocardial edema

Hearts in all the perfusion groups developed edema during ESHP as evidenced by an increase in heart weight (WM-S p=0.048, NWM-S p=0.001, WM=H p=0.004, and NWM-H p<0.001). There was no statistically significant difference in myocardial edema (weight gain percentage) between the experimental groups (WM-S: 13.74±5.13%, NWM-S: 25.33±3.31%, WM-H: 18.61±4.31, and NWM-H: 26.56±2.96%, p=0.117), and neither between the supported- and hanging-perfused hearts (S: 19.54%, H: 22.58%, p=0.494), (Figure 3.6A).

3.4.4.2. Cardiac Troponin-I

Cardiac troponin-I (cTn-I) values significantly increased in the perfusate throughout ESHP in all the perfusion groups (WM-S p<0.001, NWM-S p<0.001, WM-H p<0.001, and NWM-H p<0.001). At T0, the perfusate cTn-I was not significantly different between supported- and hanging-perfused groups (p=0.796). At T5, overall cTn-I was significantly lower in

supported-perfused compared to hanging-perfused hearts (S: 2.34 [1.98,2.67], H: 13.60 [5.25,22.92] ng/mL, p<0.001). The T5 cTn-I was similar between WM-S and NWM-S (WM: 2.18±0.23, NWM: 2.29±0.19 ng/mL, p=0.526) however, it was significantly higher in NWM compared to WM in hanging-perfused hearts (WM-H: 5.50 [4.75,8.52] ng/mL, NWM-H: 22.57 [18.74,24.02] ng/mL, p=0 .007). Similarly, at T11 the cTn-I values were significantly lower in supported-perfused hearts (S: 11.16 [7.75,16.78] ng/mL, H: 20.53 [16.30,23.36] ng/mL, p=0.001) and the values were similar between sopported-perfused WM and NWM (ng/mL WM-S: 10.75 [9.25,15.50], NWM-S: 13.72 [5.52,18.86], p=0.595). In the hanging-perfused hearts cTn-I at T11 was significantly lower in WM compared to NWM (WM-H: 17.00 [15.30,24.62] ng/mL7, NWM-H: 21.86 [19.02,25.17] ng/mL, p=0.047), (Figure 3.6B).

3.4.4.3. Histological changes

On *Hematoxylin and eosin* (H&E) staining, we found often a hypereosinophilia of the myocardial fibers in the NWM-H group (evident in the color histogram in Figure 3.6C). However, after skeletonization of the single images using the NIH-based Image-J platform, the morphologic characteristics of the nuclei of the myocardial fibers were not significantly different between the perfusion groups, or between the *ex-situ* perfused hearts and *in-vivo* controls (nuclear count: p=0.639*,* nuclear size: p=0.613, completive area of nuclei: p=0.615, circularity of nuclei: p=0.164, solidity: p=0.051, and Feret diameter: p=0.507) showing neither pyknosis nor karyorrhexis (Figure 3.6C). Wavy fibers were not clear-cut encountered, and apoptosis characterized by the detachment of scattered cardiomyocytes from neighboring cells with the formation of round-ovoidal eosinophilic globules was not seen either. Cardiomyocyte hypereosinophilia was

characterized by cardiomyocyte cytoplasm with a homogeneous appearance, which was associated with an increase in the intensity of eosin staining. Coagulative necrosis was not detected.

3.5. Discussion

To date, experimental studies reported that the function of the heart gradually declines during perfusion despite the potential and proven advantages of ESHP [3, 4]. Despite the considerable variability in methods and devices for ESHP, [5-13], a consistent attribute across these platforms is the hanging position of the heart, bearing its own empty/full weight in a considerably different status compared to its *in-vivo,* supported position in the mediastinum [17]. Similarly, the heart is also perfused in a semi-hanging state in the OCS device for clinical ESHP (not FDA approved yet), that only operates in empty beating non-working mode [1, 26].

To our knowledge, this is the first time that the effects of heart position during ESHP on the function and viability of the heart have been investigated. In the present study, the baseline functional parameters in the hanging-perfused hearts showed significantly higher values compared to the supported-perfused hearts. However, while hanging-perfused hearts experienced a significant and considerable functional decline during 12-hours of ESHP, the supported-perfused hearts preserved their function significantly better, with the WM-S group showing the best maintained functional status during the perfusion. The high values of baseline functional parameters in the hanging-perfused, compared to the supported hearts, can be explained by the increased acceleration due to gravity during early ventricular diastole. It is known that during ventricular systole, there

is compression of the extracellular matrix (series elastic component), which then recoils during early diastole, facilitating ventricular filling [27].

It was routinely observed that the unconstrained, hanging heart has considerable vertical, horizontal, and torsional movement throughout the cardiac cycle. This situation, without the support of pericardium and diaphragm, may induce augmented ventricular filling (preload), resulting in greater end-diastolic volume and, therefore overall higher cardiac output at baseline. However, this additional workload may lead to myocardial tissue stress and injury with subsequent inefficient contractile force generation (Starling forces) over time [28, 29]. Thus, it may play an essential role in tissue injury and the functional decline of these hearts.

The cardiac troponin-I values in the perfusate significantly increased throughout the ESHP regardless of the position of the heart (supported or hanging), and perfusion mode (WM or NW). The elevated concentration of troponin-I in the perfusate, generally attributed to myocyte necrosis, may be related to ischemia-reperfusion injury, surgical trauma, and possible mechanical trauma during ESHP, particularly in the non-anatomical hanging ESHP. Here, the perfusate troponin-I levels were significantly higher in hanging-perfused hearts compared to supported-perfused ones, despite enduring significantly shorter ischemic time. On the other hand, troponin-I also rose significantly overtime in the supported-perfused hearts, even in the WM-S group that showed excellent preservation of function during 12 hours of ESHP. Although longer ischemic times in supported-perfused hearts may have played a role in troponin-I secretion, the endured ischemic time in the supported groups was still shorter than the ischemic times typically seen in clinical practice and it was in a range considered highly tolerable by the heart [15]. It has been

demonstrated previously that the elevated troponin-I may not reflect the ultimate cell death, as it can be released from the cell as a response to reversible cell stress/damage [3, 30, 31]. These findings, although questioning the value of perfusate troponin-I levels as a tool for predicting the functional and vital status of the heart during ESHP in general, still suggest that the hanging-perfused heart undergo more significant mechanical stress during ESHP than the supported-perfused hearts, possibly due to the non-anatomical, weight-bearing condition of the heart during perfusion in this setting.

In accordance with our previous observations, here we have shown that hearts perfused in a loaded beating state enjoy better preservation of function compared to the empty beating hearts over 12-hours of ESHP. Interestingly, a gradual increase in LAP in supported-perfused hearts also led to a significant enhancement of myocardial performance in WM-S group at all the assessment time points, demonstrating that maximal cardiac output is not required to optimally preserve the function when the heart is kept full and beating. However, the NWM-S hearts did not show a significant improvement of the myocardial performance in response to increased LAP, except early during the perfusion (T1). Thus, similar to our previous observations [3, 32] the present study also strongly suggests that ESHP in WM not only permits functional assessment of the heart, it may hold protective effects on the functional viability of the heart during ESHP as well. The mechanism underlying this phenomenon is not yet known but is under investigation by our group.

Our findings may also introduce an avenue for improvement of clinical ESHP protocols as the currently available clinical ESHP device (not yet FDA-approved) can only perfuse the heart in the NWM. Assessment of the donor's heart on this platform relies on the

metabolic findings (lactate concentration in particular) however, it has been shown by our group and others that lactate values do not highly correlate with the functional status of the heart during ESHP, and after transplantation [2, 33, 34]. Similarly, in the present study, despite the significant difference in the functional preservation between the experimental groups during and at the end of ESHP, the venous lactate was <5 mmol/L in all of the cases except one. The veno-arterial lactate values were also similar in all the groups despite significant functional differences, suggesting that functional alongside metabolic assessment is optimal.

All the experimental groups here showed improved cardiac performance upon supplementation of pyruvate even though the WM-S had not manifested any statistical change in the functional parameters during ESHP. As we have shown in our previous work, the metabolic reserve/performance of the *ex-situ* perfused hearts may decrease during perfusion, and supplementation of pyruvate at supra-physiologic concentrations can at least partially rescue the functional decline occurring during ESHP [3]. While the recovery of function was partial in both of the hanging-perfused groups, it was statistically completed in the NWM-S group. The WM-S group showed a significant improvement of function in comparison to the baseline (T1) suggesting that perfusion in a supported position and in WM can lead to a superior preservation of metabolic and functional potential of the heart during ESHP.

In this study, the diastolic function (assessed with min dp/dt), while did not change significantly in WM-S group, declined in the remainder of the experimental groups. In contrast, edema formation was not statistically different between the groups. This observation suggests that the general extracorporeal-related factors such as activation of

inflammatory responses, may play a key role in the development of myocardial edema during ESHP [32] and that additional factors other than edema contribute to diastolic dysfunction. Interestingly, despite the changes observed in functional status, perfusate cTn-I, and edema, we did not detect any significant histological changes compared to *in-vivo* in either of the perfusion groups. This is also in accordance with the previous reports by our team, suggesting that the functional deterioration of the heart during ESHP cannot be explained by tissue injury and cell death [3, 35]. Based on the current data set, we conclude that the perfusion-related stressors, including mechanical stress, may negatively affect the mechanics of the perfusion and preservation of myocardial integrity and performance. The unnatural mechanics of the hanging heart may not only mislead the functional evaluation of the donated heart during ESHP, but it may contribute to tissue injury that may also affect early/late post-transplantation outcomes. More studies are warranted to clarify and address these issues for improving ESHP methods and protocols.

3.6. Study limitations

In general, in the present study, the ischemic times in all groups were shorter than the usual ischemic times in current clinical practice, since our main goal here was to investigate the effects of different positions during ESHP on myocardial function. Thus, we avoided longer ischemic times and severe ischemia-reperfusion injury prior to the initiation of ESHP. Mounting the heart on the supported-perfused apparatus takes a longer time compared to the hanging-perfused hearts, as cannulation of the supported-perfused heart requires more surgical manipulation before the reestablishment of the aortic perfusion (including adjustment of LA cannula, and de-airing the chambers). This

lead to longer ischemic times in supported- compared to hanging-perfused hearts; however, it was still in the ranges considered highly tolerable by the heart.

3.7. Conclusion

Ex-situ perfusion of the heart in a semi-anatomical, supported position is associated with higher functional preservation and better recovery in response to metabolic assistance when compared to a hanging-perfused position. Regardless of the perfusion position, ESHP in the physiologic WM is associated with better systolic and diastolic functional preservation of the heart. Addition of WM modality to the clinical ESHP device may represent an avenue to improve both assessment conditions, and cardio-preservation during ESHP, and may help to achieve longer safe preservation times.

3.8. Funding

This work was supported by the Canadian Institutes for Health Research/Canadian National Transplant Research Program, and the NSERC/CIHR funded Collaborative Health Research Projects Program and the University Hospital Foundation.

3.9. Conflict of interest

DF and JN are founders of Tevosol, Inc.

3.10. References

1. Ardehali A, Esmailian F, Deng M, Soltesz E, Hsich E, Naka Y, Mancini D, Camacho M, Zucker M, Leprince P *et al*: **Ex-vivo perfusion of donor hearts for human heart transplantation (PROCEED II): a prospective, open-label, multicentre, randomised non-inferiority trial**. *Lancet (London, England)* 2015, **385**(9987):2577-2584.
2. Hatami S, Freed DH: **Machine perfusion of donor heart: state of the art**. *Current Transplantation Reports* 2019, **6**(3):242-250.
3. Hatami S, White C, Shan S, Haromy A, Qi X, Ondrus M, Kinnear A, Himmat S, Michelakis E, Nagendran J *et al*: **Myocardial functional decline during prolonged ex situ heart perfusion**. *The Annals of thoracic surgery* 2019, **108**:499-507.
4. Sandha JK, White CW, Muller A, Avery E, Thliveris J, Dixon IMC, Arora RC, Tian G, Hryshko LV, Nagendran J *et al*: **Steroids limit myocardial edema during ex vivo perfusion of hearts donated after circulatory death**. *The Annals of thoracic surgery* 2018, **105**(6):1763-1770.
5. Church JT, Alghanem F, Deatrick KB, Trahanas JM, Phillips JP, Hee Song M, Perkins EM, Bartlett RH, Rojas-Pena A, Bocks ML *et al*: **Normothermic ex vivo heart perfusion: effects of live animal blood and plasma cross circulation**. *ASAIO journal (American Society for Artificial Internal Organs : 1992)* 2017, **63**(6):766-773.
6. Garbade J, Krautz C, Aupperle H, Ullmann C, Lehmann S, Kempfert J, Borger MA, Dhein S, Gummert JF, Mohr FW: **Functional, metabolic, and morphological aspects of continuous, normothermic heart preservation: effects of different preparation and perfusion techniques**. *Tissue engineering Part C, Methods* 2009, **15**(2):275-283.
7. Hatami S, White CW, Ondrus M, Qi X, Buchko M, Himmat S, Lin L, Cameron K, Nobes D, Chung HJ *et al*: **Normothermic ex situ heart perfusion in working mode: assessment of cardiac function and metabolism**. *Journal of visualized experiments : JoVE* 2019(143).
8. Ozeki T, Kwon MH, Gu J, Collins MJ, Brassil JM, Miller MB, Jr., Gullapalli RP, Zhuo J, Pierson RN, 3rd, Griffith BP *et al*: **Heart preservation using continuous ex vivo perfusion improves viability and functional recovery**. *Circulation journal : official journal of the Japanese Circulation Society* 2007, **71**(1):153-159.
9. Schraufnagel DP, Steffen RJ, Vargo PR, Attia T, Elgharably H, Hasan SM, Bribriesco A, Wierup P: **Devices for ex vivo heart and lung perfusion**. *Expert review of medical devices* 2018, **15**(3):183-191.
10. Tolboom H, Olejnickova V, Reser D, Rosser B, Wilhelm MJ, Gassmann M, Bogdanova A, Falk V: **Moderate hypothermia during ex vivo machine perfusion promotes recovery of hearts donated after cardiocirculatory deathdagger**. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* 2016, **49**(1):25-31.
11. Trahanas JM, Witer LJ, Alghanem F, Bryner BS, Iyengar A, Hirschl JR, Hoenerhoff MJ, Potkay JA, Bartlett RH, Rojas-Pena A *et al*: **Achieving 12 hour normothermic ex situ heart perfusion: an experience of 40 porcine hearts**. *ASAIO journal (American Society for Artificial Internal Organs : 1992)* 2016, **62**(4):470-476.
12. Van Caenegem O, Beauloye C, Bertrand L, Horman S, Lepropre S, Sparavier G, Vercruysse J, Bethuyne N, Poncelet AJ, Gianello P *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 : official journal of the European Association for Cardio-thoracic Surgery* 2016, **49**(5):1348-1353.

13. Xin L, Gellner B, Ribeiro RVP, Ruggeri GM, Banner D, Meineri M, Rao V, Zu J, Badiwala MV: **A new multi-mode perfusion system for ex vivo heart perfusion study**. *Journal of Medical Systems* 2017, **42**(2):25.
14. Cobert ML, Peltz M, West LM, Merritt ME, Jessen ME: **Glucose is an ineffective substrate for preservation of machine perfused donor hearts**. *The Journal of surgical research* 2012, **173**(2):198-205.
15. Iyer A, Gao L, Doyle A, Rao P, Jayewardene D, Wan B, Kumarasinghe G, Jabbour A, Hicks M, Jansz PC *et al*: **Increasing the tolerance of DCD hearts to warm ischemia by pharmacological postconditioning**. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2014, **14**(8):1744-1752.
16. White CW, Hasanally D, Mundt P, Li Y, Klein J, Xiang B, Müller A, Ambrose E, Ravandi A, Arora RC *et al*: **A wholeblood–based perfusate provides superior preservation of myocardial function during ex vivo heart perfusion**. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* 2014, **S1053-2498**(14):01356-01354.
17. Rehman I, Rehman A: **Anatomy, Thorax, Pericardium**. Treasure Island (FL): StatPearls Publishin StatPearls Publishing LLC.; 2019.
18. Kumar P, Morton JS, Shah A, Do V, Sergi C, Serrano-Lomelin J, Davidge ST, Beker D, Levasseur J, Hornberger LK: **Intrauterine exposure to chronic hypoxia in the rat leads to progressive diastolic function and increased aortic stiffness from early postnatal developmental stages**. 2020, **8**(1):e14327.
19. Schneider CA, Rasband WS, Eliceiri KW: **NIH Image to ImageJ: 25 years of image analysis**. *Nature Methods* 2012, **9**(7):671-675.
20. Faa A, Iacovidou N, Xanthos T, Locci A, Pampaloni P, Aroni F, Papalois A, Faa G, Fanos V: **Hypoxia/reoxygenation-induced myocardial lesions in newborn piglets are related to interindividual variability and not to oxygen concentration**. *Clinics (Sao Paulo, Brazil)* 2012, **67**(5):503-508.
21. Lemler MS, Bies RD, Frid MG, Sastravaha A, Zisman LS, Bohlmeyer T, Gerdes AM, Reeves JT, Stenmark KR: **Myocyte cytoskeletal disorganization and right heart failure in hypoxia-induced neonatal pulmonary hypertension**. *American journal of physiology Heart and circulatory physiology* 2000, **279**(3):H1365-1376.
22. McGuire M, Bradford A: **Chronic intermittent hypoxia increases haematocrit and causes right ventricular hypertrophy in the rat**. *Respiration physiology* 1999, **117**(1):53-58.
23. Baddeley AJ, Gundersen HJG, Cruz-Orive LM: **Estimation of surface area from vertical sections**. *Journal of Microscopy* 1986, **142**(3):259-276.
24. Gundersen HJG, Jensen EB: **The efficiency of systematic sampling in stereology and its prediction***. *Journal of Microscopy* 1987, **147**(3):229-263.
25. Templeton GF, Burney LL: **Using a two-step transformation to address non-normality from a business value of information technology perspective**. *Journal of Information Systems* 2017, **31**(2):149-164.
26. Messer S, Ardehali A, Tsui S: **Normothermic donor heart perfusion: current clinical experience and the future**. *Transplant international : official journal of the European Society for Organ Transplantation* 2015, **28**(6):634-642.
27. Robinson TF, Factor SM, Sonnenblick EH: **The heart as a suction pump**. *Scientific American* 1986, **254**(6):84-91.
28. Hinghofer-Szalkay H: **Gravity, the hydrostatic indifference concept and the cardiovascular system**. *European journal of applied physiology* 2011, **111**(2):163-174.

29. Malanga CJ: **Structure and function of the heart**. In: *XPharm: The Comprehensive Pharmacology Reference.* edn. Edited by Enna SJ, Bylund DB: Elsevier; 2007: 1-5.
30. Hickman PE, Potter JM, Aroney C, Koerbin G, Southcott E, Wu AH, Roberts MS: **Cardiac troponin may be released by ischemia alone, without necrosis**. *Clinica chimica acta; international journal of clinical chemistry* 2010, **411**(5-6):318-323.
31. Tanindi A, Cemri M: **Troponin elevation in conditions other than acute coronary syndromes**. *Vascular health and risk management* 2011, **7**:597-603.
32. Hatami S, White C, Qi X, Buchko M, Ondrus M, Kinnear A, Himmat S, Sergi C, Nagendran J, Freed D: **Immunity and stress responses are induced during ex-situ heart perfusion**. *Circ Heart Fail* 2020, **13**(6):e006552.
33. Stamp NL, Shah A, Vincent V, Wright B, Wood C, Pavey W, Cokis C, Chih S, Dembo L, Larbalestier R: **Successful heart transplant after ten hours out-of-body time using the TransMedics Organ Care System**. *Heart, lung & circulation* 2015, **24**(6):611-613.
34. White CW, Ambrose E, Muller A, Li Y, Le H, Hiebert B, Arora R, Lee TW, Dixon I, Tian G *et al*: **Assessment of donor heart viability during ex vivo heart perfusion**. *Canadian journal of physiology and pharmacology* 2015, **93**(10):893-901.
35. White CW, Ali A, Hasanally D, Xiang B, Li Y, Mundt P, Lytwyn M, Colah S, Klein J, Ravandi A *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 : the official publication of the International Society for Heart Transplantation* 2013, **32**(7):734-743.

3.11. Tables and figures:

Table 3.1. Left ventricular function during *ex-situ* heart perfusion in experimental groups

Parameter		WM-S	NWM-S	WM-H	NWM-H
CI mL.min^{-1}. g^{-1}	**T1**	4.63 [4.48,6.09]	5.54 [4.69,6.47]	15.87 [13.13,20.99]	16.68 [12.58,20.00]
	T5	4.19 [3.94,6.09]	3.59 [3.10,5.10]	12.35 [9.07,15.35]	5.77 [4.35,7.71]
	T11	4.40 [4.07,6.12]	3.68 [1.37,4.48]	6.37 [4.00,7.50]	1.08 [0.18, 4.02]
	p-value	0.506	0.028	0.002	<0.001
SW mmHg.mL	**T1**	768 [635,1406]	802 [680,1196]	4896 [4059,5530]	4725 [2504,6703]
	T5	836 [661,1207]	604 [461,861]	2735 [1331,3621]	880 [537,1810]
	T11	882 [638,1295]	339 [198,662]	1102 [518,1744]	256 [0.75,622]
	p-value	0.831	0.008	<0.001	<0.001
max dp/dt mmHg.s^{-1}	**T1**	1084 [819,1304]	1085 [1042,1203]	3046 [2450,3555]	3441 [2504,5052]
	T5	1285 [1156,1359]	1190 [1033,1512]	1948 [1707,2088]	2079 [952,2922]
	T11	1342 [1147,1492]	1111 [893,1347]	1709 [1428,1928]	798 [155,1548]
	p-value	0.026	0.937	<0.001	<0.001
min dp/dt mmHg.s^{-1}	**T1**	-992 [-702,-1072]	-1090 [-953,-1176]	-2659 [-2511,-2895]	-3038 [-2031,-3762]
	T5	-816 [-753,-988]	-798 [-655,-1000]	-1783 [-1649,-2270]	-1509 [-1125,-1674]
	T11	-767 [-700,-1070]	-634 [-515,-975]	-1248 [-864,-1505]	-828 [-353,-953]
	p-value	0.366	0.003	<0.001	<0.001

Data are presented as median [IQR]. CI, cardiac index; max dP/dT, maximum rate of pressure change; min dP/dT, minimum rate of pressure change; NWM-S, non-working mode perfusion in supported position; NWM-H, non-working mode perfusion in hanging position; SW, stroke work; T(x); x: hours after the start of the perfusion*;* WM-S, working

mode perfusion in supported position; WM-H, working mode perfusion in hanging position.

Table 3.2. Left ventricular functional preservation during *ex-situ* heart perfusion

preserved function	group	T5	T11
CI	**WM-S**	89.17 [86.93,103.31]	94.77 [85.07,103.52]
(%)	**NWM-S**	67.49 [59.11,80.11]	62.80 [31.17,69.03]
	WM-H	78.82 [51.51,86.66]	36.18 [22.87,51.86]
	NWM-H	39.75 [28.17,45.77]	9.75 [1.00,21.55]
	p-value	<0.001	<0.001
SW	**WM-S**	103.28 [76.71,139.52]	104.33 [90.49,134.79]
(%)	**NWM-S**	73.52 [52.99,78.40]	33.28 [26.98,64.04]
	WM-H	51.29 [32.54,73.38]	22.31 [14.65,31.54]
	NWM-H	30.38 [8.04,40.52]	9.47 [0,12.65]
	p-value	<0.001	<0.001
dP/dT max	**WM-S**	110.66 [97.71,158.46]	115.40 [112.34,156.97]
(%)	**NWM-S**	108.41 [94.97,125.60]	98.63 [83.02,112.27]
	WM-H	64.40 [45.42,93.15]	63.29 [38.04,74.37]
	NWM-H	56.10 [34.56,70.91]	25.28 [6.15,38.35]
	p-value	<0.001	<0.001
dP/dT min	**WM-S**	89.36 [82.99,114.63]	101.82 [71.00,107.50]
(%)	**NWM-S**	72.05 [68.90,80.53]	58.33 [47.56,87.01]
	WM-H	70.81 [61.46,79.59]	47.98 [34.96,54.30]
	NWM-H	44.67 [41.80,59.95]	28.32 [16.60,36.01]

	p-value	<0.001	<0.001

Data are presented as median [IQR]. CI, cardiac index; max dP/dT, maximum rate of pressure change; min dP/dT, minimum rate of pressure change; NWM-S, non-working mode perfusion in supported position; NWM-H, non-working mode perfusion in hanging position; SW, stroke work; T(x); x: hours after the start of the perfusion*;* WM-S, working mode perfusion in supported position; WM-H, working mode perfusion in hanging position.

Figures:

Figure 3.1. Hanging versus supported ex-situ heart perfusion. A: Schematic diagram of the hanging *ex-situ* heart perfusion B: Pig heart perfused in hanging position C: Schematic diagram of the supported *ex-situ* heart perfusion D: Pig heart perfused in supported position

A, organ chamber and Sorting silicone membrane; B, venous reservoir; C, arterial line filter; D, left atrial pump; E, Aortic pump; F, oxygenator; G, heat exchanger; H, medical gas mixer; I, infusion line; J, pressure sensor; K, Flow sensor; Ao, aorta; LA, left atrium; PA, pulmonary artery.

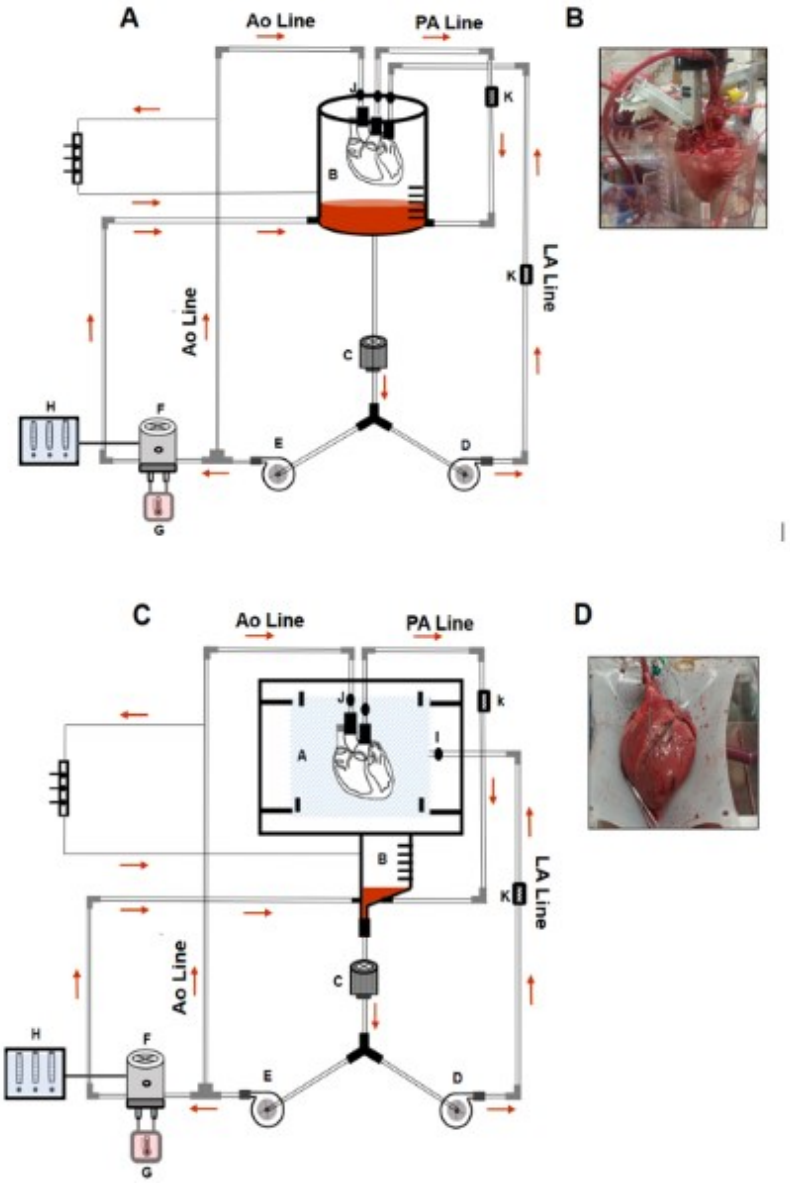

A
Ao Line
PA Line
B
K
LA Line
Ao Line
C
H
F
E
D
G
C
Ao Line
PA Line
D
k
A
I
B
K
LA Line
Ao Line
C
H
F
E
D
G

Figure 3.2. Myocardial functional parameters during ex-situ heart perfusion at the left atrial pressure of 6 mmHg (A-D), and cardiac index change over a gradual increase of the left atrial pressure from 6 to 12 mmHg in Sorted perfused hearts (E).

Data are presented as median [IQR]. LAP; left atrial pressure, NWM-S; non-working mode perfusion in supported position, NWM-H; non-working mode perfusion in hanging position; WM-S, working mode perfusion in supported position; WM-H, working mode perfusion in hanging position, T(x); x: hours after the start of the perfusion, Ω; significant overtime change in WM-S, Ø; significant overtime change in NWM-S, ■; significant overtime change in WM-H; □; significant overtime change in NWM-H; Ω; significant increase over left atrial pressure change in WM-S, error bars: IQR

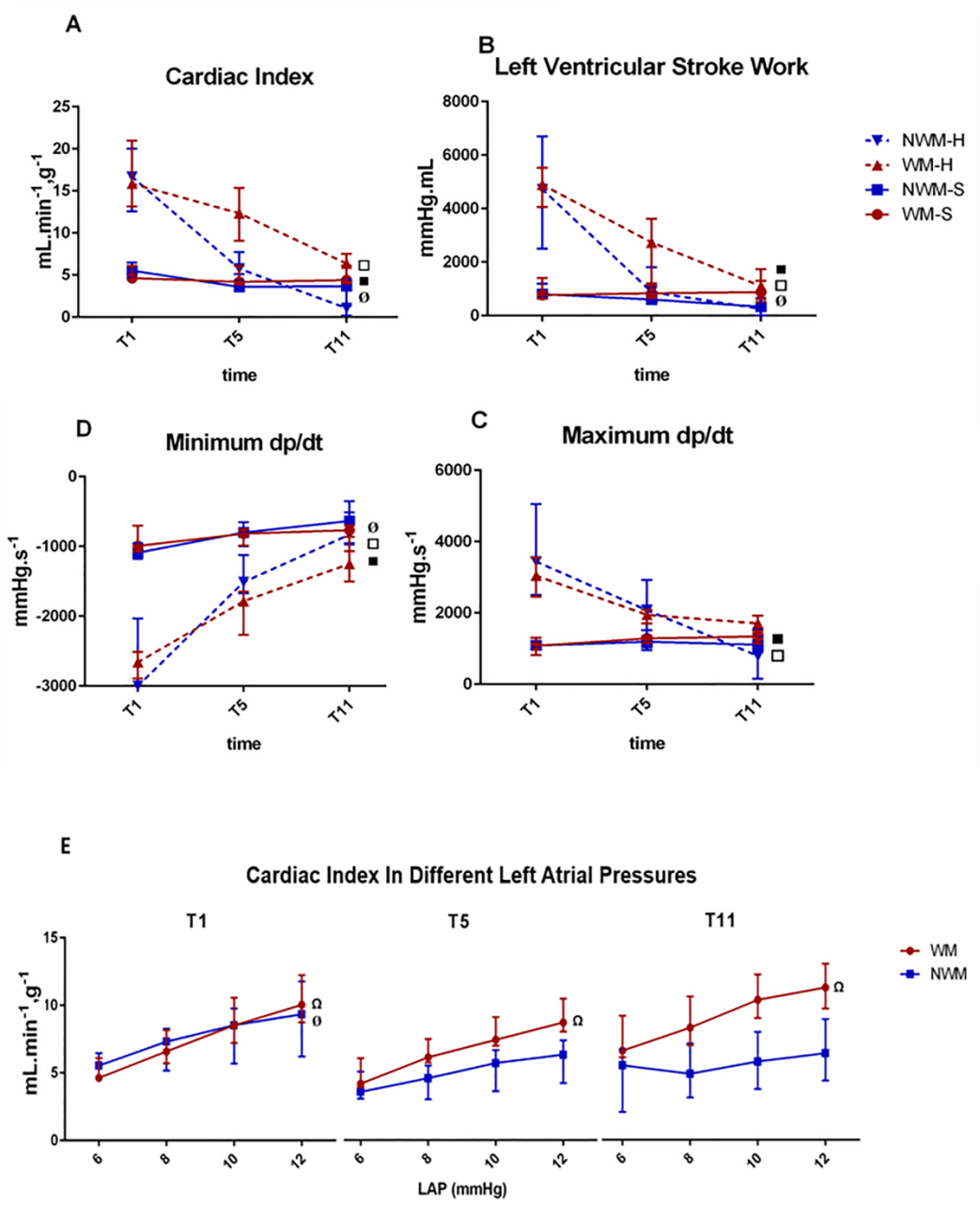

A
Cardiac Index
mL.min-1.g-1
time
T1
T5
T11
B
Left Ventricular Stroke Work
mmHg.mL
NWM-H
WM-H
NWM-S
WM-S
D
Minimum dp/dt
mmHg.s-1
C
Maximum dp/dt
E
Cardiac Index In Different Left Atrial Pressures
LAP (mmHg)
WM
NWM

Figure 3.3. Preserved (relative to the baseline) myocardial functional parameters during ex-situ heart perfusion (A-D),

Data are presented as median [IQR]. NWM-S; non-working mode perfusion in supported position, NWM-H; non-working mode perfusion in hanging position; WM-S, working mode perfusion in supported position; WM-H, working mode perfusion in hanging position, T(x); x: hours after the start of the perfusion, *; p- value<0.05, **; p-value<0.01, ***; p-value<0.001, error bars: IQR

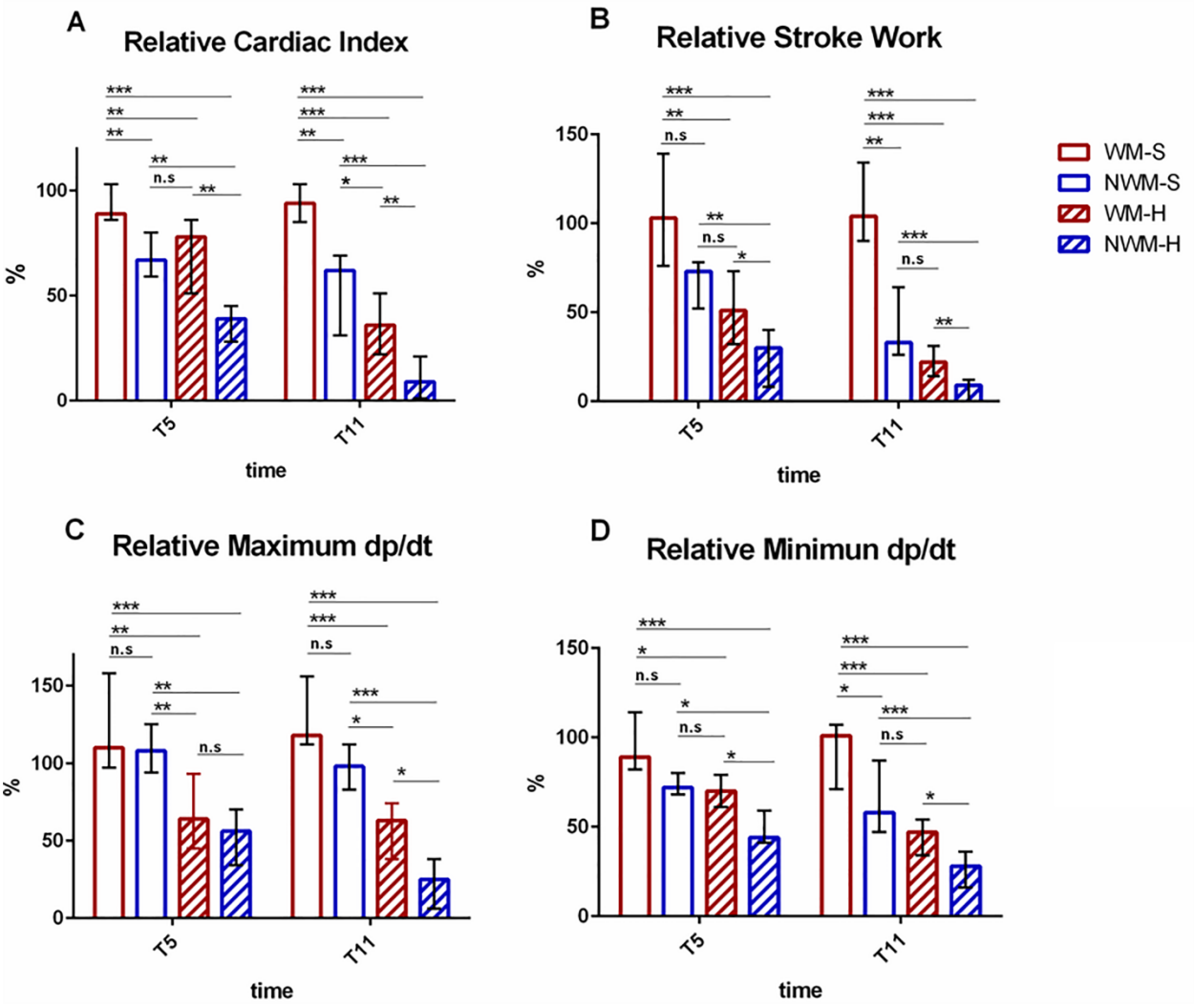


Figure 3.4. Metabolic parameters during ex-situ heart perfusion. A: myocardial oxygen consumption B: Venous lactate values during the whole duration of the perfusion

Data are presented as median [IQR]. MVO_2; myocardial oxygen consumption; NWM-S; non-working mode perfusion in supported position, NWM-H; non-working mode perfusion in hanging position; WM-S, working mode perfusion in supported position; WM-H, working mode perfusion in hanging position, T(x); x: hours after the start of the perfusion, Ø; significant overtime change in NWM-S, □; significant overtime change in NWM-H, ■; significant overtime change in WM-H; —, median venous lactate, error bars; SD

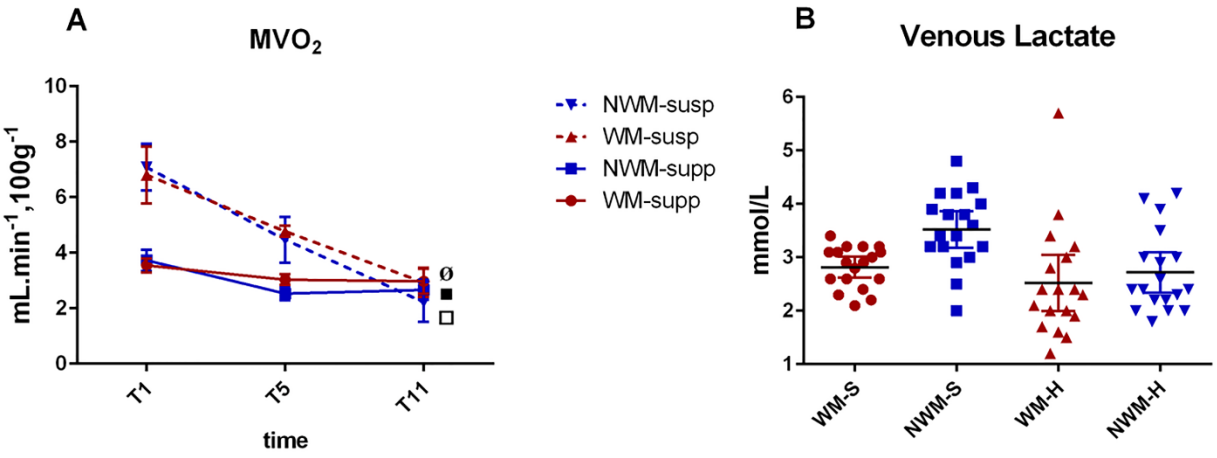


Figure 3.5. Absolute and relative cardiac index during the perfusion and after pyruvate supplementation

Data are presented as median [IQR]. NWM-S; non-working mode perfusion in supported position, NWM-H; non-working mode perfusion in hanging position; post-pyruvate, after administration of pyruvate, WM-S, working mode perfusion in supported position; WM-H, working mode perfusion in hanging position, T(x); x: hours after the start of the perfusion, Ω; significant change after pyruvate supplementation in WM-S, Ø; significant change after pyruvate supplementation in NWM-S, ■; significant change after pyruvate supplementation in WM-H, □; significant change after pyruvate supplementation in NWM-H, *; significantly different compared to other experimental groups, error bars; IQR

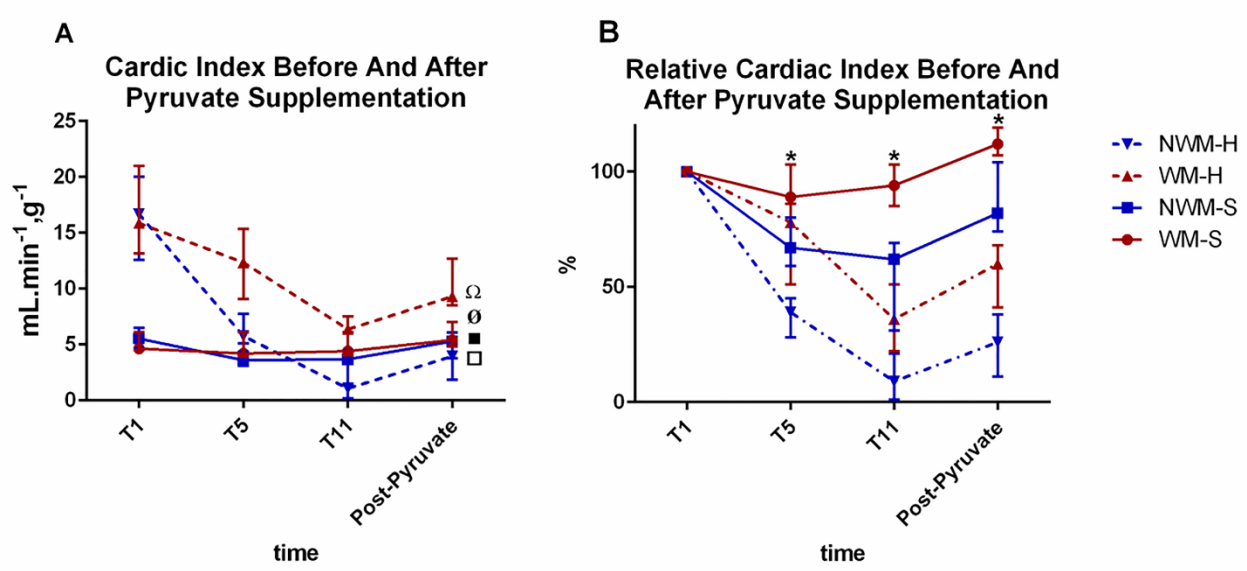
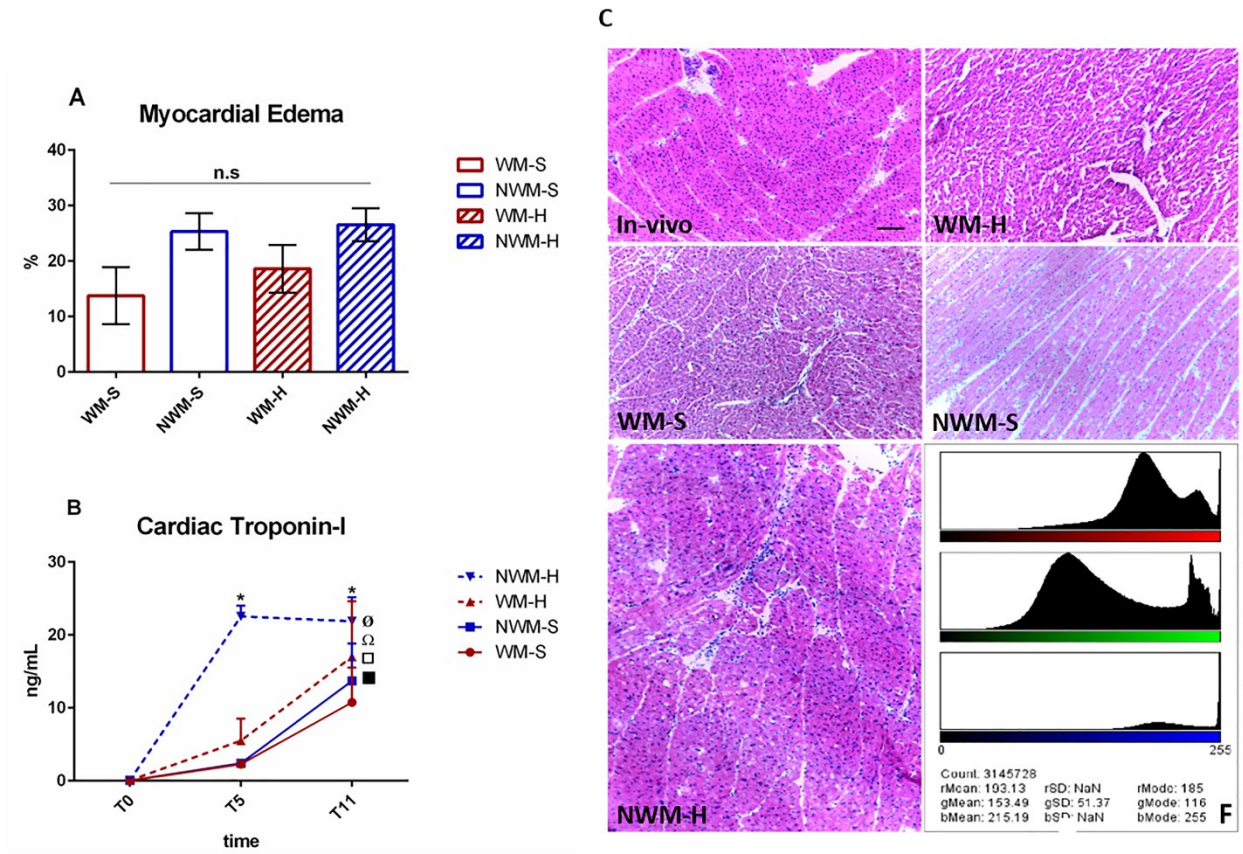


Figure 3.6. Myocardia tissue injury. Edema (A), perfusate cardiac troponin-I (B) , and histological evaluation (C) in *ex-situ*-perfused hearts. All microphotographs were stained with hematoxylin and eosin and taken at 100x magnification (Bar, 50 micrometers).

Data are presented as mean±SD (A) or median [IQR], (B). n.s.; non-significant difference, NWM-S; non-working mode perfusion in supported position, NWM-H; non-working mode perfusion in hanging position; WM-S, working mode perfusion in supported position; WM-H, working mode perfusion in hanging position, T(x); x: hours after the start of the perfusion, Ω; significant change after pyruvate supplementation in WM-S, Ø; significant change after pyruvate supplementation in NWM-S, ■; significant change after pyruvate supplementation in WM-H, □; significant change after pyruvate supplementation in NWM-H, error bars; SD or IQR



3.12. Supplementary material

Supplementary Table s3.1. Basic characteristics of perfusion and animal weight

Perfusate characteristics	WM-S	NWM-S	WM-H	NWM-H	*p-value*
***Na^+* (mmol/L)**	136.50±4.46	135.00±6.03	141.00±2.54	140.16±4.26	0.289
***K^+* (mmol/L)**	4.33±0.27	4.25±0.25	4.04±.36	3.98±0.17	0.118
***Ca^{2+}* (mmol/L)**	0.97±0.05	0.97±0.05	1.01±0.06	0.96±0.04	0.358
pH	7.33±0.07	7.33±0.07	7.33±0.08	7.29±0.13	0.848
***Glucose* (mmol/L)**	6.78±0.44	6.90±0.54	7.46±0.51	7.30±0.23	0.064
***Hemoglobin* (g/L)**	43.83±2.48	43.40±5.17	39.80±2.58	45.00±5.17	0.224
***Heart Weight* (g)**	235.66±10.78	225.0±22.48	220.33±22.36	217.83±15.56	0.375
Defibrillation	5	4	5	4	0.832

Data are presented as mean±SD. NWM-S, non-working mode perfusion in supported position; NWM-H, non-working mode perfusion in hanging position*;* WM-S, working mode perfusion in supported position; WM-H, working mode perfusion in hanging position.

Chapter 4: The Functional Decline of the *Ex-situ* Perfused Heart is not Due to Cell Death

Sanaz Hatami, MD,[1,6] Christopher W. White, MD, PhD,[1] Shubham Shan, BSc[2], Alois Haromy, MSc,[3] Xiao Qi, MD, MSc,[1,6] Martin Ondrus, BSc[1], Alexandra Kinnear, BSc[1], Sayed Himmat, MD,[1,6] Evangelos Michelakis, MD,[3] Jayan Nagendran, MD, PhD,[1,5,6] Darren H. Freed, MD, PhD,[1,2,4,5,6]*

Affiliations:

Departments of Surgery[1], Physiology[2,] Medicine[3], and Biomedical Engineering[4], University of Alberta, Edmonton, AB, Canada

Alberta Transplant Institute[5], Edmonton, AB, Canada

Canadian Transplant Research Program[6], Canada

This work has been published as: **Hatami S**, White CW, Shan S, Haromy A, Qi X, Ondrus M, Kinnear A, Himmat S, Michelakis E, Nagendran J *et al*: **Myocardial Functional Decline During Prolonged Ex Situ Heart Perfusion**. *The Annals of thoracic surgery* 2019, 108(2):499-507.

4.1. Abstract

Background: Myocardial function declines in a time dependent fashion during *ex-situ* heart perfusion. Cell death and metabolic alterations may contribute to this phenomenon, limiting the safe perfusion period and the potential of *ex-situ* heart perfusion to expand the donor pool. Our aim is to investigate the etiology of myocardial functional decline in *ex-situ* perfused hearts. **Methods:** Cardiac function, apoptosis, effectors and markers of cell death, and metabolic function were assessed in healthy pig hearts, perfused for 12 hours. These hearts were perfused either in non-working mode (NWM), or working mode (WM). **Results**: Cardiac function declined during *ex-situ* heart perfusion regardless of perfusion mode but was significantly better preserved in the hearts perfused in WM (11-hour cardiac index / 1-hour cardiac index: WM=33 vs. NWM=10%, p=0.025). The rate of apoptosis was higher in the *ex-situ* perfused hearts compared to *in vivo* samples (% apoptotic cells: *in vivo*=0.13, WM=0.54, NWM=0.88%, p<0.001) but the absolute values were low and out of proportion to the decline in function in either group. Myocardial dysfunction at the end of the perfusion interval was partially rescued by delivery of a pyruvate bolus. **Conclusions:** Hearts preserved *ex-situ* experience a significant decline in myocardial function over time that is out of proportion to the magnitude of myocyte cell death present in dysfunctional hearts. Alterations in myocardial substrate utilization during prolonged *ex-situ* heart perfusion may contribute to this phenomenon, and represent an avenue to improve donor heart preservation.

4.2. Introduction

The clinical standard for donor heart preservation is static cold storage (CS) [1], which exposes the organ to a period of cold ischemia that limits the safe preservation interval (4-6 hours) and increases the risk of primary graft dysfunction [2]. *Ex-situ* heart perfusion (ESHP) is a novel technique of extracorporeal organ perfusion that minimizes exposure to cold ischemia by providing oxygenated, nutrient-rich perfusate to the heart during preservation. ESHP has been shown to be non-inferior to CS in the preservation of standard-criteria donor hearts [1] and has facilitated the clinical transplantation of hearts donated after circulatory death [3, 4].

Experimental models of ESHP have demonstrated that myocardial function declines in a time-dependent fashion during normothermic perfusion [5] and the mechanisms responsible for this phenomenon have not been elucidated. Perfusate markers of myocyte injury increase during ESHP; however, the degree of myocardial dysfunction appears to be out of proportion to the severity of myocardial injury observed histologically [6]. Alternatively, the development of myocardial dysfunction during ESHP may have a metabolic origin. Current clinical protocols for ESHP rely on exogenous glucose as a metabolic substrate [1]; however, experimental data suggest that glucose may not participate efficiently in energy production in the ESHP setting [7, 8]. Overall, there is very little evidence regarding the optimal metabolic support for the beating heart during ESHP. Therefore, we sought to quantify the magnitude of cell death that occurs during prolonged ESHP and investigate alterations in myocardial energy metabolism that might contribute to the myocardial dysfunction developed during ESHP.

4.3. Material and Methods

After approval of institutional animal care committee of the University of Alberta, hearts from 17 female domestic pigs were utilized in this study. Animals received humane care in compliance with the guidelines of the Canadian Council on Animal Care and the guide for the care and use of laboratory animals.

4.3.1. Heart procurement and preparation:

An intramascular dose of ketamine (20mg/kg) was injected as premedication then animals were intubated and mechanically ventilated with isoflurane (1-2%). After surgical plane was confirmed, a standard median sternotomy was performed and 6F catheters were placed into the internal jugular vein and common carotid artery to monitor central venous and aortic pressures respectively. 1000 U/kg of heparin was delivered intravenously. The animals were infused with 1000 mL of Lactated Ringer's solution to facilitate the harvest of 1000 ml of whole blood to prime the ESHP circuit prior to exsanguination. After priming the circuit, a two-stage venous cannula was placed into right atrium and animal was exsanguinated (euthanized). The hearts were then excised and used either as *in vivo* controls (n=4), or were immediately mounted on the ESHP apparatus and perfused for 12 hours (n=13).

4.3.2. *Ex-situ* heart perfusion and sample collection:

The ESHP apparatus was constructed as described previously [9]. A membrane oxygenator (D 905, LivaNova, UK), venous reservoir, two centrifugal pumps (ROTAFLOW, MAQUET, US), and an arterial blood leukocyte filter (LeukoGuard LG, PALL Medical, US) were used to construct the ESHP circuit (Figure 4.1A). A custom-

designed computer system was used here to control the pump speed to maintain the desired aortic diastolic pressure (40 mm Hg) and left atrial pressure. A combination (1:1) of whole donor blood and STEEN solution (XVIVO Perfusion, Goteborg, Sweden) was used for the perfusate [9]. Glucose (1 grams/hour), insulin (2 units/hour), and epinephrine (0.04 micrograms/minute) were infused continuously. The coronary sinus blood flow and left atrial flow were measured using a TS410 *Tubing Flow Module and* two flow meters (Transonic, US). Perfusion was initiated in a Langendorff, non-working mode (NWM). The transition from an empty beating, non-working state (left atrial pressure = 0 mmHg) into a physiologic working mode (WM) was achieved by increasing the left atrial pump revolutions per minute to maintain desired left atrial pressure (6 mmHg). At hours 1 (T1), 5 (T5), and 11 (T11) after the start of the perfusion, perfusate samples were collected from the aortic root and were stored in -80 ˚C. Also, blood gas analysis was performed on the perfusate samples collected from aortic root, and venous line (pulmonary artery), (ABL 800 blood gas analyser, Radiometer, US). At the end of ESHP, samples were taken from the anterolateral wall of the left ventricle and were snap frozen in liquid nitrogen and stored at -80˚C for later assessments.

4.3.3. Experimental groups

ESHP was initiated in the non working mode (NWM) with isolated aortic root perfusion. The perfusate was warmed from room temperature to 37˚C over a 30-minute period.

4.3.3.1. Working mode (loaded) perfusion group (n=7)

Hearts were transitioned to a working mode (WM), (left atrial pressure=6 mmHg), AAI paced at 100 beats/minute, and were maintained in a loaded state over the remainder of the preservation period (Figure 4.1B).

4.3.3.2. Non-working mode (unloaded) perfusion group (n=6)

Hearts were maintained in an unloaded state over the 12-hour preservation period except for brief transitions into working mode to facilitate assessments of myocardial function and metabolism at T1, T5, and T11 (Figure 4.1B).

4.3.3.3. *In vivo* group (n=4)

Samples from normal *in vivo* porcine left ventricles were obtained to serve as normal heart control samples for subsequent analyses.

4.3.4. Myocardial functional assessment

Myocardial functional parameters were assessed at T1, T5, and T11 in a physiologic working mode (left atrial pressure=6 mmHg). The cardiac index (CI) was determined by measuring the blood flow through the left atrial line divided by the heart weight (mL/minute/gram). To assess maximum (dP/dt max) and minimum (dP/dt min) rates of pressure change in the left ventricle, a 5F pigtail catheter was placed in the left ventricle via the subclavian artery.

Calculation of left ventricular SW:

The SW (mmHg*ml) was calculated as the product of LV developed pressure (mmHg): {mean arterial pressure (mmHg) – left atrial pressure (mmHg)}, and SV (ml/beat): {cardiac output (ml/min)/heart rate (beats/min)}.

Calculation of left ventricular preload recruitable stroke work (PRSW):

PRSW was assessed during a computer controlled linear reduction in left atrial pressure, during which the linear regression of left ventricular stroke work and left atrial pressure was performed.

4.3.5. Cell death effectors and apoptosis

The expression of cleaved caspase-3 as a downstream effector of apoptosis, CCAAT-enhancer-binding protein homologous protein (CHOP) as the major *effector* of the endoplasmic reticulum (ER) stress-mediated apoptosis, and apoptosis-inducing factor (AIF) as a caspase-independent effector of apoptosis were assessed in the left ventricular (LV) tissue samples with western blot analysis and were compared between the *ex-situ* perfused hearts and *in vivo* samples.

The number of apoptotic cells in the LV tissues was determined using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay.

4.3.6. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay:

DNA fragmentation was measured on 4% paraformaldehyde-fixed, and sectioned left ventricle (6 μm thickness) with a colorimetric apoptosis detection system (TUNEL assay) (Promega, US) according to the manufacturer's instructions. Slices were examined in a confocal microscope (Zeiss, LSM 710). Cells with condensed chromatin were imaged with a 40x objective with zoom of 1, in 25 distinct fields for each slice. The extent of TUNEL was normalized to total nuclei count with 4, 6-diamidino-2-phenylindole (DAPI) staining (Molecular Probes, US) using ZEN software (Zeiss, DE).

4.3.7. Immunoblots:

In vivo and samples obtained from the anterolateral wall of the left ventricle at the end of ESHP were lysed in cold RIPA buffer system (Santa Cruz Biotechnology, US) containing protease and phosphatase inhibitors (Santa Cruz, US and Thermo Fischer, CA). Similar amounts of denatured protein samples were separated by SDS-PAGE and were transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, US). Samples were blocked (1 hour at room temperature) in 5% bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween (TBS-T), and the membranes were incubated overnight at 4˚ with primary antibodies targeting cleaved caspase-3 (Cell Signalling, US), apoptosis-inducing factor (AIF, Novus Biologicals, US), CCAAT-enhancer-binding protein homologous protein (CHOP ,Novus Biologicals, US), pyruvate kinase M1 (PKM1, Abcam, US), pyruvate kinase M2 (PKM2, Abcam, US), and phosphorylated pyruvate kinase M2 (Tyr105) (p-PKM2, Cell Signalling, US) in 5% BSA in TBST. TBS-T washed membranes were then probed with the appropriate horseradish peroxidase (HRP)-conjugated secondary anti-rabbit (BioRad, US) or anti-mouse (Cell Signalling, US) antibody for 60 min at room temperature. Target proteins were visualized with peroxidase substrate for enhanced chemiluminescence (ECL) substrate (Pierce Biotechnology, USA) and 4000MMpro imaging system (Carestream, US). Band densities were analyzed with Quantity One software (Bio-Rad, US) and normalized with *β*-tubulin as the loading control (Abcam, US). Caspase-3 and pyruvate kinase activity were assessed using a colorimetric and fluorometric assay kit respectively (Abcam, US, and Biovision, US), according to manufacturers' instructions. The results were compared between ESHP groups and *in vivo* samples.

4.3.8. Metabolic assessment

Non-esterified free fatty acid (FFA) and triglyceride (Tg) concentrations in the perfusate were assessed in T1, T5, and T11 perfusate samples using colorimetric assay kits (Wako, US). The enzymatic activity of pyruvate kinase (PK), the catalyzer of the final step of glycolysis, as well as the LV expression of PKM1 as the constitutively active adult isoform of PK, PKM2 as the fetal less active isoform, and its phosphorylated (Tyr105) inactivated form (p-PKM2) were assessed in the LV samples using a fluorometric assay (Biovision, US) and western blot analysis respectively.

4.3.8.1. Calculation of glucose utilization:

Glucose utilization between T1-T3 (hour-1 and hour-3 after the start of the perfusion, representing early perfusion), T5-T7 (hour-5 and hour-7 after the start of the perfusion, representing mid-perfuion), and T9-T11 (hour-9 and hour-11 after the start of the perfusion, representing late perfusion) was calculated using blood gas analysis (ABL 800 blood gas analyser, Radiometer, US), as below:

{the difference in the venous glucose concentrations between two consecutive assessment with two-hour interval (g/dl) + the amount of glucose delivered to the perfusate during the two-hour interval (g)} / 2 (h)

4.3.8.2. Calculation of mechanical efficiency:

Mechanical efficiency (ME) was calculated as follows:

Left ventricular stroke work (joules/beat) / MVO_2 (joules/beat) where

Stroke work = *{mean arterial pressure (mmHg) - left atrial pressure (mmHg)}* {left atrial flow (ml/min)/ heart rate (beats/min)}* 0.0001334 (joules/ml/mmHg),* and

MVO_2 (joules/beat) = {MVO_2 (ml/min)/heart rate (beats/min)}* 20 (joules/ml)

4.3.9. Tissue injury

4.3.9.1. Troponin-I

The concentration of cardiac troponin-I was determined in T1, T5, and T11 perfusate samples using a porcine ELISA Kit (Life Diagnostics, US).

4.3.9.2. Myocardial edema

The heart weight gain (edema formation) over the 12-hour ESHP was calculated as a percentage of initial heart weight as described previously [5].

4.3.10. Pyruvate challenge

The impact of exogenous pyruvate supplementation on myocardial function was assessed after T11 functional and metabolic assessments. Sodium pyruvate (Fisher Scientific, CA) was added to the perfusate solution to achieve a concentration of 5 mmol/L. Assessments of myocardial function were undertaken when the cardiac output reached a plateau.

4.3.11. Statistical analysis

The normally distributed data are reported as mean ± standard error and compared using the paired t-test or analysis of variance. The non-normally distributed variables are reported as median and were compared using the Mann-Whitney U test. Data analysis was performed with the IBM SPSS statistics software (Ver. 21.0), and a 2-sided p-value <0.05 was considered statistically significant.

4.4. Results

There was no difference in donor animal weight or heart weight, donor organ ischemic time, defibrillation requirements for reanimation, and perfusion duration between the

experimental groups (Supplemental Table s4.1). Similarly, the perfusate characteristics were comparable between experimental groups (Supplemental Table s4.2)

4.4.1. Myocardial function:

Myocardial function declined significantly over time in both treatment groups (Table 4.1); however, WM hearts exhibited significantly better preservation of CI, SW, dP/dt max, dP/dt min, and ME, and a trend towards better preservation of PRSW (Table 4.2).

4.4.2. Cell death effectors and apoptosis:

While cleaved caspase-3 was significantly higher in NWM hearts compared to *in vivo* hearts (p=0.009), the difference was not significant between WM and *in vivo* hearts (p=0.108). CHOP (p= 0.11) and AIF (p=0.07) were comparable between *in vivo*, WM and NWM groups (Figure 4.2A-D).

The percentage of TUNEL positive cells in myocardial tissue was higher in *ex-situ* perfused hearts compared to *in vivo* samples (*in vivo*=0.13±0.01, WM=0.54±0.05, NWM=0.88±0.15 %, p<0.001); however, the values were negligible (<1%) in both WM and NWM hearts. There was no significant difference in the percentage of apoptotic cells between WM and NWM hearts (Figure 4.3A-B).

4.4.3. Metabolic parameters

Coronary vascular resistance did not change over the 12-hour perfusion period in either of the two groups and was not different between WM and NWM groups (data not shown). MVO_2 decreased significantly during ESHP in NWM group but not in WM hearts (WM: 7.41 mL/min/100g±1.06 at T1 vs. 2.70 mL/min/100g±0.40 at T11, p=0.07 and NWM: 7.08 mL/min/100g±0.84 at T1 vs. 2.22 mL/min/100g±0.72 at T11, p<0.001); however, MVO_2 was not significantly different between WM and NWM groups (Figure 4.4A). Venous

lactate concentration and arteriovenous lactate difference did not change significantly throughout the perfusion period and it was not different between WM and NWM hearts (Figure 4.4B).

Glucose utilization increased during ESHP in both groups, compared to baseline values at T1-T3 (WM p=0.04, NWM p=0.04. Glucose utilization was not different between WM and NWM during early perfusion (T1-T3), but was significantly higher in WM hearts at later time points (Figure 4.4C). FFA concentration declined considerably overtime in both groups (WM p=0.009, NWM p=0.036) and perfusate concentrations were not different between WM and NWM hearts at any time point (Figure 4.5A). Tg concentrations in the perfusate did not change overtime in WM hearts (p= 0.662), but increased in NWM hearts (p=0.047). The perfusate Tg concentration was higher in NWM at T1 (WM=0.09±0.02 vs. NWM=0.20±0.03 mmol/L, p=0.032), T5 (WM=0.14±0.04, NWM=0.33±0.02 mmol/L, p=0.019) and T11 (WM=0.16±0.06, NWM=0.39±0.02 mmol/L, p=0.047) (Figure 4.5B).

Despite the increase in the glucose utilization over time, PKM1 protein expression was significantly lower in WM hearts (p=0.001) and trended towards being lower in NWM hearts (p=0.073) when compared to *in vivo* samples. PKM1 was significantly higher in NWM compared to WM (p=0.013) hearts. PKM2 protein expression was significantly higher in WM and NWM hearts compared to *in vivo* samples (WM p<0.001, NWM p=0.015) and was higher in WM compared to NWM (p=0.009) hearts. Phosphorylated (inactive) PKM2 (p-PKM2) was significantly higher in WM and NWM hearts compared to *in vivo* samples (WM p<0.001, NWM p=0.008). The p-PKM2 was not different between WM and NWM (p=0.069) hearts (Figure 4.6A-D). The general enzymatic activity of PK was significantly lower in WM and NWM hearts compared to *in vivo* samples (*in*

vivo=2.56±0.09, WM=1.77±0.31, NWM=1.66±0.34 nmol/min/mL, p=0.001), while the pyruvate kinase activity was not different between WM and NWM hearts (Figure 4.7).

4.4.4. Tissue injury:

Hearts in both groups developed edema during ESHP; however, there was no difference between experimental groups (WM 19%±3.6, NWM 26%±2.9 weight gain, p=0.14). The concentration of troponin-I in the perfusate increased significantly overtime (WM p=0.026, NWM p<0.001), and was significantly higher in the NWM group at T5 (WM 12.70±3.03, NWM 31.96±0.92 ng/ml, p<0.001) and T11 (WM 19.45±4.38, NWM 32.84±1.77 ng/ml, p=0.022).

4.4.5. Pyruvate challenge:

The addition of pyruvate to the perfusate solution led to a significant improvement in myocardial function in both WM and NWM groups compared to T11 (Figure 4.8). There was no significant difference between the time to reach the peak of the effect between WM and NWM (data not shown), and duration of the effect between WM and NWM (WM 54.85±3.88, NWM 58.33±3.51 minutes, p=0.52).

4.5. Discussion

The only normothermic ESHP apparatus available for clinical use supports the heart in NWM [1]. Consequently, evaluation of the myocardial function is not possible and assessments of organ viability are based primarily on lactate profiles during organ perfusion. The limitations of this approach have been recently highlighted by Messer et al. [4], who demonstrated that up to one-third of DCD hearts deemed viable based on *in vivo* assessments of myocardial function during normothermic regional perfusion, exhibited lactate profiles that would have labeled the organ as non-viable [4, 10]. These

results highlight the need for an ESHP device capable of assessing myocardial function in a physiologic WM; however, little is known about the impact of prolonged ESHP on donor heart function. The current study highlights 2 important points regarding the preservation of donor heart function during prolonged ESHP: 1) myocardial function declines significantly during prolonged preservation, 2) the decline in myocardial function may partially be mitigated by preserving the heart in a WM and optimizing metabolic substrate provision.

The myocardial functional decline during ESHP has been observed in previous studies [5]; however, the severity of myocyte necrosis and apoptosis that occur in this context have not been previously characterized. In this study, the proportion of TUNEL positive cells was significantly higher in the myocardium after 12 hours of ESHP compared to *in vivo* samples; however, the overall frequency of apoptotic cells was low (<1%) and out of proportion to the observed decline in myocardial function over time. We observed increased cleaved caspase-3 in NWM hearts and a trend for higher AIF in *ex-situ* perfused hearts compared to *in vivo* samples. Upregulation of these proteins has been shown to alter energy metabolism, induce NF-κB-regulated inflammation, and cause myocardial dysfunction independent of apoptosis induction [11, 12]. This may partially account for the decline in myocardial function over time and the difference in function observed between WM and NWM hearts; however, the mechanism leading to the increased level of cleaved caspase-3 requires further investigation.

We also characterized the trend in troponin-I during ESHP, and observed an increase in the perfusate concentration over time. Although troponin levels are generally attributed to myocyte necrosis, recent evidence suggest that cardiomyocytes can develop blebs and

release cytoplasmic contents without undergoing necrosis during conditions of stress [13, 14]. In the setting of ESHP, troponin-I release may at least partly result from donor heart handling during cardiectomy and cannulation, ischemia-reperfusion injury, the lack of renal clearance [14], and the inflammatory response during ESHP [15, 16]. However, perfusate troponin-I may also be used as a prognostic tool [17] as it was higher in NWM hearts which also showed a greater functional decline during ESHP. Delineating the clinical significance of troponin-I concentration during extended, closed extracorporeal perfusion is challenging and requires further study.

Current clinical ESHP protocols preserve the heart in NWM; however, the data presented here suggest that perfusion in a loaded and working state provides superior preservation of myocardial function. The mechanisms by which perfusion in working mode leads to improved functional preservation requires further investigation. In our study, analogous to the clinical trials on ESHP, glucose and insulin were provided for metabolic support [1]. Interestingly, studies on *ex-situ* perfused rat hearts have shown that exogenous glucose minimally participates in energy-yielding metabolism compared to exogenous pyruvate. Further, very low rates of glycolysis have been observed in rat hearts perfused with widely-used clinical preservation solutions [7, 18]. Although the measured glucose consumption was predictably higher in the WM hearts, the increase in consumption over time could reflect utilization of other substrates that were present in the perfusate at the start of perfusion (FFAs and ketone bodies), with a transition to glucose utilization as these metabolites were depleted. This conclusion is supported by the rapid depletion of FFAs that was observed in the perfusate over time, despite steady perfusate Tg levels. This suggests that Tg hydrolysis and FFA provision during ESHP may be dysregulated,

which may be related to the effect of insulin (delivered in a constant dose throughout the perfusion) downregulating lipoprotein lipase enzymatic activity [19]. Lipid metabolism has a crucial role in myocardial metabolic substrate provision and energy metabolism [20], and a shortage of FFAs may significantly impact the function of *ex-situ* perfused hearts.

PK is the enzyme that catalyzes the final step of glycolysis converting phosphoenolpyruvate and adenosine diphosphate (ADP) to pyruvate and adenosine triphosphate (ATP). We observed that PK activity was lower in *ex-situ* perfused hearts compared to *in vivo* samples. Further, the expression of PKM1 (the constitutively active and predominant isoform of PK in the heart) was lower in WM hearts and had a trend toward being lower in NWM hearts compared to *in vivo* samples. Conversely, the expression of PKM2 (the fetal and less active isoform of PK), and its phosphorylated (inactive) form was higher in *ex-situ* perfused hearts compared to *in vivo* samples. While the role of PKM2 as a master regulator of cancer cell metabolism has been well documented, recent evidence suggests that a switch from PKM1 to PKM2 may occur in non-neoplastic diseases such as muscular dystrophy and heart failure [21, 22]. This PK isoform switch and post-translational modification lead to a transition between glycolysis and accessory/anabolic pathways such as pentose phosphate and hexosamine biosynthesis pathways [23]. Such alterations may occur during ESHP in response to inflammatory processes and oxidative stressors that are an inevitable consequence of extracorporeal circulation [16].

The significant recovery of function in WM and NWM hearts following the addition of a lower level metabolic intermediate (pyruvate) suggests that the PK isoform switch may be limiting glucose metabolism and contributing to the myocardial functional decline that

occurs during ESHP. Pyruvate is not usually considered as an important source of energy for myocardial tissue and its effect *in vivo* has been modest. This may be due to the availability of alternative substrates (FFAs, ketone bodies, etc.) in the *in vivo* setting [24, 25]. Conversely, isolated myocardium and perfused hearts display a robust increase in function in response to pyruvate, a response that may be related to the reliance on glucose oxidation in an environment where FFAs and other substrates may be rapidly depleted. Overall, the partial functional recovery observed in response to pyruvate suggests that alterations in normal myocardial energy metabolism contribute to the decline in myocardial function that occurs during prolonged ESHP. Furthermore, these results suggest that a significant proportion of this functional decline is reversible, and provide an avenue to optimize donor heart preservation and maximize the potential for ESHP to expand the donor pool.

4.6. Study limitations:

The ischemic times in this study were negligible and do not reflect current clinical practice; however, our main goal which was to investigate ESHP-related alterations in myocardial function and metabolism that could have been impacted by ischemia-reperfusion injury. Therefore, we chose to not expose donor hearts tolonger ischemic times prior to initiation of ESHP. The *ex-situ* perfused hearts were not matched with *in vivo* LV samples from the same animal due to the negative effects of repeated needle-biopsies on myocardial function. Alternatively, we utilized a group of *in vivo* LV samples obtained from non-ischemic, non-perfused control porcine hearts.

4.7. Conclusion

Myocardial function declines significantly during prolonged ESHP, which limits the potential of this technology to expand the donor pool. This functional decline appears to be out of proportion to the severity of cell death and was partially reversible through optimization of metabolic substrate provision. Further research investigating the optimal metabolic support for the *ex-situ* perfused heart is warranted.

4.8. Financial disclosure

This work was supported by the Canadian Institutes for Health Research/Canadian National Transplant Research Program and the University Hospital Foundation. DF and JN are founders of Tevosol, Inc.

4.9. References:

1. Ardehali A, Esmailian F, Deng M, Soltesz E, Hsich E, Naka Y, Mancini D, Camacho M, Zucker M, Leprince P *et al*: **Ex-vivo perfusion of donor hearts for human heart transplantation (PROCEED II): a prospective, open-label, multicentre, randomised non-inferiority trial**. *Lancet (London, England)* 2015, **385**(9987):2577-2584.
2. Freed DH, White CW: **Donor heart preservation: straight up, or on the rocks?** *Lancet (London, England)* 2015, **385**(9987):2552-2554.
3. Dhital KK, Iyer A, Connellan M, Chew HC, Gao L, Doyle A, Hicks M, Kumarasinghe G, Soto C, Dinale A *et al*: **Adult heart transplantation with distant procurement and ex-vivo preservation of donor hearts after circulatory death: a case series**. *Lancet (London, England)* 2015, **385**(9987):2585-2591.
4. Messer SJ, Axell RG, Colah S, White PA, Ryan M, Page AA, Parizkova B, Valchanov K, White CW, Freed DH *et al*: **Functional assessment and transplantation of the donor heart after circulatory death**. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* 2016, **35**(12):1443-1452.
5. Sandha JK, White CW, Muller A, Avery E, Thliveris J, Dixon IMC, Arora RC, Tian G, Hryshko LV, Nagendran J *et al*: **Steroids limit myocardial edema during ex vivo perfusion of hearts donated after circulatory death**. *The Annals of thoracic surgery* 2018, **105**(6):1763-1770.
6. White CW, Ali A, Hasanally D, Xiang B, Li Y, Mundt P, Lytwyn M, Colah S, Klein J, Ravandi A *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 : the official publication of the International Society for Heart Transplantation* 2013, **32**(7):734-743.
7. Cobert ML, Peltz M, West LM, Merritt ME, Jessen ME: **Glucose is an ineffective substrate for preservation of machine perfused donor hearts**. *The Journal of surgical research* 2012, **173**(2):198-205.
8. Khairallah M, Labarthe F, Bouchard B, Danialou G, Petrof BJ, Des Rosiers C: **Profiling substrate fluxes in the isolated working mouse heart using 13C-labeled substrates: focusing on the origin and fate of pyruvate and citrate carbons**. *American journal of physiology Heart and circulatory physiology* 2004, **286**(4):H1461-1470.
9. White CW, Hasanally D, Mundt P, Li Y, Klein J, Xiang B, Müller A, Ambrose E, Ravandi A, Arora RC *et al*: **A wholeblood–based perfusate provides superior preservation of myocardial function during ex vivo heart perfusion**. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* 2014, **S1053-2498**(14):01356-01354.
10. White CW, Messer SJ, Large SR, Conway J, Kim DH, Kutsogiannis DJ, Nagendran J, Freed DH: **Transplantation of hearts donated after circulatory death**. *Frontiers in cardiovascular medicine* 2018, **5**:8.
11. Neviere R, Fauvel H, Chopin C, Formstecher P, Marchetti P: **Caspase inhibition prevents cardiac dysfunction and heart apoptosis in a rat model of sepsis**. *American journal of respiratory and critical care medicine* 2001, **163**(1):218-225.
12. Sevrioukova IF: **Apoptosis-inducing factor: structure, function, and redox regulation**. *Antioxidants & redox signaling* 2011, **14**(12):2545-2579.
13. Hickman PE, Potter JM, Aroney C, Koerbin G, Southcott E, Wu AH, Roberts MS: **Cardiac troponin may be released by ischemia alone, without necrosis**. *Clinica chimica acta; international journal of clinical chemistry* 2010, **411**(5-6):318-323.

14. Tanindi A, Cemri M: **Troponin elevation in conditions other than acute coronary syndromes**. *Vascular health and risk management* 2011, **7**:597-603.
15. Inbar R, Shoenfeld Y: **Elevated cardiac troponins: the ultimate marker for myocardial necrosis, but not without a differential diagnosis**. *The Israel Medical Association journal : IMAJ* 2009, **11**(1):50-53.
16. Millar JE, Fanning JP, McDonald CI, McAuley DF, Fraser JF: **The inflammatory response to extracorporeal membrane oxygenation (ECMO): a review of the pathophysiology**. *Critical care (London, England)* 2016, **20**(1):387.
17. Wettersten N, Maisel A: **Role of Cardiac Troponin Levels in Acute Heart Failure**. *Cardiac failure review* 2015, **1**(2):102-106.
18. Peltz M, He TT, Adams GAt, Koshy S, Burgess SC, Chao RY, Meyer DM, Jessen ME: **Perfusion preservation maintains myocardial ATP levels and reduces apoptosis in an ex vivo rat heart transplantation model**. *Surgery* 2005, **138**(4):795-805.
19. Jindrichova E, Kratochvilova S, Kovar J: **Glucose administration downregulates lipoprotein lipase activity in vivo: a study using repeated intravenous fat tolerance test**. *Physiological research* 2007, **56**(2):175-181.
20. Lopaschuk GD, Ussher JR, Folmes CD, Jaswal JS, Stanley WC: **Myocardial fatty acid metabolism in health and disease**. *Physiological reviews* 2010, **90**(1):207-258.
21. Gao Z, Cooper TA: **Reexpression of pyruvate kinase M2 in type 1 myofibers correlates with altered glucose metabolism in myotonic dystrophy** *Proceedings of the National Academy of Sciences of the United States of America* 2013, **110**(33).
22. Rees ML, Subramaniam J, Li Y, Hamilton DJ, Frazier OH, Taegtmeyer H: **A PKM2 signature in the failing heart**. *Biochemical and biophysical research communications* 2015, **459**(3):430-436.
23. Morgan HP, O'Reilly FJ, Wear MA, O'Neill JR, Fothergill-Gilmore LA, Hupp T, Walkinshaw MD: **M2 pyruvate kinase provides a mechanism for nutrient sensing and regulation of cell proliferation**. *Proceedings of the National Academy of Sciences of the United States of America* 2013, **110**(15):5881-5886.
24. Mallet RT, Sun J, Knott EM, Sharma AB, Olivencia-Yurvati AH: **Metabolic cardioprotection by pyruvate: recent progress**. *Experimental biology and medicine (Maywood, NJ)* 2005, **230**(7):435-443.
25. Ochiai K, Zhang J, Gong G, Zhang Y, Liu J, Ye Y, Wu X, Liu H, Murakami Y, Bache RJ *et al*: **Effects of augmented delivery of pyruvate on myocardial high-energy phosphate metabolism at high workstate**. *American journal of physiology Heart and circulatory physiology* 2001, **281**(4):H1823-1832.

4.10. Tables and figures

Table 4.1. Left ventricular function during *ex-situ* heart perfusion in working mode and non-working mode experimental groups

			T5	T11	*p-value*
CI (mL/min/g)	**WM**	17.40 (1.75)	11.48 (1.77)	5.39 (1.01)	0.008
	NWM	16.36 (1.65)	6.12 (0.91)	1.66 (0.80)	0.005
SW (mmHg*mL)	**WM**	4444.53 (448.16)	2394.47 (531.19)	1012.46 (245.72)	<0.001
	NWM	4655.05 (784.93)	1111.28 (365.11)	303.41 (121.647)	0.017
dP/dT max (mmHg/s)	**WM**	2750.52 (341.91)	1871.49 (124.53)	1616.40 (131.77)	0.015
	NWM	3722.80 (526)	2068.81 (476.08)	893.27 (353.81)	0.012
Sys p (mmHg)	**WM**	180.50 (9.83)	132.66 (8.32)	117.66 (7.80)	0.007
	NWM	179.83 (16.28)	137.30 (12.87)	89.85 (10.23)	0.020
ME (%)	**WM**	14.90 (1.60)	13.2 (1.90)	15 (3.60)	0.155
	NWM	15.20 (1.90)	6.3 (0)	3.90 (1.90)	0.056
PRSW	**WM**	702.09 (81.68)	357.04 (58.31)	197.81 (52.10)	<0.001
	NWM	729.72 (107.52)	255.13 (36.88)	89.29 (28.99)	0.011
dP/dT min (mmHg/s)	**WM**	-2675.56 (81.59)	-1940.53 (153.94)	-1190.28 (126.51)	<0.001
	NWM	-2923.68 (364.81)	-1423.50 (133.81)	-693.16 (164.62)	0.020

CI, cardiac index; dP/dT max, maximum rate of pressure change; dP/dT min, minimum rate of pressure change; LV, left ventricle; ME, mechanical efficiency; mmHg/s, millimeter of mercury per seconds; ms, milliseconds; NWM, non-working mode perfusion; PRSW, preload recruitable stroke work; SW, stroke work; sys p, systolic pressure*;* T(x), x-hours after the start of the perfusion; WM, working mode perfusion. Values are presented as mean (standard error of the mean).

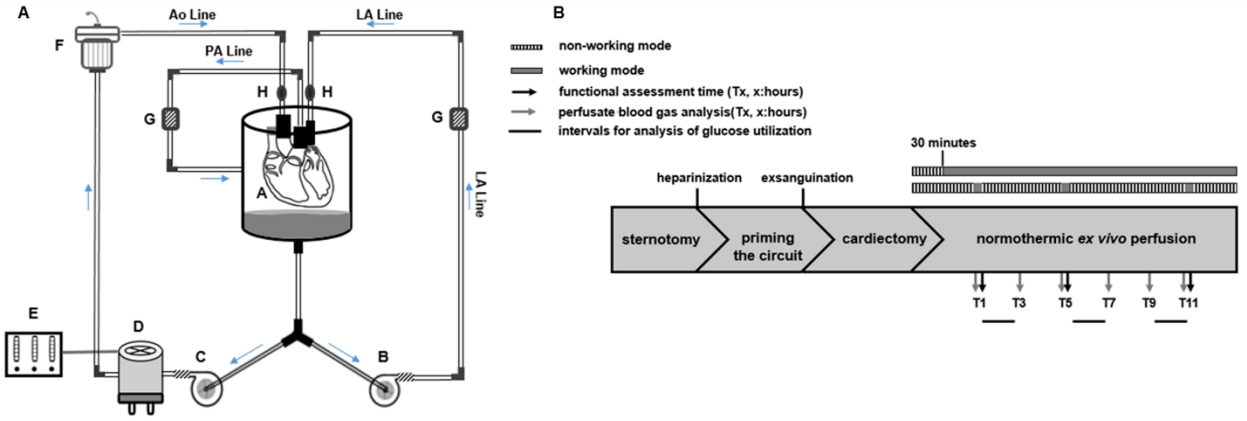
Table 4.2. Left ventricular function preservation over perfusion time, compared to baseline assessment at T1, compared between WM and NWM groups

preserved function % (SE)	group	T5	T11
CI (%), mean	**WM**	66 (8)	33 (6)
	NWM	38 (4)	10 (4)
	p-value	0.013	0.025
SW (%), mean	**WM**	51 (7)	21 (3)
	NWM	26 (6)	7 (2)
	p-value	0.035	0.010
dP/dT max (%), mean	**WM**	77 (12)	65 (8)
	NWM	54 (8)	23 (7)
	p-value	0.162	0.004
PRSW (%), mean	**WM**	59 (7)	26 (4)
	NWM	32 (9)	14 (5)
	p-value	0.299	0.114
ME (%), mean	**WM**	87 (6)	95 (18)
	NWM	46 (7)	31 (14)
	p-value	0.002	0.024
dP/dT min (%)	**WM**	73 (5)	44 (3)
	NWM	51 (6)	26 (5)
	p-value	0.033	0.020

CI, cardiac index; dP/dT max, maximum rate of pressure change; dP/dT min, minimum rate of pressure change; LV, left ventricle; ME, mechanical efficiency; NWM, non-working mode perfusion; PRSW, preload recruitable stroke work; SE, standard error of the mean;

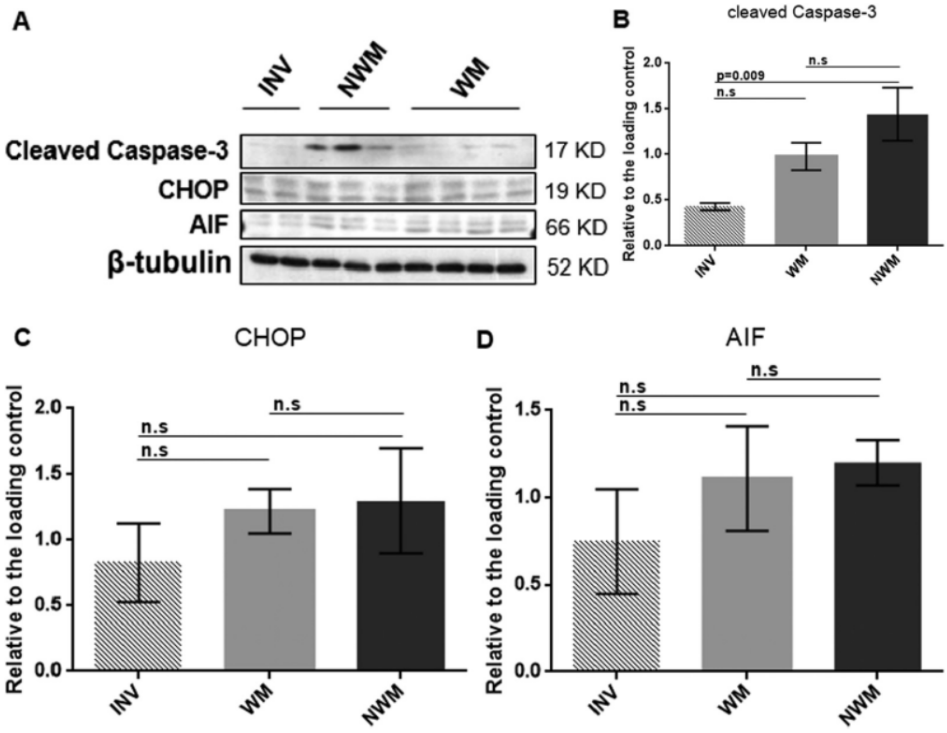
SW, stroke work; T(x), x-hours after the start of the perfusion; WM, working mode perfusion. Values are presented as mean (standard error of the mean).

Figure 4.1. *Ex-situ* heart perfusion system and protocol A: Diagram of the *ex-situ* heart perfusion circuit. B: Experimental protocol.



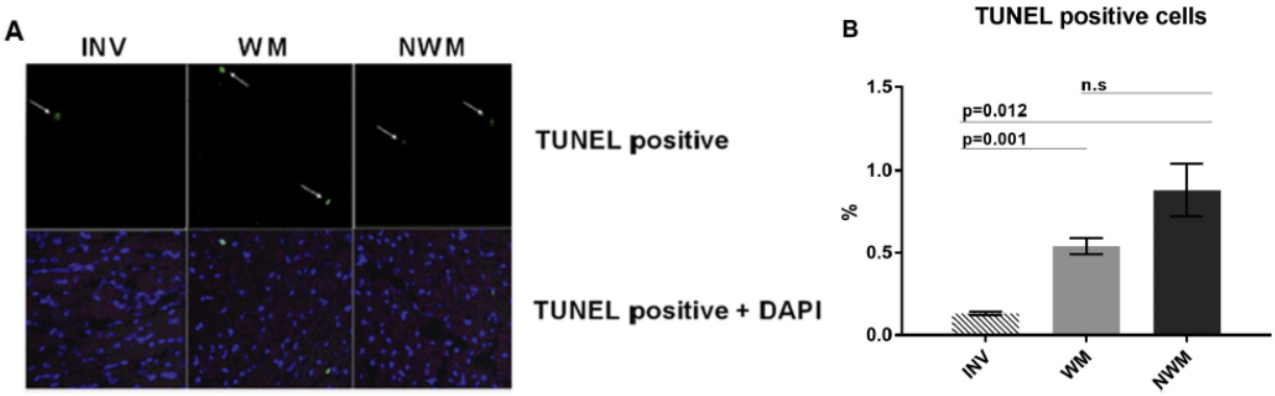
T(x); x-hours after the start of the perfusion; A, venous reservoir; B, left atrial pump; C, Aortic pump; oxygenator/heat exchanger; D, oxygen/heat exchanger; E, medical gas mixer; F, leukocyte filter; G, Flow sensor; H, Pressure sensor; Ao, Aorta; LA, left atrium; PA, pulmonary artery.

Figure 4.2. Cell death effectors in the left ventricle tissue of in vivo and ex-situ perfused hearts. A: Immunoblotting of cell lysate prepared from LV samples, using the indicated antibodies. B: Cleaved caspase-3 in ESHP hearts compared *to in vivo*. C: Immunoblotted CHOP in ESHP hearts compared *to in vivo*. D: Immunoblotted AIF in ESHP hearts compared *to in vivo*.



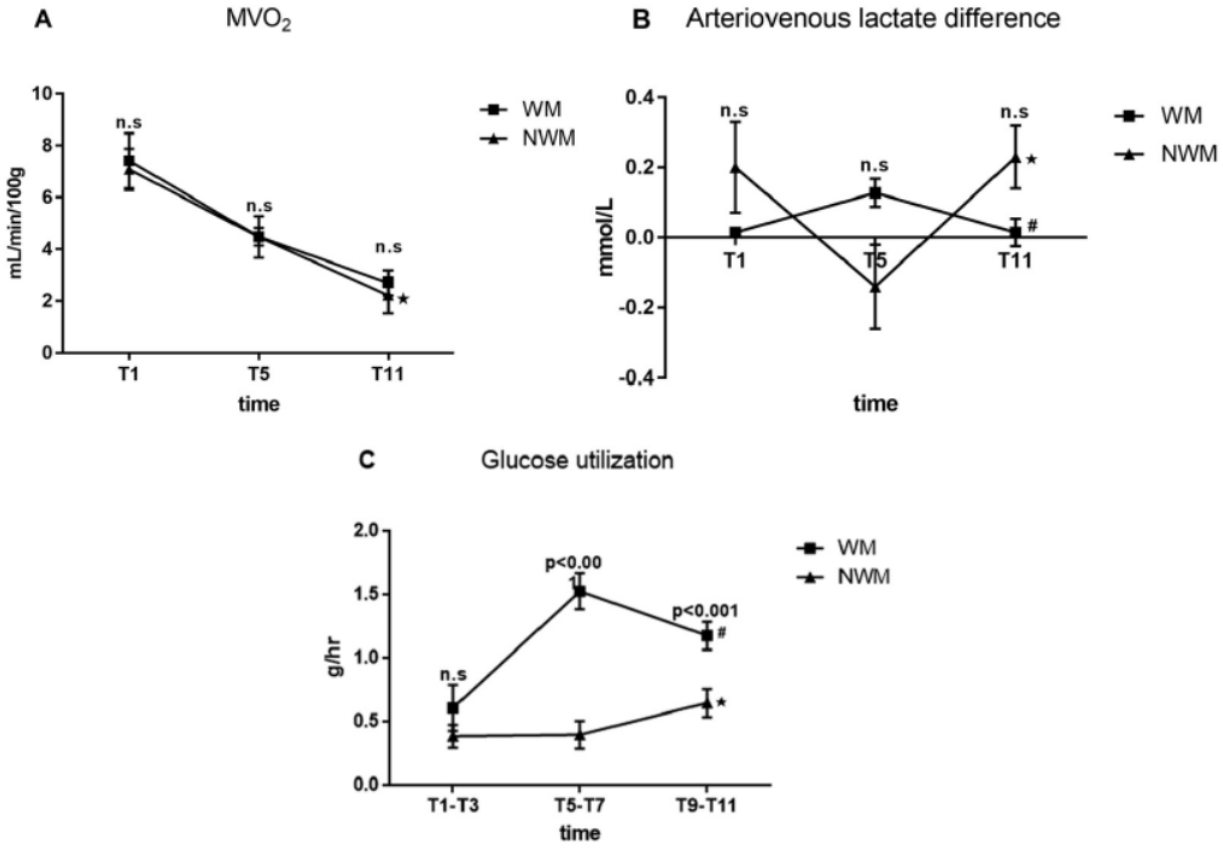
AIF, apoptosis inducing factor; CHOP, CCAAT-enhancer-binding protein homologous protein; INV, *in vivo*; n.s, no significant difference between the groups; NWM, non-working mode perfusion; WM, working mode perfusion

Figure 4.3. Apoptosis in the left ventricle tissue of in vivo and ex-situ perfused hearts. **A**: DNA fragmentation measured on left ventricle sections with TUNEL assay. The arrays are pointing to the TUNEL positive cells (40x magnification). **B**: The percentage of apoptotic cells in ESHP hearts compared *to in vivo*.



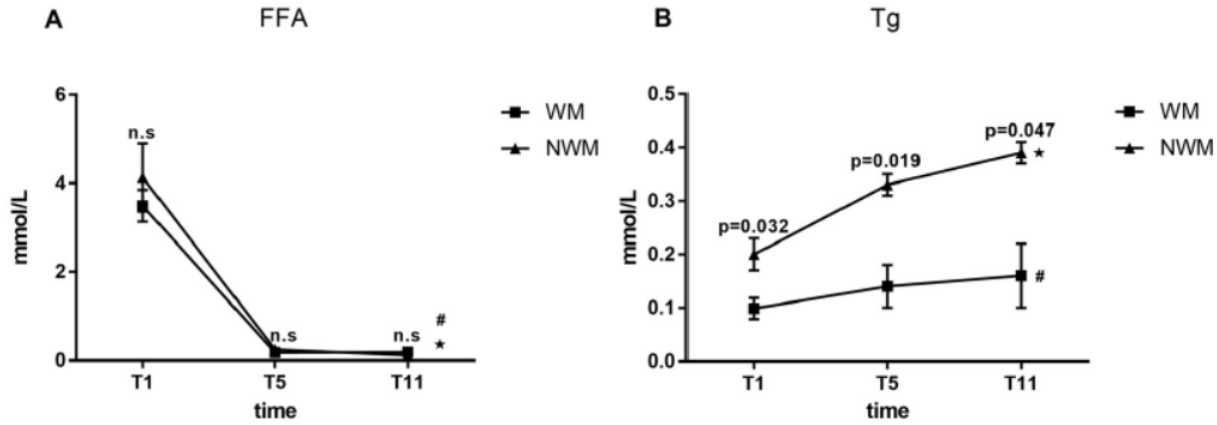
DAPI, 4, 6-diamidino-2-phenylindole staining; INV, *in vivo*; n.s, no significant difference between the groups; NWM, non-working mode perfusion; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; WM, working mode perfusion

Figure 4.4. Metabolic changes during ESHP A: MVO2 preservation during ESHP B: Arteriovenous lactate difference during ESHP C: Glucose utilization during ESHP



MVO_2, myocardial oxygen consumption; n.s: no significant difference between the groups; NWM, non-working mode perfusion; T(x); x-hours after the start of the perfusion; WM, working mode perfusion; #: significant change during perfusion in WM; ★: significant change during perfusion in NWM

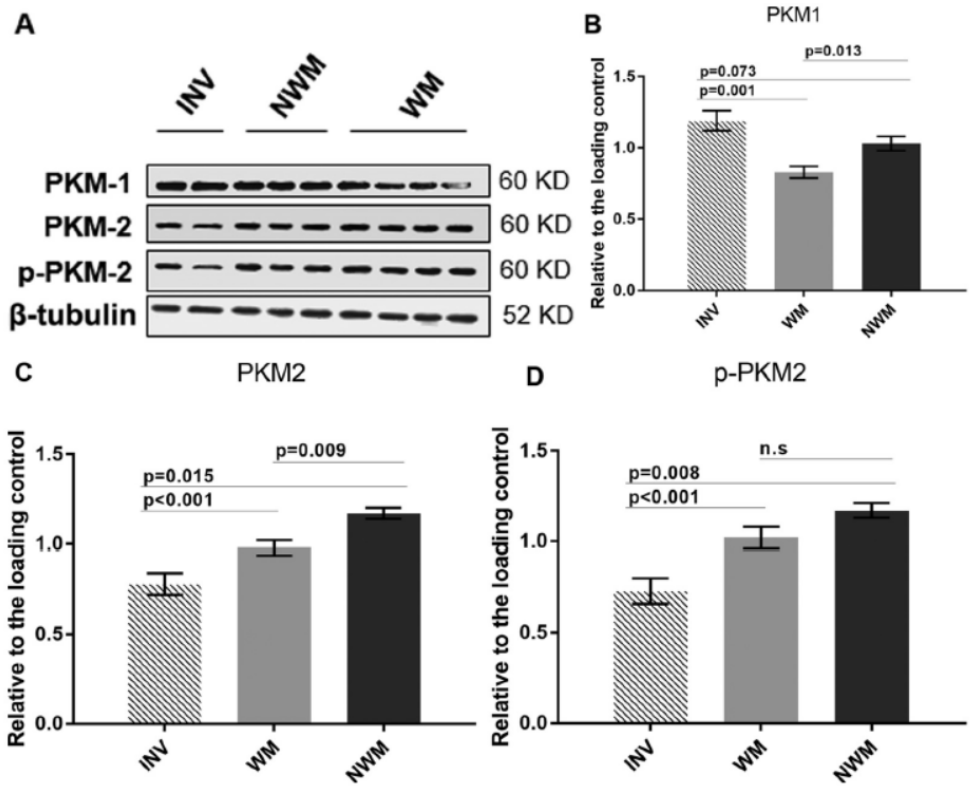
Figure 4.5. Non-esterified fatty acid and triglyceride levels during ESHP A: FFA concentration in the perfusate during ESHP B: Tg concentration in the perfusate during ESHP



FFA, free fatty acid; n.s: no significant difference between the groups; NWM, non-working mode perfusion; Tg, triglyceride; T(x), x-hours after the start of the perfusion; WM, working mode perfusion; #, significant change during perfusion in WM; ★, significant change during perfusion in NWM

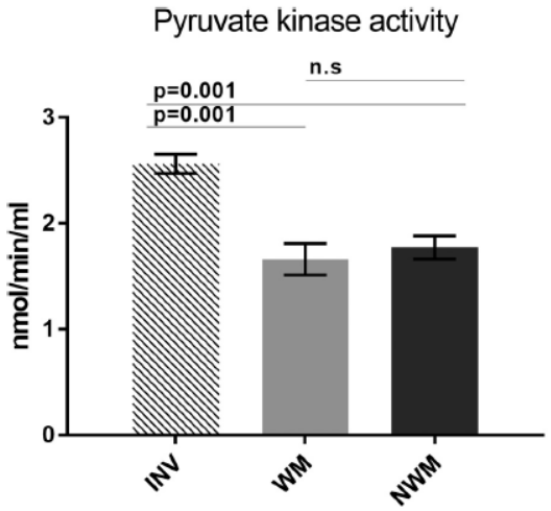
Figure 4.6. Pyruvate kinase isoform expression in ex-situ perfused hearts compared to in vivo.

A: Immunoblotting of total cell lysate prepared from LV samples, using the indicated antibodies B: Expression of PKM1 in ESHP hearts compared to in vivo C: Expression of PKM2 in ESHP hearts compared to in vivo. D: Phosphorylated PKM2 in ESHP hearts compared to in vivo.



INV, *in vivo*; n.s, no significant difference between the groups; NWM, non-working mode perfusion; PKM1, pyruvate kinase M1; PKM2, pyruvate kinase M2; p-PKM2, phosphorylated PKM2; WM, working mode perfusion

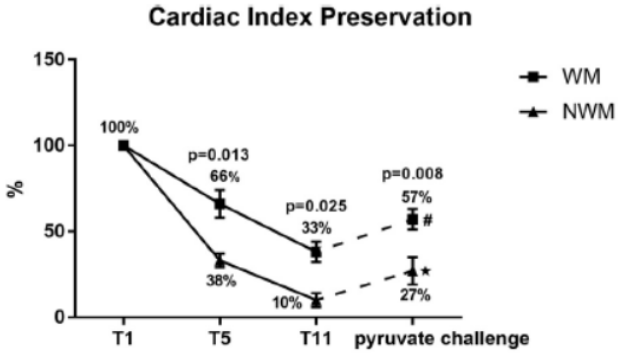
Figure 4.7. Pyruvate kinase enzymatic activity in *ex-situ* perfused hearts compared to *in vivo*.



INV, *in vivo*; n.s, no significant difference between the groups; NWM, non-working mode perfusion; WM, working mode perfusion

Figure 4.8. Partial recovery of function in *ex-situ* perfused hearts with addition of pyruvate.

The Y access represents the preserved cardiac index during perfusion (T5 and T11) and after addition of pyruvate, compared to baseline (T1).



NWM, non-working mode perfusion; T(x); x: hours after the start of the perfusion; WM, working mode perfusion; #, significant Change compared to T11 assessment in WM; ★; significant change compared to T11 assessment in NWM

4.11. Supplementary material

Supplementary Table s4.1. Basic characteristics of perfusion and animal weight

	WM	NWM	*p-value*
Animal Weight (kg)	42 (0.6)	43 (0.8)	0.72
Heart Weight (g)	168 (11.43)	175 (8.49)	0.47
Ischemic time (min)	5.57 (0.61)	5.1 (0.30)	0.58
Perfusion time	12.08 (0.04)	12.21 (0.05)	0.11
Defibrillation (yes)	6	3	0.21

NWM, non-working mode perfusion; WM, working mode perfusion

The continuous values are presented as mean (standard error of the mean)

Supplementary Table s4.2. Perfusate characteristics

Perfusate characteristics	WM	NWM
Na^+ (mmol/L)	145.42 (1.36)	141.16 (2.92)
Cl^- (mmol/L)	114.42 (1.1)	109 (2.36)
K^+ (mmol/L)	3.71 (0.11)	4.08 (0.21)
Ca^{2+} (mmol/L)	1.06 (0)	1.04 (0)
pH	7.33 (02)	7.29 (05)
Glucose (mmol/L)	10 (0.8)	8.5 (0.39)
Hemoglobin (g/L)	37.14 (2.05)	43.33 (1.80)
Osmotic Pressure (mOsmol/kg)	301.5 (3.86)	290.83 (6.01)

NWM, non-working mode perfusion; WM, working mode perfusion

The values are presented as mean (standard error of the mean)

Chapter 5: Inflammation, Oxidative And Endoplasmic Reticulum Stress Responses Are Induced During *Ex-situ* Heart Perfusion Regardless of The Left Ventricular Load

Sanaz Hatami, MD,[1,6] Christopher W. White, MD, PhD,[7] Xiao Qi, MD, MSc,[1,6], Max Buchko MD, [1] Martin Ondrus, BSc, [1] Alexandra Kinnear, BSc, [1] Sayed Himmat, MD,[1,6] Consolato Sergi, MD, PhD, MPH, [4] Jayan Nagendran, MD, PhD,[1,5,6] Darren H. Freed, MD, PhD,[1,2,3,5,6]

Affiliations:

Departments of Surgery[1], Physiology[2], and Biomedical Engineering[3], Laboratory Medicine and Pathology[4] University of Alberta, Edmonton, AB, Canada

Alberta Transplant Institute[5], Edmonton, AB, Canada

Canadian Transplant Research Program[6], Canada

Current Address: New Brunswick Heart Centre, Saint John, NB[7], Canada

This work has been published as: **Hatami S**, White CW, Qi X, Buchko, M. Ondrus M, Kinnear A, Himmat S, Nagendran J *et al*: **Immunity and Stress Responses Are Induced During Ex Situ Heart Perfusion.** *CIRCHF*, 2019 (006552R3) [accepted on Apr 23, 2020].

5.1. Abstract:

Aims: *Ex-situ* heart perfusion (ESHP) preserves the donated heart in a perfused, beating condition preventing cold storage-related ischemia, and provides a platform to evaluate myocardial viability during preservation. However, myocardial function declines gradually during ESHP. Extracorporeal circulation systems are associated with the induction of systemic inflammatory and stress responses. Our aim was to evaluate the incidence of inflammation and induction of endoplasmic reticulum (ER) stress responses during an extended period of ESHP. **Methods:** Cardiac function, myocardial tissue injury, markers of inflammation, oxidative stress, and ER stress were assessed in healthy pig hearts, perfused for 12 hours either in non-working mode (NWM, n=7), or working mode (WM, n=6). **Results:** Cardiac function declined during ESHP, but was significantly better preserved in the hearts perfused in WM (median 11-hour cardiac index / 1-hour cardiac index: WM= 27% vs. NWM= 9.5%, p=0.022). Myocardial markers of ER stress were expressed higher in ESHP hearts compared to *in vivo* samples. The pro-inflammatory cytokines and oxidized low-density lipoprotein significantly increased in the perfusate throughout the perfusion in both perfusion groups. The left ventricular expression of the cytokines and malondialdehyde was induced in NWM while it was not different between WM and *in vivo*. **Conclusions:** Myocardial function declines during ESHP regardless of perfusion mode. However, ESHP in WM may lead to superior preservation of myocardial function and viability. Both inflammation and ER stress responses are significantly induced during ESHP and may contribute to the myocardial functional decline, representing a potential therapeutic target to improve the clinical donor heart preservation.

5.2. Introduction

Ex-situ heart perfusion (ESHP) is a novel technique of extracorporeal organ preservation that minimizes exposure of the donated heart to cold ischemia by providing oxygenated, nutrient-rich perfusion to the heart during preservation [1]. Besides being non-inferior to static cold storage for preservation of standard criteria donor hearts, ESHP has also facilitated the clinical transplantation of organs donated after circulatory death [2]. However, the gradual functional decline of the heart during ESHP has limited the potential of this technology to further expand the donor pool [3], and existing data about the effects of extracorporeal circuits and extended perfusion on myocardial function and viability is limited.

Extracorporeal circulation systems (e.g., cardiopulmonary bypass and extracorporeal membrane oxygenation) are known to induce oxidative stress and a systemic inflammatory response (SIRS) which negatively impact patient outcomes [4]. Activation of the innate immune system, the elevation of circulating pro-inflammatory cytokines and oxidative stress are associated with mitochondrial dysfunction and cardiomyocyte damage/death leading to myocardial dysfunction [5, 6].

Inflammation can trigger several signaling pathways, including endoplasmic reticulum (ER) stress pathways [7]. The ER is responsible for various vital cellular processes, including protein folding, post-translational modification, and Ca^{2+} homeostasis. Pathophysiologic stress conditions such as inflammation can disturb ER homeostasis and induce unfolded protein responses (UPR) via activation of three adaptive signaling pathways; protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and Inositol-requiring enzyme-1 (IRE-1) pathways [8]. ER

stress and UPR, although essential for cell viability, if severe and prolonged, may lead to irreversible cell damage and death [9, 10]. In this study, we have assessed inflammatory, oxidative, and ER stress responses occurring during extended *ex-situ* perfusion of porcine hearts.

5.3. Methods:

5.3.1. Animals

Seventeen female domestic pigs weighing 41-54 kg were used as heart and blood donors. The institutional animal care committee of the University of Alberta approved the experimental protocol as compliant with the guidelines of the Canadian Council on Animal Care Guidelines and federal and provincial regulations/legislation.

5.3.2. Heart procurement and preparations

The porcine hearts were procured and prepared, as described previously [11]. Briefly, an intramuscular dose of ketamine (20 mg/kg) and atropine (0.05 mg/kg) was injected as premedication, then orotracheal intubation was established, and general anesthesia was maintained with isoflurane (1-2%). A standard median sternotomy was performed under general anesthesia, and the heart was exposed. After intravenous delivery of heparin (1000 U/kg bolus) and Ringer's lactate solution (1000 mL), 1000 ml of whole blood was retrieved through a two-stage venous cannula placed into the right atrium and was used to prime the ESHP circuit. The pigs were then exsanguinated (euthanized), and the hearts were excised, weighed, and immediately mounted on a custom ESHP apparatus and perfused for 12 hours (n=13). Four new hearts were used as healthy controls.

5.3.3. *Ex-situ* heart perfusion and sample collection:

A combination (1:1) of whole donor blood and modified Krebs-Henseleit solution containing 8% albumin (to decrease myocardial edema) was used for the perfusate. The ESHP apparatus was constructed as previously described [11] (Figure 5.1A&B). Custom-designed software controlled the pump speed to maintain the desired aortic (Ao) and left atrial (LA) pressures during the perfusion, and enabled preservation and evaluation in different conditions [Langendorff mode (non-working, NWM), and working mode (WM)]. Perfusion was initiated in NWM with isolated aortic root perfusion in all hearts. The Ao pressure was maintained at 40 mm Hg during perfusion in all the hearts. The transition from an empty, beating, non-working state into a physiologic WM was achieved with an automatic increase of the left atrial pump speed to achieve a left atrial pressure of 6 mmHg. Coronary sinus and LA flow were recorded with a TS410 Tubing Flow Module and flow meters (Transonic, US). The perfusate was continuously infused with glucose (1 gram/hour), insulin (2 units/hour), and epinephrine (0.04 micrograms/minute) for metabolic and inotropic support.

After initiation of ESHP, perfusate samples were collected from the aortic root at baseline (T0), hour-5 (T5, mid-perfusion), and hour-11 (T11, late perfusion) and plasma were then extracted and frozen at -80°C for later analysis. Blood gas analysis of the samples was performed (ABL 800 analyzer, Radiometer, Ca) at regular intervals, and a pH of 7.35-7.45, and arterial partial pressure of oxygen (PaO_2) of 100-150 mmHg were maintained. At the end of ESHP, samples were taken from the anterolateral wall of the left ventricle and immediately snap-frozen in liquid nitrogen and stored at -80˚C for later analysis

5.3.4. Experimental groups

ESHP was initiated in NWM, and the perfusate was warmed from room temperature to 38˚C over 30-minutes (in NWM, LAP=0mmHg). All hearts were paced with atrial wires at 100 beats/minute. The animals were randomly assigned to different experimental groups.

5.3.4.1. Working mode perfusion group (n = 7)

After the initial perfusion in NWM (30-minutes), the hearts were transitioned into WM by increasing LA pressure to 6 mmHg and were maintained in WM over the remainder of the 12-hour perfusion.

5.3.4.2. Non-working mode (unloaded) perfusion group (n = 6)

The hearts were maintained in the unloaded, NWM (LA pressure=0 mmHg) over the 12-hour preservation period except for the brief transitions into WM at T1, T5, and T11 to assess myocardial function and metabolism.

5.3.4.3. In vivo tissue samples (n = 4)

Samples from healthy, non-perfused porcine left ventricles were obtained to serve as control samples for subsequent analysis.

5.3.5. Functional assessment of the *ex-situ*-perfused heart

Myocardial function parameters were assessed at T1 (representing early perfusion), T5 (representing mid-perfusion), and T11 (representing late perfusion) in a physiologic WM (left atrial pressure = 6 mmHg). The maximum (dP/dt max) and minimum (dP/dt min) rates of pressure change were assessed through a pigtail catheter (5F) placed in the left ventricle via the subclavian branch of the aortic arch. The ESHP software collected atrial, ventricular, and aortic pressure in real-time, and calculated the ventricular stroke volume (SV) and stroke work (SW). The SW (mmHg.ml) was calculated as the product

of LV developed pressure (mmHg)*: {mean aortic pressure (mmHg) – left atrial pressure (mmHg}, and SV (ml.beat^{-1}): {cardiac output (ml.min^{-1})/heart rate (beat.min^{-1}).* The cardiac index (CI) was determined by measuring flow through the left atrial line divided by the heart weight (mL·minute^{-1}·gram^{-1}).

5.3.6. Assessment of the markers of inflammation and oxidative stress

The concentrations of pro-inflammatory cytokines, anti-inflammatory IL-10, and oxidized low-density lipoprotein (oxLDL), a marker of oxidative stress were measured in the perfusate samples using commercially available enzyme-linked immunosorbent assay (ELISA) kits for semi-quantification of tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1α, IL-1β, IL-6, IL-8, and IL-10 (R&D Systems, MN, US), and IL-18 (Reddot Biotech Inc, BC, CA), and oxLDL (MyBioSource, CA, US). The myocardial tissue expression of pro-inflammatory cytokines TNF-a, IL-6, and IL-1β, and a marker of oxidative stress, malondialdehyde (MDA) as well as p65 (RelA) subunit of the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB-p65), suggested as the most active, canonical NFκB subunit [12] were assessed in LV tissue samples with western blot analysis and were compared between the *ex-situ*-perfused hearts, and *in vivo* LV samples.

5.3.7. Evaluation of endoplasmic reticulum stress

Activation of the three signaling pathways of ER stress were examined with western blot assessment of the expression of the alpha subunit of eukaryotic initiation factor 2 (EIF-2α), activated, phosphorylated EIF-2α (pEIF-2α), (effectors of the protein kinase RNA-like endoplasmic reticulum kinase, PERK pathway), activating transcription factor 6 (ATF 6), (ATF 6 pathway), and alpha subunit of inositol-requiring enzyme 1 (IRE-1α) and activated phosphorylated IRE-1α (p-IRE-1α) (IRE-1 pathway). The ER stress-induced apoptotic

factor [CCAAT/enhancer-binding protein homologous protein (CHOP)], and ER-associated proteins involved in protein folding (protein disulfide isomerase; PDI and alpha isoform of the ER oxidoreductase 1; ERO-1α) were also assessed.

5.3.8. Western blotting

In vivo and ESHP samples (obtained from the anterolateral wall of the left) were lysed in cold RIPA buffer system (Santa Cruz Biotechnology, TX, US) with protease and phosphatase inhibitors (Santa Cruz Biotechnology, TX, US, and Thermo Fischer, ON, CA). Similar amounts (20 µg) of denatured protein samples were separated by SDS-PAGE and were transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, ON, CA). Samples were blocked for 1 hour at room temperature in a solution of 5% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween (TBS-T). The membranes were incubated overnight at 4˚C with primary antibodies targeting IRE-1α, eIF-2α, p-eIF-2α, ERO-1α, PDI (Cell Signalling, MA, US), p-IRE, IL-6, TNF-α (Abcam, ON,CA), ATF-6 (Aviva Systems Biology, CA, US), CHOP (Novus Biologicals, CO, US), IL-1β (Antibodies Online, PA, US), NFκB-p65 (Santa Cruz Biotechnology, TX, US) and MDA (MyBioSource, CA, US) in a solution of 5% BSA in TBST. TBS-T washed membranes were then probed with the appropriate horseradish peroxidase (HRP)-conjugated secondary anti-rabbit (BioRad, CA, US) or anti-mouse (Cell Signalling, MA, US) antibody for 60 min at room temperature. Target proteins were visualized with peroxidase substrate for enhanced chemiluminescence (ECL) substrate (Pierce Biotechnology, MA, USA) and 4000MMpro imaging system (Carestream, NY, US). Band intensities were analyzed with ImageJ software. Membrane protein, stained using a

reversible total protein stain system (MemCode, Thermo Fischer, ON, Ca), was used as the loading control.

5.3.9. Metabolic parameters

Coronary vascular resistance and myocardial oxygen consumption (MVO_2) were calculated as described previously [13]. Mechanical efficiency (ME) was calculated as:

Left ventricular stroke work (joules·beat^{-1}) / MVO_2 (joules·beat^{-1}) where

Stroke work = *[mean arterial pressure (mmHg) - left atrial pressure (mmHg)] × [left atrial flow (ml·min^{-1})/ heart rate (beats·min^{-1})] × 0.0001334 (joules·ml^{-1}·mmHg^{-1}),* and

MVO_2 (Joules·beat^{-1}) = *[MVO_2 (ml·min^{-1})/heart rate (beats·min^{-1})] × 20 (joules·ml^{-1})*

5.3.10. Myocardial injury

5.3.10.1 Myocardial edema

The formation of myocardial edema over the 12-hour ESHP was evaluated using myocardial weight gain relative to the initial heart weight according to the equation described previously [14]

5.3.10.2. Cardiac troponin-I

The concentration of cardiac troponin-I (cTn-I) was determined in T0, T5, and T11 perfusate samples using a commercial porcine ELISA Kit (Life Diagnostics, PA, US).

5.3.10.3. Soluble adhesion molecules

Soluble intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) were measured in the perfusate during ESHP as markers of endothelial cell activation [15] using commercial ELISA kits (MyBioSource, CA, US). The values were compared overtime in each group, and also between the two perfusion groups.

5.3.10.4. Myocardial leukocytes

Histologic sections (6 μm thick) of the OCT-embedded samples collected from the LV anterolateral wall of the *ex-situ* perfused, and in-vivo hearts were stained with hematoxylin and eosin. The leukocyte count in the LV tissue sections was determined by a blinded clinical pathologist (C.S) using Image J technology as previously published [16]. To determine the sub-populations of the leukocytes, the histologic sections of OCT-embedded LV samples (6 μm thick), were fixed in 4% paraformaldehyde, permeabilized with 0.25% Triton X-100, blocked (for one hour at room temperature) and probed (incubated overnight at 4º C) for cluster of differentiation 3 (CD 3), (marker of T lymphocyte, Novus Biologicals, CO, US) and porcine CD68 (a marker of monocyte, macrophage and neutrophil, clone: BA4D5, BioRad, CA, US) [17, 18].Secondary antibodies Alexa Flour 488 (goat anti-rabbit, Abcam, ON, CA) and Alexa Flour 647 (donkey anti-mouse, Thermo Fischer, ON, CA) were used against CD3 and CD68 antibodies respectively (incubated for one hour at room temperature). The microscopic immunofluorescence evaluation was performed by a blinded clinical pathologist (C.S) using a Zeiss Axioscope at 200x (objective 20x). Quantification was performed based on analyzing 6 fields of view of the LV sections for each case.

5.3.11. Statistical analysis

The statistical analysis was performed with the IBM SPSS statistics software (Version 21.0). Continuous data were reported as median [Interquartile Range (IQR)], or mean±Standard Deviation (SD) and were analyzed between the groups using Kruskal-Wallis/ one-way ANOVA (followed by Dunn's / Fisher's Least Significant Difference, LSD posthoc tests), or Mann-Whitney U test/ independent sample t-test where appropriate.

Over time changes within each group were assessed using Jonckheere–Terpstra trend test/ linear regression where appropriate. Significance was set at p=0.05 for all statistical tests.

5.4. Results

The donor animal weight, heart weight, ischemic time, defibrillation requirement, and duration of perfusion were not significantly different between WM and NWM perfusions (Supplemental Table s5.1). Similarly, the perfusate characteristics, including perfusate pH, arterial partial pressure of oxygen (PaO_2), and ionic composition, were comparable between experimental groups (data not shown).

5.4.1. Myocardial function

During ESHP, myocardial function declined significantly in both WM and NWM groups (Table 5.1). At the T11 assessments, while the WM hearts preserved 27% [22,50] of the baseline cardiac index, the hearts in the NWM group preserved only 9.5% [0,21], (p=0.022). Similarly, the left ventricular stroke work (SW), the maximum rate of pressure change (dP/dt max), the minimum rate of pressure change (dP/dt min), and mechanical efficiency (ME) were also better preserved throughout ESHP in the WM group (Table 5.2).

5.4.2. Markers of inflammation and oxidative stress

5.4.2.1. Perfusate markers

Relative to the baseline (T0) values, the perfusate tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, IL-8, IL-1β, IL-18, and IL-10 significantly increased during ESHP in both groups. IL-1α did not change over time significantly in WM or NWM. TNF-α and IL-8 perfusate concentrations significantly increased over time in both perfusion groups. Perfusate IL-1β, and IL-18 concentrations, only showed a significant increase between

T5-T11 assessments. IL-6 continuously increased throughout the perfusion (Figure 5.2). There was no significant difference between the perfusate concentration of any of the cytokines mentioned above between WM and NWM at T0, T5, or T11 (Supplemental Table s5.2). While oxidized LDL (oxLDL, marker of oxidative stress), was not detectible in the perfusate at T0 (considered 0 according to the utilized kit instructions), it significantly increased throughout ESHP in both groups (Figure 5.2) and the values at T0, T5, or T11 were not significantly different between WM and NWM (Supplemental Table s5.2). In summary, inflammation and oxidative stress developed significantly in the perfusate during ESHP.

5.4.2.2. Left ventricular tissue markers

The LV tissue expression of mature, active IL-1β, TNF-α, and p65 (RelA) subunit of the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB-p65), suggested to be the most dynamic, canonical NFκB subunit [12], were all significantly higher in NWM compared to *in vivo* (p=0.013, p=0.049, and p=0.029 respectively) and WM (p=0.019, p=0.029, and p=0.018 respectively). The expression of IL-1β, TNF-α, and NFκB-p65 was not different between *in vivo* and WM (p=1, p=1, and p=1 respectively). IL-6 expression was not significantly different between *in vivo*, WM, and NWM (p=0.483). The LV tissue MDA was significantly higher in NWM compared to both *in vivo* and WM (p=0.004 and p=0.028 respectively), while the values were not significantly different between WM and *in vivo* (p=0.201), (Figure 5.3A-E). In general, LV myocardial tissue of the hearts perfused in NWM (but not WM) expressed markers of inflammation and oxidative stress during ESHP.

5.4.3. Left ventricular expression of the endoplasmic reticulum stress markers

The total IRE-1α and its phosphorylated active form (pIRE-1α) were both significantly higher in NWM compared to *in vivo* (p=0.015 and p= 0.013 respectively) but the values of IRE-1α and pIRE-1α were not significantly different between WM and NWM (p=0.090 and p=0.471 respectively). No significant difference was detected in the total alpha subunit of eukaryotic initiation factor 2 (EIF-2α), (the effector of the PERK pathway) between *in vivo*, and *ex-situ*-perfused hearts (p=0.384). The active, phosphorylated EIF-2α (pEIF-2α), was significantly higher in NWM compared to *in vivo* and WM (p=0.042 and p=0.005 respectively) but it was not different between *in vivo* and WM (p=1). The ATF-6 was significantly higher in NWM compared to *in vivo* and WM (p=0.001 and p=0.014, respectively) and no significant difference was detected between WM and *in vivo* (p=0.084). The protein disulfide isomerase (PDI) was significantly higher in both WM and NWM compared to *in vivo* (p=0.013 and p=0.033, respectively). However, it was not significantly different between WM and NWM (p=0.657). Similarly, the endoplasmic reticulum oxidoreductase 1 alpha (ERO-1α), was significantly higher in WM and NWM compared to *in vivo* (p=0.046 and p=0.008 respectively), and no significant difference was detected between WM and NWM. The ER stress-induced apoptotic factor [CCAAT/enhancer-binding protein homologous protein (CHOP) was not significantly different between *in-vivo*, NWM, and WM (P=0.330), (Figure 5.4A-B). In summary, the ER stress responses were induced in LV myocardial tissue of *ex-situ*-perfused hearts, but in a higher degree in NWM compared to WM.

5.4.3. Metabolic assessment

Coronary vascular resistance did not change significantly during perfusion in either of the two groups (WM T1: 0.040 ± 0.002, T5: 0.030 ± 0.003, T11: 0.030 ± 0.004, p=0.12 and NWM T1: 0.030 ± 0003, T5: 0.030 ± 0.004, and T11: 0.030 ± 0.004, p=0.65) and there was not a significant difference between WM and NWM at T1 (WM: 0.04 ± 0.002, NWM: 0.030 ± 0003, p=0.22), T5 (WM: 0.030 ± 0.003, NWM: 0.030 ± 0.004, p=0.61), or T11 (WM: 0.030 ± 0.004, NWM: 0.030 ± 0.004 p=0.77). Myocardial oxygen consumption (MVO_2) significantly declined over time in both WM and NWM (WM T1: 7 ± 1, T5: 4 ± 0.34, T11: 2 ± 0.49 $mL.min^{-1}.100g^{-1}$, p=0.022, NWM T1: 7 ± 0.84, T5: 4 ± 0.82, T11: 2 ± 0.72 $mL.min^{-1}.100g^{-1}$, p<0.001). MVO_2 was not significantly different between WM and NWM at at T1 (WM: 7 ± 1, NWM: 7 ± 0.84 $mL.min^{-1}.100g^{-1}$, p=0.81), T5 (WM: 4 ± 0.34, NWM: 4 ± 0.82, $mL.min^{-1}.100g^{-1}$ p=0.99), or T11 (WM: 2 ± 0.49, NWM: 2 ± 0.72, $mL.min^{-1}.100g^{-1}$, p=0.58).

5.4.4. Myocardial injury

5.4.4.1. Edema formation

Hearts in both groups developed edema during ESHP, as evidenced by a significant increase in heart weight compared to the start of perfusion (WM start weight: 196 [191,238], end weight: 246 [232,255], p=0.018, and NWM start weight:195 [185,208], end weight: 244 [228,275], p=0.028). Myocardial weight gain percentage was not significantly different between WM and NWM hearts (WM: 19% ± 9, NWM: 26% ± 7, p=0.14) (Figure 5.5A).

5.4.4.2. Soluble adhesion molecules

The soluble intercellular adhesion molecule-1 (ICAM-1) values significantly increased during ESHP in both WM and NWM (WM T0: 0, T5: 2 [2,3], T11: 5 [3,6] ng/mL, p<0.001 and NWM T0: 0, T5: 4 [4,5], T11: 4 [2,5] ng/mL, p=0.021). The perfusate ICAM-1 concentration at T5 was higher in NWM (WM: 2 [2,3] ng/mL, NWM: 4 [4,5] ng/mL, p=0.010), but it was not significantly different between WM and NWM at T11 (WM: 5 [3,6] ng/mL, NWM: 4 [2,5] ng/mL p=0.476). Similarly, the perfusate vascular cell adhesion molecule-1 (VCAM-1) concentration also significantly increased during the perfusion in both WM and NWM (WM T0: 77± 1, T5: 97 ± 2, T11: 101 ± 4 µg/mL, p=0.003 and NWM T0: 73 ± 1, T5: 89 ± 3, T11: 99 ± 3 µg/mL, p=0.007). There was not a significant difference in VCAM-1 between WM and NWM at T0 (WM: 77 ± 1 µg/mL, NWM: 73 ± 1 µg/mL, p=0.078), T5 (WM: 97 ± 2 µg/mL, NWM: 89 ± 3 µg/mL, p=0.089), or T11 (WM: 101 ± 4 µg/mL, NWM: 99 ± 3 µg/mL, p=0.775) (Figure 5.5B&C). In summary, the markers of endothelial activation significantly increased in the perfusate during ESHP in both WM and NWM.

5.4.4.3. Cardiac Troponin-I

The perfusate concentration of cardiac troponin-I (cTn-I) increased significantly during ESHP in both groups (WM T0: 0 [0,1], T5: 8 [7,18], T11:12 [11,21] ng/mL, p=0<001, NWM T0: 0 [0,1], T5: 32 [30,34], T11: 32 [30,35] ng/mL, p=0.001). While the cTn-I concentration in the perfusate at T0 was not significantly different between WM and NWM (WM: 0 [0,1] ng/mL, NWM: 0 [0,1] ng/mL, p=0.485), it was significantly higher in the NWM group at T5 (WM: 8 [7,18] ng/mL, NWM: 32 [30,34] ng/mL, p=0.001) and T11 (WM: 12 [11,21] ng/mL, NWM: 32 [30,35] ng/mL, p=0.015), (Figure 5.5D). In brief, during ESHP

the perfusate cTn-I increased in both WM and NWM, but the values were significantly lower in WM group in extended-perfusion times.

5.4.4.4. LV myocardial leukocytes

The number of leukocytes in each assessed microscopic field was higher in WM compared to *in vivo* (WM: 67 [62,73], *In vivo* 38 [35,38], p=0.003). There was not a significant difference in leukocyte count between NWM and *in vivo* (*in vivo*: 38 [35,38], NWM: 44 [38,55], p=0.813), or WM (WM: 67 [62,73], NWM: 44 [38,55], p=0.051) (Figure 5.6A&B). The number of the cluster of differentiation (CD) 3-positive cells (T cells) was not significantly different between *in vivo*, WM, and NWM (*in vivo*: 4 [3,4], WM: 5 [5,7], NWM: 4 [3,8], p=0.97). The mean number of CD68-positive cells (monocytes, macrophages, and neutrophils) was significantly lower in *in vivo* compared to both WM (*in vivo*: 23 ± 4, WM: 37 ± 5, p=0.005) and NWM (*in vivo*: 23 ± 4, NWM: 35 ± 6, p=0.010). The values were not significantly different between WM and NWM (WM: 37 ± 5, NWM: 35 ± 6 p=0.683), (Figure 5.6A,C&D). In summary, the leukocytes subtypes mainly involved in innate immunity were higher in the LV tissue of the *ex-situ*-perfused hearts compared to *in vivo* myocardium.

5.5. Discussion

Extracorporeal circulation is associated with activation of the innate immune responses, and the development of oxidative stress, which negatively impacts patient outcomes [4, 19-21]. Ischemia-reperfusion injury (IRI), hemolysis, and the contact of the blood cells with artificial, non-endothelialized surfaces of the circuit are among the most important underlying factors [4, 20, 22]. Conditions related to acute systemic inflammation cause coronary endothelial damage, edema, systolic and diastolic cardiac malfunction, and

accumulation of markers of myocardial injury such as cTn-I in the circulation [23]. In this study, the perfusate IL-6, IL-8, and TNF-α increased considerably by T5. Perfusate IL-6 and IL-8 increased throughout the entire perfusion time, but levels of TNF-α reached a plateau at T5 and did not change significantly after that. The perfusate cytokine level trends observed here followed similar patterns as those reported during cardiopulmonary bypass (CPB) and extracorporeal membrane oxygenation (ECMO) [24, 25], suggesting that while early-onset stimuli such as myocardial IRI prominently trigger production/release of TNF-α during perfusion, IL-6, and IL-8 production may be related to persistent ESHP-related stimuli such as artificial surfaces and activation of complement and contact systems [20]. On the other hand, the perfusate active IL-1β and IL-18 was either very low or undetectable at T5 and only increased after T5, through T11. Activation of these cytokines is mainly dependent on the assembly of multi-protein intracellular complexes called the inflammasome, and subsequent activation of caspase-1[26] [27]. Our findings suggest that inflammasome formation and activation may occur slower, or be triggered by stimuli developed later in ESHP such as increased level of other pro-inflammatory cytokines in the perfusate, and persistent oxidative stress and damage. Therefore, while perfusate IL-1β and IL-18 may not have an important contribution to the functional decline occurring during early hours of ESHP, increased levels of these cytokines later during perfusion can further disrupt viability and function of the heart and negatively affect the outcomes of donor heart preservation and transplantation [28, 29]. Similar to the pro-inflammatory cytokines, the perfusate Ox-LDL also increased significantly throughout ESHP with comparable values in WM and NWM, suggesting that increased perfusate pro-inflammatory cytokines cause/exacerbate the exposure of the

heart to oxidative stress during ESHP. Ox-LDL can also directly induce negative inotropic and lusitropic effects on heart, independent of the risk factors such as age, blood pressure, and cholesterol levels [30, 31].

In addition to immune cells, different cardiac cells, including macrophages, cardiomyocytes, and endothelial cells also produce pro-inflammatory cytokines in various pathological conditions (sepsis, ischemia-reperfusion injury, graft rejection, and heart failure) [32, 33]. Myocardial expression of pro-inflammatory cytokines may be a sign of extreme stress and can lead to the progression of cardiac dysfunction and failure [34, 35]. The LV tissue expression of NFκB-p65, known as the most active, canonical subunit of NFκB (transcription factor necessary for induction of inflammatory molecules) [12], IL-1β and TNF-α, while comparable between WM and *in vivo,* was significantly higher in NWM hearts. This observation may suggest a higher degree of tissue stress/damage that may have contributed to the more profound functional decline we observed in this group compared to the WM hearts. Moreover, the LV tissue malondialdehyde (MDA, a marker of oxidative stress), that can also directly suppress the contractile function of the heart via p38 mitogen-activated protein kinase (MAPK) phosphorylation (activation) [36], was significantly higher in NWM compared to both *in vivo,* and WM.

Previous reports have suggested that IL-6 is produced in cardiomyocytes as a response to IL-1β. Although IL-1β was significantly elevated in the perfusate in both perfusion groups, and in LV tissue in NWM, the LV active IL-6 content was comparable between the *ex-situ*-perfused hearts and *in vivo samples*. This observation suggests that LV tissue expression of IL-6 may occur as a result of greater/longer exposure to stress conditions and circulating proinflammatory cytokines.

In this study, we observed a higher concentration of leukocytes in the hematoxylin/eosin-stained LV tissue of WM hearts, however, the immunofluorescence staining suggested that the number of LV CD3-positive cells (T cells) was not significantly different between *in vivo* and *ex-situ*- perfused hearts. The number of LV CD68-positive cells (monocytes, macrophages, and neutrophils), while significantly higher in *ex-situ*-perfused hearts compared to *in vivo*, was not significantly different between WM and NWM. The number of monocyte, macrophages and neutrophils were increased mild/moderately in the LV tissue of both WM and NWM with the absolute terms being negligible in all groups and not consistent with the rest of our findings, particularly the higher expression of IL-1β and TNF-α in NWM. Studies with larger sample size and evaluation of possible production of cytokines by myocardial cells may be beneficial for further evaluation of leukocyte migration into the myocardium during ESHP, and determining the source of pro-inflammatory cytokines in this setting.

Inflammation, oxidative stress, and ER stress are interconnected through various mechanisms and are associated with cardiac disease and failure [37, 38]. In the present study, we observed the activation of the ER stress and UPR in the *ex-situ*-perfused hearts, mainly in the hearts perfused in NWM, in which all the three main pathways of ER stress responses were induced during ESHP. The effectors of the PERK pathway, active isozyme (peIF-2α), ATF-6 (effector of the ATF-6 pathway), and total IRE-1 α and its active isozyme (p-IRE-1α) while were not significantly different between *in vivo a*nd WM, were significantly higher in the NWM compared to both *in vivo* and WM suggesting that activation of these pathways may play a role in the inferior function of *ex-situ*-perfused hearts in NWM and/or be induced by conditions related to perfusion in NWM including

sub-physiological myocardial wall stress and preload, as a part of adaptive mechanisms [39-41]. Meanwhile, the expression of ER protein folding-related disulfide bond-forming elements (ERO-1 and PDI) were significantly higher in *ex-situ*-perfused hearts(WM and NWM) compared to *in vivo*. The activity of these proteins, while an essential component of maintaining ER homeostasis, may create a source of reactive oxygen species (hydrogen peroxide) as a by-product of the folding process, and can cause/exacerbate oxidative stress in cells [42].

Activation of ER stress and UPR has been observed in many pathologic cardiac conditions [37], and may also contribute to the functional decline that occurs in *ex-situ*-perfused hearts. Therapeutic interventions aimed to reduce ER stress may ameliorate cardiovascular diseases, but paradoxically, activation of UPR has been observed to induce cardioprotective effects as well [40, 41, 43]. In the present study, activated UPR in the *ex-situ*-perfused hearts was not accompanied with increased expression of CHOP, the mediator of ER stress-related cell death, suggesting that the activation of UPR in the setting of ESHP may be either pro-survival and adaptive, or need stronger/longer exposure to mediate cell death [40, 44]. This is in keeping with our previous observation that there is a very low level of cell death during ESHP [44].ER stress can significantly affect cellular processes, including redox homeostasis, energy production, and inflammation [10]. The interaction of inflammation, oxidative stress, ER stress, and UPR during ESHP and the effects of their induction or inhibition on the preservation of cardiac function in this setting and on the outcomes of heart transplantation is not clear and needs further investigation.

Regardless of the perfusion mode, the *ex-situ*-perfused hearts developed significant edema during perfusion, which was not significantly different in WM and NWM. At the same time, despite higher expression of IL-1β, TNF-α, and MDA in LV tissue in NWM group, the soluble, circulating markers of endothelial activation, ICAM-1 and VCAM-1 increased comparably between WM and NWM during ESHP, suggesting that the extracorporeal perfusion-related inflammatory and oxidative stress environment of the perfusate may be playing the pivotal role in the activation of the coronary endothelial cells in this setting, leading to vasodilation, impaired barrier function and increased permeability of microvasculature and thus, development of myocardial edema during ESHP. The factors associated with activation of coronary endothelial cells during ESHP, and related effects on the outcomes of cardiac preservation and transplantation are under-studied and require further clarification of the outcomes of this phenomenon and how to address them in this setting [45, 46].

The perfusate cTn-I concentration increased during ESHP in both WM and NWM. However, the values while not significantly different at baseline (T0), were significantly higher in NWM at both T5 and T11 compared to WM. Although cTn-I is generally accepted as a marker of cardiomyocyte necrotic death, according to our previous observations, and studies by others, secretion of cTn-I during ESHP into the perfusate may be related to reversible stress and damage, rather than ultimate cell necrosis, [44, 47, 48] and higher inflammatory/oxidative status of the myocardial tissue in NWM may have contributed to the higher cTn-I secretion in this group [44].

In this study, similar to our previous observations [44], ESHP in WM, not only provided the opportunity for continuous monitoring of cardiac function but also lead to better

functional preservation when compared to NWM. This is a critical finding, as currently the only clinical ESHP device, Organ Care System device (OCS), which isnot FDA approved yet, can only operate in empty beating NWM, and quality assessment of the heart in OCS relies on metabolic findings (lactate concentration in particular), which may not correlate accurately with the functional status of the donor heart [13, 49]. Normothermic Regional Perfusion (NRP), which refers to *in-situ* reperfusion of thoracic organs after declaration of death based on circulatory criteria (DCD) using veno-arterial extracorporeal membrane oxygenation (ECMO) can be used to revive the DCD heart following which assessment of function can occur *in-situ* prior to *ex-situ* heart perfusion. However, the safety, cost-effectiveness, and the ethics of NRP are still a matter of debate since it takes place for a short time, involves an extra episode of extracorporeal circulation and exposes the heart to high levels of catecholamines in donor's blood, increasing the risk of inflammation and tissue damage. It may also lead to partial reperfusion of donor's brain through collateral circulation [20, 50, 51]. Thus, *ex-situ* perfusion of the DCD donor heart in WM may offer a safer, more reliable approach for both monitoring and protecting the heart during preservation. The mechanisms related to better functional preservation in WM are unknown yet and are under investigation by our group, but the cardioprotective effects of a physiologic wall stretch and contraction may be involved [39, 52].

5.6. Study limitations

Our study has some important limitations. Here, hearts were exposed to negligible episodes of ischemia, in contrast to the current clinical practice. Also, we employed hearts from normal healthy donor animals without replicating donation after neurologic determination of death (NDD), or donation after circulatory death (DCD) conditions. The process of NDD and DCD both lead to myocardial dysfunction and damage [50, 53], and our aim here was to focus on the impact of the extracorporeal circuit on the heart without a confounder such as IRI, and hormonal imbalance. Due to the very short, negligible ischemic times in this study, we avoided using cardioplegic solution, to be able to focus on ESHP-related alterations only.

Due to the negative effects of repeated needle-biopsies on coronary flow and myocardial function, the *ex-situ*-perfused hearts were not matched with *in vivo* LV samples from the same pig. Alternatively, we used a group of *in vivo* LV samples obtained from healthy non-ischemic, non-perfused, control porcine hearts.

5.7. Conclusion

Current clinical ESHP protocols preserve the heart in NWM; however, perfusion in a loaded and working state provides superior preservation of myocardial function. Myocardial functional decline occurs during prolonged ESHP, creating an obstacle for this technology to expand the donor pool. Activation of innate immune, oxidative, and ER stress responses may play an important role in the functional decline of the *ex-situ*-perfused heart. Further investigation is warranted to clarify the role of these phenomena on myocardial functional preservation and the impact of targetting them during ESHP.

5.8. Funding

This work was supported by the Canadian Institutes for Health Research/Canadian National Transplant Research Program and the University Hospital Foundation.

5.9. Conflict of interest

DF and JN are founders of Tevosol, Inc., Edmonton, AB, Canada (https://www.tevosol.com/)

5.10. References

1. Ardehali A, Esmailian F, Deng M, Soltesz E, Hsich E, Naka Y, Mancini D, Camacho M, Zucker M, Leprince P *et al*: **Ex-vivo perfusion of donor hearts for human heart transplantation (PROCEED II): a prospective, open-label, multicentre, randomised non-inferiority trial**. *Lancet (London, England)* 2015, **385**(9987):2577-2584.
2. Dhital KK, Iyer A, Connellan M, Chew HC, Gao L, Doyle A, Hicks M, Kumarasinghe G, Soto C, Dinale A *et al*: **Adult heart transplantation with distant procurement and ex-vivo preservation of donor hearts after circulatory death: a case series**. *Lancet (London, England)* 2015, **385**(9987):2585-2591.
3. Sandha JK, White CW, Muller A, Avery E, Thliveris J, Dixon IMC, Arora RC, Tian G, Hryshko LV, Nagendran J *et al*: **Steroids limit myocardial edema during ex vivo perfusion of hearts donated after circulatory death**. *The Annals of thoracic surgery* 2018, **105**(6):1763-1770.
4. McDonald CI, Fraser JF, Coombes JS, Fung YL: **Oxidative stress during extracorporeal circulation**. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* 2014, **46**(6):937-943.
5. Drosatos K, Lymperopoulos A, Kennel PJ, Pollak N, Schulze PC, Goldberg IJ: **Pathophysiology of sepsis-related cardiac dysfunction: driven by inflammation, energy mismanagement, or both?** *Current heart failure reports* 2015, **12**(2):130-140.
6. Zanotti-Cavazzoni SL, Hollenberg SM: **Cardiac dysfunction in severe sepsis and septic shock**. *Current opinion in critical care* 2009, **15**(5):392-397.
7. Kolattukudy PE, Niu J: **Inflammation, endoplasmic reticulum stress, autophagy, and the monocyte chemoattractant protein-1/CCR2 pathway**. *Circulation research* 2012, **110**(1):174-189.
8. Khan MM, Yang WL, Wang P: **Endoplasmic reticulum stress in sepsis**. *Shock (Augusta, Ga)* 2015, **44**(4):294-304.
9. Sano R, Reed JC: **ER stress-induced cell death mechanisms**. *Biochimica et biophysica acta* 2013, **1833**(12):3460-3470.
10. Cao SS, Kaufman RJ: **Endoplasmic reticulum stress and oxidative stress in cell fate decision and human disease**. *Antioxidants & redox signaling* 2014, **21**(3):396-413.
11. White CW, Hasanally D, Mundt P, Li Y, Klein J, Xiang B, Müller A, Ambrose E, Ravandi A, Arora RC *et al*: **A wholeblood–based perfusate provides superior preservation of myocardial function during ex vivo heart perfusion**. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* 2014, **S1053-2498**(14):01356-01354.
12. Zhang XQ, Tang R, Li L, Szucsik A, Javan H, Saegusa N, Spitzer KW, Selzman CH: **Cardiomyocyte-specific p65 NF-kappaB deletion protects the injured heart by preservation of calcium handling**. *American journal of physiology Heart and circulatory physiology* 2013, **305**(7):H1089-1097.
13. White CW, Ambrose E, Muller A, Li Y, Le H, Hiebert B, Arora R, Lee TW, Dixon I, Tian G *et al*: **Assessment of donor heart viability during ex vivo heart perfusion**. *Canadian journal of physiology and pharmacology* 2015, **93**(10):893-901.
14. Hatami S, White CW, Ondrus M, Qi X, Buchko M, Himmat S, Lin L, Cameron K, Nobes D, Chung HJ *et al*: **Normothermic ex situ heart perfusion in working mode: assessment of cardiac function and metabolism**. *Journal of visualized experiments : JoVE* 2019(143).

15. Videm V, Albrigtsen M: **Soluble ICAM-1 and VCAM-1 as markers of endothelial activation**. *Scandinavian journal of immunology* 2008, **67**(5):523-531.
16. Amella C, Cappello F, Kahl P, Fritsch H, Lozanoff S, Sergi C: **Spatial and temporal dynamics of innervation during the development of fetal human pancreas**. *Neuroscience* 2008, **154**(4):1477-1487.
17. Kunisch E, Fuhrmann R, Roth A, Winter R, Lungershausen W, Kinne RW: **Macrophage specificity of three anti-CD68 monoclonal antibodies (KP1, EBM11, and PGM1) widely used for immunohistochemistry and flow cytometry**. *Annals of the rheumatic diseases* 2004, **63**(7):774-784.
18. Laumet G, Ma J, Robison AJ, Kumari S, Heijnen CJ, Kavelaars A: **T cells as an emerging target for chronic pain therapy**. *Frontiers in molecular neuroscience* 2019, **12**:216.
19. Clermont G, Vergely C, Jazayeri S, Lahet JJ, Goudeau JJ, Lecour S, David M, Rochette L, Girard C: **Systemic free radical activation is a major event involved in myocardial oxidative stress related to cardiopulmonary bypass**. *Anesthesiology* 2002, **96**(1):80-87.
20. Millar JE, Fanning JP, McDonald CI, McAuley DF, Fraser JF: **The inflammatory response to extracorporeal membrane oxygenation (ECMO): a review of the pathophysiology**. *Critical care (London, England)* 2016, **20**(1):387.
21. Zakkar M, Guida G, Suleiman MS, Angelini GD: **Cardiopulmonary bypass and oxidative stress**. *Oxidative medicine and cellular longevity* 2015, **2015**:189863.
22. Chen Q, Yu W, Shi J, Shen J, Hu Y, Gong J, Li J, Li N: **The effect of extracorporeal membrane oxygenation therapy on systemic oxidative stress injury in a porcine model**. *Artificial organs* 2014, **38**(5):426-431.
23. Li Y, Ge S, Peng Y, Chen X: **Inflammation and cardiac dysfunction during sepsis, muscular dystrophy, and myocarditis**. *Burns & trauma* 2013, **1**(3):109-121.
24. Frering B, Philip I, Dehoux M, Rolland C, Langlois JM, Desmonts JM: **Circulating cytokines in patients undergoing normothermic cardiopulmonary bypass**. *The Journal of thoracic and cardiovascular surgery* 1994, **108**(4):636-641.
25. Mc IRB, Timpa JG, Kurundkar AR, Holt DW, Kelly DR, Hartman YE, Neel ML, Karnatak RK, Schelonka RL, Anantharamaiah GM *et al*: **Plasma concentrations of inflammatory cytokines rise rapidly during ECMO-related SIRS due to the release of preformed stores in the intestine**. *Laboratory investigation; a journal of technical methods and pathology* 2010, **90**(1):128-139.
26. Chiu B, Jantuan E, Shen F, Chiu B, Sergi C: **Autophagy-inflammasome interplay in heart failure: a systematic review on basics, pathways, and therapeutic perspectives**. *Annals of clinical and laboratory science* 2017, **47**(3):243-252.
27. van de Veerdonk FL, Netea MG, Dinarello CA, Joosten LA: **Inflammasome activation and IL-1beta and IL-18 processing during infection**. *Trends in immunology* 2011, **32**(3):110-116.
28. Weigt SS, Palchevskiy V, Belperio JA: **Inflammasomes and IL-1 biology in the pathogenesis of allograft dysfunction**. *The Journal of clinical investigation* 2017, **127**(6):2022-2029.
29. Mantovani A, Dinarello CA, Molgora M, Garlanda C: **Interleukin-1 and related cytokines in the regulation of inflammation and immunity**. *Immunity* 2019, **50**(4):778-795.
30. Ho E, Karimi Galougahi K, Liu CC, Bhindi R, Figtree GA: **Biological markers of oxidative stress: Applications to cardiovascular research and practice**. *Redox biology* 2013, **1**:483-491.
31. Rietzschel ER, Langlois M, De Buyzere ML, Segers P, De Bacquer D, Bekaert S, Cooman L, Van Oostveldt P, Verdonck P, De Backer GG *et al*: **Oxidized low-density lipoprotein cholesterol is associated with decreases in cardiac function independent of vascular alterations**. *Hypertension (Dallas, Tex : 1979)* 2008, **52**(3):535-541.
32. Kumar S, Maulik S: **Role of cytokines in heart failure**. *PHARMACOL REP* 2017, **2**(1).

33. Aoyagi T, Matsui T: **The cardiomyocyte as a source of cytokines in cardiac injury**. *Journal of cell science & therapy* 2011, **2012**(S5).
34. Avlas O, Fallach R, Shainberg A, Porat E, Hochhauser E: **Toll-like receptor 4 stimulation initiates an inflammatory response that decreases cardiomyocyte contractility**. *Antioxidants & redox signaling* 2011, **15**(7):1895-1909.
35. Kubota T, Miyagishima M, Alvarez RJ, Kormos R, Rosenblum WD, Demetris AJ, Semigran MJ, Dec GW, Holubkov R, McTiernan CF *et al*: **Expression of proinflammatory cytokines in the failing human heart: comparison of recent-onset and end-stage congestive heart failure**. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* 2000, **19**(9):819-824.
36. Folden DV, Gupta A, Sharma AC, Li SY, Saari JT, Ren J: **Malondialdehyde inhibits cardiac contractile function in ventricular myocytes via a p38 mitogen-activated protein kinase-dependent mechanism**. *British journal of pharmacology* 2003, **139**(7):1310-1316.
37. Groenendyk J, Lee D, Jung J, Dyck JR, Lopaschuk GD, Agellon LB, Michalak M: **Inhibition of the unfolded protein response mechanism prevents cardiac fibrosis**. *PloS one* 2016, **11**(7):e0159682.
38. Zhang C, Syed TW, Liu R, Yu J: **Role of endoplasmic reticulum stress, autophagy, and inflammation in cardiovascular disease**. *Frontiers in cardiovascular medicine* 2017, **4**:29.
39. Hao J, Kim HS, Choi W, Ha TS, Ahn HY, Kim CH: **Mechanical stretch-induced protection against myocardial ischemia-reperfusion injury involves AMP-activated protein kinase**. *The Korean journal of physiology & pharmacology : official journal of the Korean Physiological Society and the Korean Society of Pharmacology* 2010, **14**(1):1-9.
40. Liu M, Dudley SC, Jr.: **Role for the unfolded protein response in heart disease and cardiac arrhythmias**. *International journal of molecular sciences* 2015, **17**(1).
41. Martindale JJ, Fernandez R, Thuerauf D, Whittaker R, Gude N, Sussman MA, Glembotski CC: **Endoplasmic reticulum stress gene induction and protection from ischemia/reperfusion injury in the hearts of transgenic mice with a tamoxifen-regulated form of ATF6**. *Circulation research* 2006, **98**(9):1186-1193.
42. Benham AM, van Lith M, Sitia R, Braakman I: **Ero1-PDI interactions, the response to redox flux and the implications for disulfide bond formation in the mammalian endoplasmic reticulum**. *Philosophical transactions of the Royal Society of London Series B, Biological sciences* 2013, **368**(1617):20110403.
43. Minamino T, Komuro I, Kitakaze M: **Endoplasmic reticulum stress as a therapeutic target in cardiovascular disease**. *Circulation research* 2010, **107**(9):1071-1082.
44. Hatami S, White C, Shan S, Haromy A, Qi X, Ondrus M, Kinnear A, Himmat S, Michelakis E, Nagendran J *et al*: **Myocardial functional decline during prolonged ex situ heart perfusion**. *The Annals of thoracic surgery* 2019, **108**:499-507.
45. Sutton NR, Baek A, Pinsky DJ: **Endothelial cells and inflammation**. In: *Encyclopedia of Medical Immunology: Autoimmune Diseases.* edn. Edited by Mackay IR, Rose NR, Diamond B, Davidson A. New York, NY: Springer New York; 2014: 367-381.
46. Zhang C: **The role of inflammatory cytokines in endothelial dysfunction**. *Basic research in cardiology* 2008, **103**(5):398-406.
47. Hickman PE, Potter JM, Aroney C, Koerbin G, Southcott E, Wu AH, Roberts MS: **Cardiac troponin may be released by ischemia alone, without necrosis**. *Clinica chimica acta; international journal of clinical chemistry* 2010, **411**(5-6):318-323.
48. Tanindi A, Cemri M: **Troponin elevation in conditions other than acute coronary syndromes**. *Vascular health and risk management* 2011, **7**:597-603.

49. Hatami S, Freed DH: **Machine perfusion of donor heart: state of the art**. *Current Transplantation Reports* 2019, **6**(3):242-250.
50. White CW, Messer SJ, Large SR, Conway J, Kim DH, Kutsogiannis DJ, Nagendran J, Freed DH: **Transplantation of hearts donated after circulatory death**. *Frontiers in cardiovascular medicine* 2018, **5**:8.
51. Ribeiro R, Gomes B, Hondjeu A, Yu F, Adamson M, Ribeiro R, Alvarez J, Bissoondath V, Meineri M, Rao V *et al*: **Assessment of cerebral perfusion and activity during normothermic regional perfusion in a porcine model of donation after circulatory death**. *Canadian Journal of Cardiology* 2019, **35**(10):S8-S9.
52. Bittl JA, Ingwall JS: **The energetics of myocardial stretch. Creatine kinase flux and oxygen consumption in the noncontracting rat heart**. *Circulation research* 1986, **58**(3):378-383.
53. Wilhelm MJ, Pratschke J, Laskowski IA, Paz DM, Tilney NL: **Brain death and its impact on the donor heart-lessons from animal models**. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* 2000, **19**(5):414-418.

5.11. Tables and figures

Table 5.1. Left ventricular function during *ex-situ* heart perfusion

Functional parameter		T1	T5	T11	*p-value*
CI **,**	**WM**	16 [13,19]	11 [6,14]	5 [3,7]	<0.001
(ml/min/g)	**NWM**	16 [12,20]	5 [4,7]	0.96 [0,4]	<0.001
SW	**WM**	4600 [3200,5400]	2200 [930,3200]	700 [500,1700]	<0.001
(mmHg*ml)	**NWM**	4700 [2500,6700]	880 [530,1800]	250 [1,622]	<0.001
dP/dT max	**WM**	2800 [2100,3400]	1940 [1600,1970]	1600 [1200,1800]	0.005
(mmHg/s)	**NWM**	3400 [2500,5000]	2000 [950,2900]	790 [150,1500]	0.001
ME	**WM**	15 [11,20]	15 [9,17]	13 [5,25]	0.723
(%)	**NWM**	14 [10,20]	6 [5,7]	2 [0,8]	0.001
dP/dT min	**WM**	-2600 [-2500,-2800]	-1800 [-1700,-2100]	- 1100 [-890,-1400]	<0.001
(mmHg/s)	**NWM**	-3000 [-2000,-3700]	-1500 [-1100,-1600]	-820 [-350,-950]	<0.001

Data reported as median [IQR]. CI, cardiac index; dP/dT max, maximum rate of pressure change; dP/dT min, minimum rate of pressure change; ME, mechanical efficiency; NWM, non-working mode perfusion; SW, stroke work; T(x), x: hours after the start of the perfusion; WM, working mode perfusion.

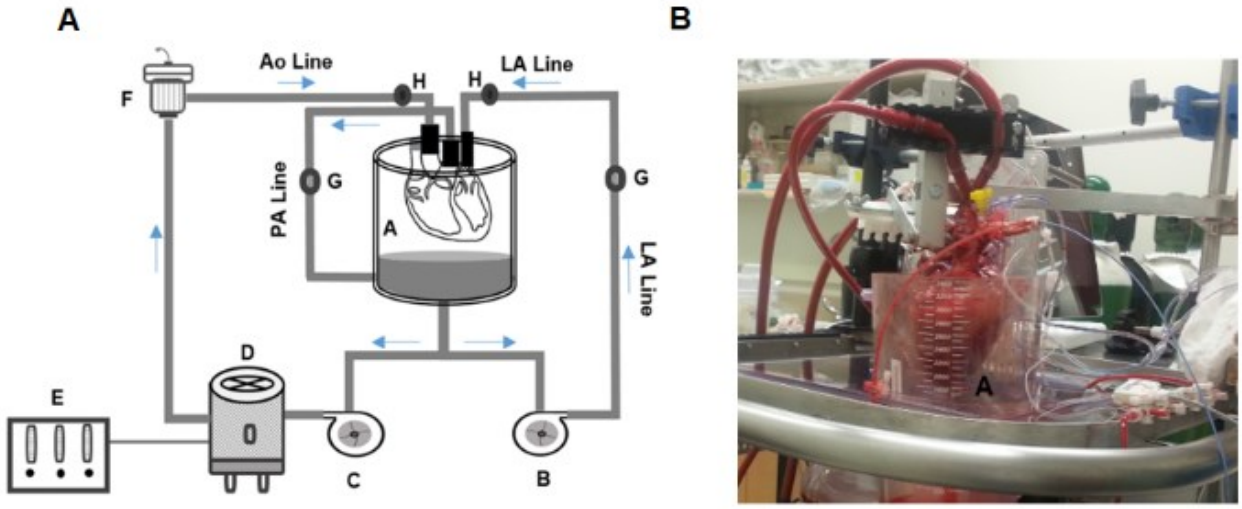
Table 5.2. Left ventricular function preservation during *ex-situ* heart perfusion

preserved function	group	T5	T11
CI	**WM**	75 [49,86]	27 [22,50]
(%)	**NWM**	40 [28,45]	9.50 [0,21]
	p-value	0.022	0.022
SW	**WM**	44 [34,71]	16 [12,31]
(%)	**NWM**	30 [8,40]	9 [0,13]
	p-value	0.051	0.014
dP/dT max	**WM**	70 [47,95]	65 [39,79]
(%)	**NWM**	56 [34,71]	25 [6,38]
	p-value	0.181	0.014
ME	**WM**	95 [83,98]	100 [42,125]
(%)	**NWM**	49 [27,58]	20 [0,72]
	p-value	0.008	0.022
dP/dT min	**WM**	71 [66,84]	46 [38,53]
(%)	**NWM**	44 [41,60]	28 [16,36]
	p-value	0.035	0.022

Data reported as median [IQR]. CI, cardiac index; dP/dT max, maximum rate of pressure change; dP/dT min, minimum rate of pressure change; ME, mechanical efficiency; NWM, non-working mode perfusion; SW, stroke work; T(x), x: hours after the start of the perfusion; WM, working mode perfusion

Figure 5.1. *Ex-situ* heart perfusion device.

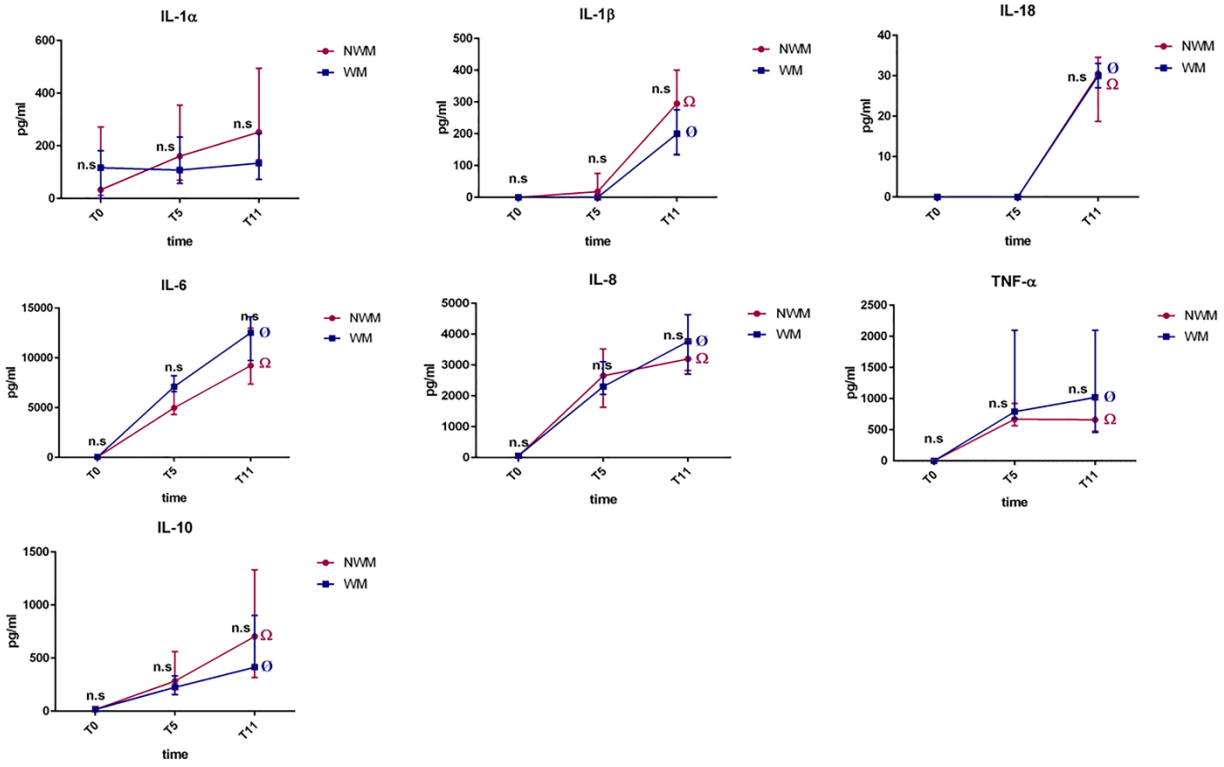
A: Diagram of the *ex-situ* heart perfusion circuit B: Porcine heart perfused on the *ex-situ* perfusion apparatus



A, venous reservoir; B, left atrial pump; C, Aortic pump; D, oxygen/heat exchanger; E, medical gas mixer; F, leukocyte filter; G, Flow sensor; H, Pressure sensor; Ao, Aorta; LA, left atrium; PA, pulmonary artery.

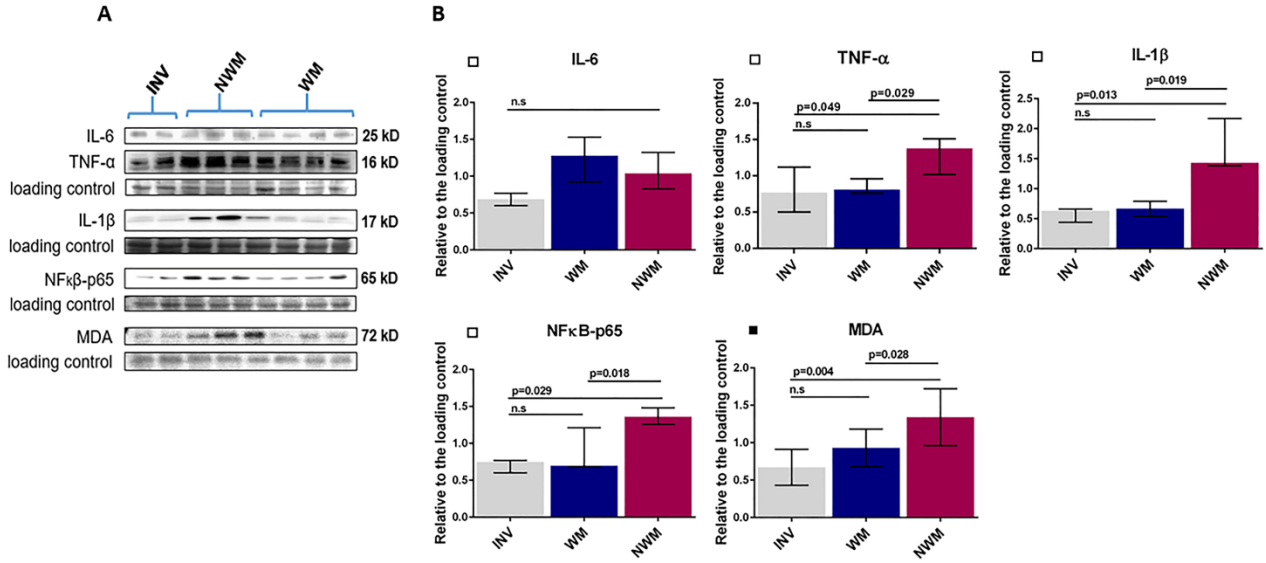
Figure 5.2. Perfusate cytokine and oxidized low density lipoprotein accumulation during *ex-situ* heart perfusion

[tested over time within WM (n=7) and NWM (n=6) groups with Jonckheere–Terpstra trend test, and between WM and NWM with Mann-Whitney U test]. Data are presented as median [IQR]. Error bars: IQR.



IL-1α, interleukine-1 alpha; IL-1β, interleukin 1B; IL-18, interleukin-18; IL-6, interlekin-6; IL-8, interleukin-8; IL-10, interleukin-10; LDL, low density lipoprotein; n.s, no significant difference between the groups; NWM, non-working mode perfusion; TNF-α, tumour necrosis factor-alpha; T(x), x: hours after the start of the perfusion; WM, working mode perfusion; Ø, significant over time change within WM group; Ω, significant change within NWM group.

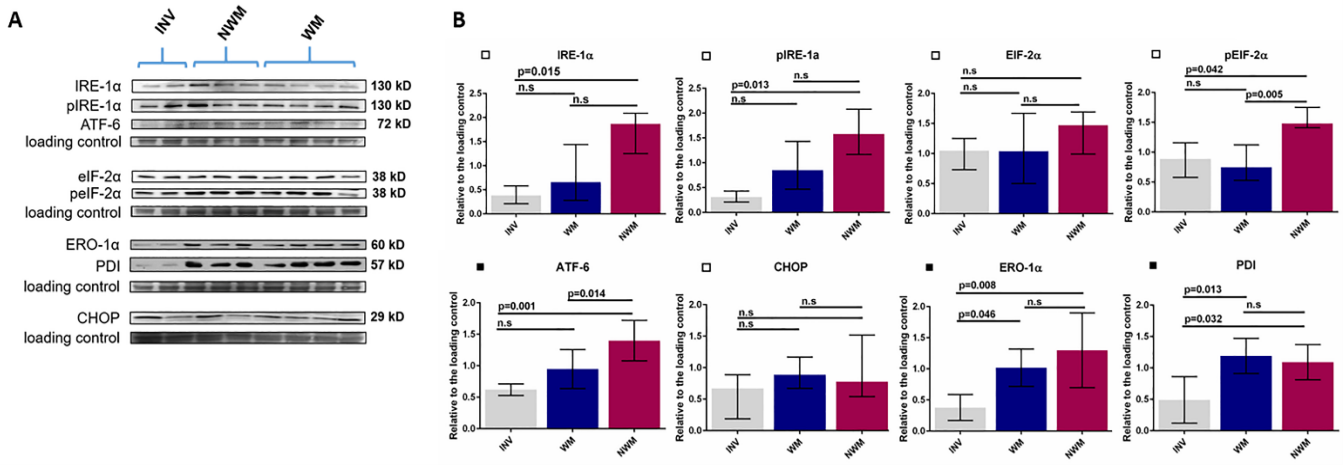
Figure 5.3. Myocardial expression of the markers of inflammation and oxidative stress A: Immunoblotting of total cell lysate prepared from LV samples taken either from *in vivo* non-perfused hearts, or at the end of ESHP, using the indicated antibodies. Total protein values (reversibly-stained) were used as loading control B: Expression of pro-inflammatory cytokines and transcription factor, and a marker of oxidative stress in ESHP hearts compared *to in vivo* [tested between WM (n=7), NWM (n=6) and *in vivo* (n=4) groups with ■: one-way ANOVA (followed by Fisher's Least Significant Difference posthoc test), or □: Kruskal-Wallis (followed by Dunn's posthoc test)]. Data are presented as ■: mean±SD, or □: median [IQR]. Error bars ■: SD, □: IQR.



IL-1β, interleukin 1β; IL-6, interleukin 6; INV, in vivo; MDA, malondialdehyde; NFκB-p65, p65 subunit of the nuclear factor kappa-light-chain-enhancer of activated B cells; n.s, no significant difference between the groups; NWM, non-working mode perfusion; TNF-α, tumour necrosis factor-alpha; WM, working mode perfusion

Figure 5.4. Endoplasmic reticular stress responses in myocardium

A: Immunoblotting of total cell lysate prepared from LV samples taken either from *in vivo* non-perfused hearts, or at the end of ESHP using the indicated antibodies. Total protein values (reversibly-stained) were used as loading control B: Expression of markers/effectors of ER in ESHP hearts compared *to in vivo* [tested between WM (n=7), NWM (n=6) and *in vivo* (n=4) groups with ■: one-way ANOVA (followed by Fisher's Least Significant Difference posthoc test), or □: Kruskal-Wallis (followed by Dunn's posthoc test). Data are presented as ■: mean±SD, or □: median (IQR). Error bars ■: SD, □: IQR].

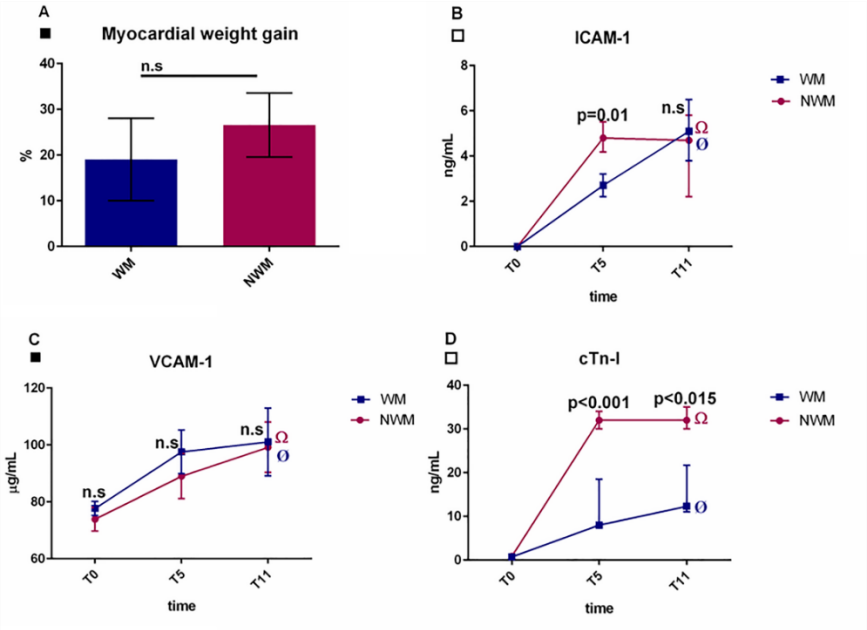


ATF-6, activating transcription factor 6; CHOP, CCAAT/enhancer-binding protein homologous protein; EIF-2α, the alpha subunit of the eukaryotic Initiation Factor 2; pEIF-2α, phosphorylated, active EIF-2α; ERO- 1α, the alpha isoform of the endoplasmic reticulum oxidoreductase 1; IRE-1α, the alpha subunit of the Inositol requiring enzyme-1; pIRE-1α, phosphorylated, active IRE-1α; n.s, no significant difference between the

groups; NWM, non-working mode perfusion; PDI, protein disulfide isomerase; WM, working mode perfusion

Figure 5.5. Myocardial and endothelial injury during *ex-situ* heart perfusion

A: myocardial edema formation measured by weight gain percentage after *ex-situ* perfusion B: perfusate Intracellular Adhesion Molecule-1 C: perfusate Vascular Cell Adhesion Molecule D: perfusate cardiac troponin-I concentrations [tested over time within WM (n=7) and NWM (n=6) groups with ■: linear regression or paired sample t-test, □: Jonckheere–Terpstra trend test, and between WM and NWM with ■: independent samples t-test, □: Mann-Whitney U test]. Data are presented as ■: mean±SD, or □: median (IQR). Error bars ■: SD, □: IQR].

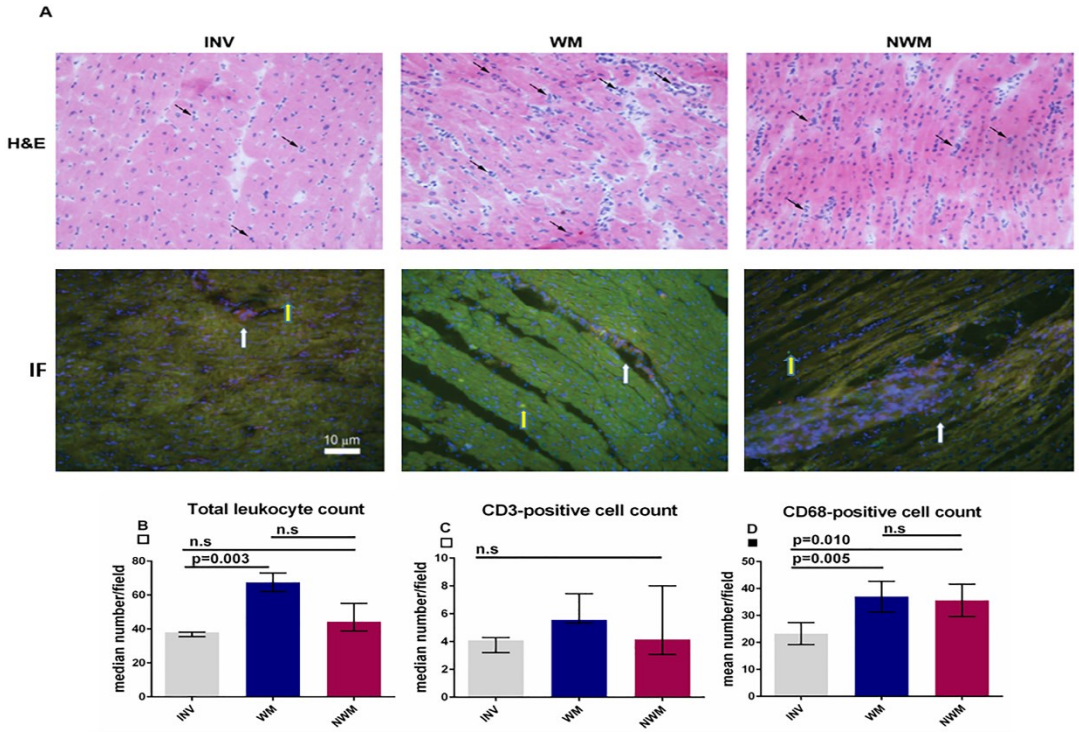


cTn-I, cardiac troponin I; ICAM-1, Intracellular Adhesion Molecule-1; n.s, no significant difference between the groups; NWM, non-working mode perfusion; T(x), x: hours after the start of the perfusion; VCAM-1, Vascular Cell Adhesion Molecule-1; WM, working

mode perfusion; Ø, significant over time change within WM group; Ω, significant change within NWM group.

Figure 5.6. Histological evaluation of residing leukocytes in LV tissue in *in vivo* controls and *ex-situ*-perfused hearts

A: Hematoxylin/eosin staining (magnification 10x) in the upper panels, and immunofluorescence staining of CD3 positive cells (T cells, bright green), and CD68-positive cells (monocytes, macrophages, and neutrophils, red) in the lower panels. The black arrows in the upper panels in the photograph point to the leukocytic recruitment observed in this group. The yellow and white arrows in the lower panels point to CD3-positive, and CD68-positive cells respectively B: Total number of leukocytes residing in the left ventricular tissue C: Number of CD3-positive T cells residing in the left ventricular tissue D: Number of CD68-positive monocytes, macrophages, and neutrophils residing in the left ventricular tissue [tested between WM (n=7), NWM (n=6) and *in vivo* (n=4) groups with ■: one-way ANOVA (followed by Fisher's Least Significant Difference posthoc test), or □: Kruskal-Wallis (followed by Dunn's posthoc test)]. Data are presented as ■: mean±SD, or □: median [IQ]. Error bars ■: SD, □: IQR.



CD, cluster of differentiation; H&E, hematoxylin/eosin staining; IF, immunofluorescence staining; INV, *in vivo;* n.s, no significant difference between the groups; NWM, non-working mode perfusion; WM, working mode perfusion

5.12. Supplementary material

Supplementary Table s5.1. Basic characteristics of perfusion and animal weight

	WM **N=7**	**NWM** **N=6**	***p-value***
Defibrillation (yes)	6	3	0.217
Ischemic time (min)	5 [5,9]	5 [4.75,6]	0.945
Perfusion time (h)	12 [12,12.15]	12.23 [12,12.33]	0.138
Animal Weight (kg)	42 ± 2.61	43 ± 1.78	0.725
Heart Weight (g)	196 [191,238]	195 [185,208]	0.628

The continuous values are presented as median [interquartile range], or mean ± Standard Deviation. NWM, non-working mode perfusion; WM, working mode perfusion

Supplementary Table s5.2. Concentration of the circulating cytokines in the perfusate during *ex-situ* heart perfusion

Interleukins		**T0**	**T5**	**T11**	***p-value***
IL-1α	**WM**	116 [0,181]	108 [57,233]	134 [72,246]	0.370
pg/mL	**NWM**	33 [11,271]	160 [69,354]	252 [146,494]	0.064
IL-1β	**WM**	0 [0,0]	0 [0,0]	200 [134,275]	0.002
pg/mL	**NWM**	0 [0,0]	18 [0,75]	295 [135,400]	<0.001
IL-18	**WM**	0 [0,0]	0 [0,0]	30 [27,33]	0.001
pg/mL	**NWM**	0 [0,0]	0 [0,0]	30 [18,34]	0.001
IL-6	**WM**	42 [0,171]	7100 [6600,8200]	12500 [900,14000]	<0.001
pg/mL	**NWM**	46 [12,93]	4900 [4300,8100]	9200 [7300,12900]	<0.001
IL-8	**WM**	62 [0,72]	2300 [2000,3100]	3700 [2700,4600]	<0.001
pg/mL	**NWM**	56 [40,78]	2600 [1600,3500]	3100 [2800,3800]	0.001
TNF-α	**WM**	0 [0,0]	790 [680,2000]	1000 [450,2000]	0.004
pg/mL	**NWM**	0 [0,20]	670 [560,920]	660 [470,1000]	0.005
IL-10	**WM**	17 [12,38]	250 [150,330]	400 [390,900]	<0.001
pg/mL	**NWM**	17 [14,42]	280 [200,560]	700 [300,1300]	<0.001
Ox-LDL	**WM**	0 [0,0]	16 [13,20]	51 [27,54]	<0.001
ng/mL	**NWM**	0 [0,0]	27 [10,57]	40 [14,65]	<0.001

Data reported in pg/mL as median [interquartile range]. IL-1α, interleukine-1 alpha; IL-1β, interleukin 1B; IL-18, interleukin-18; IL-6, interlekin-6; IL-8, interleukin-8; IL-10, interleukin-10; NWM, non-working mode perfusion; ox-LDL, oxidized low density lipoprotein; TNF-α, tumor necrosis factor-alpha; T(x), x: hours after the start of the perfusion; WM, working mode perfusion. Overtime trends have been tested using *Jonckheere-Terpstra trend test.*

Chapter 6: Oxidative stress and related metabolic responses are induced in *Ex-situ* heart perfusion regardless of the ventricular load, or leukocyte depletion

Sanaz Hatami, MD,[1,7] Xiao Qi, MD, MSc,[1,7] Christopher W. White, MD, PhD,[8] Sayed Himmat, MD,[1,7] Mobashir Khan, MD,[1] Bryce Tkachuk, [1] Consolato Sergi, MD, PhD, MPH, [5] Jayan Nagendran, MD, PhD,[1,6,7] Darren H. Freed, MD, PhD,[1,2,4,6,7]*

Affiliations:

Departments of Surgery[1], Physiology[2,] Medicine[3], and Biomedical Engineering[4], Laboratory Medicine and Pathology[5] University of Alberta, Edmonton, AB, Canada

Alberta Transplant Institute[6], Edmonton, AB, Canada

Canadian Transplant Research Program[7], Canada

Current address: New Brunswick Heart Centre[8], Saint John, NB, Canada

This work is ready for submission as Sanaz Hatami, Xiao Qi, Christopher W. White, Sayed Himmat, Mobashir Khan, Bryce Tkachuk, Consolato Sergi, Jayan Nagendran, Darren H. Freed: *Ex-situ* heart perfusion in the semi-physiological working mode improves functional preservation of the heart and does not exacerbate the perfusion-induced oxidative stress

6.1. Abstract

Background: Normothermic *ex-situ* heart perfusion allows preservation of donated heart in a perfused, dynamic state, preventing cold ischemia. However, the function of the *ex-situ* perfused heart declines in a time-related fashion during perfusion, limiting the potential of this method for improvement of preservation, and expanding donor pool. The underlying mechanisms for the functional decline may be associated with the extracorporeal circulation-related oxidative stress and related modifications. **Methods:** Hearts from domestic pigs were either perfused for 12 hours in working mode (WM, whole blood-based or leukocyte-depleted blood-based perfusate) or non-working mode (NWM), or were used as *in vivo* healthy left ventricular (LV) tissue. **Results:** Different markers of oxidative stress (including protein carbonyl and malondialdehyde) appeared in higher values in ex-situ perfused hearts compared to *in vivo* controls. The oxidative stress responsive pathways (including pentose phosphate pathway and protein kinase B) were significantly higher in ex-situ perfused hearts compared to *in vivo*. While the hearts prerfused in WM had a adenosine monophosphate (AMPK) kinase and creatine kinase (CK) activation similar to *in vivo*, NWM hearts had significantly lower AMPK and CK activity compared to *in vivo* and WM. The function of ex-situ-perfused hearts significantly declined during perfusion regardless of the perfusion mode but the myocardial function was significantly better preserved in the hearts perfused in WM. Leukocyte depletion in the perfusate (WM-Lred) did not improve inflammation or oxidative stress, or the functional preservation of the hearts. Interestingly, the myocardial expression of the pro inflammatory genes was lower in the perfused hearts compared to *in vivo***.** The myocardial expression of pro-inflammatory cytokines was similar between WM, and WM-Lred.

Conclusion: Ex-situ perfusion exposes the heart to ongoing oxidative stress and damage. Ex-situ perfusion of heart in WM in associated with better functional preservation that may be related to preserved AMPK and CK activity. Depletion of leukocyte in the perfusate may not attenuate the inflammation and oxidative stress effectively.

6.2. Introduction

Normothermic *ex-situ* heart perfusion is an emerging technology which allows preservation of donated heart in a perfused, beating state, preventing preservation-related cold ischemia [1]. Thus, it may protect the donated heart better and provide a platform for therapeutic reconditioning, and functional and metabolic assessment of donor hearts (particularly sub-optimal hearts) during preservation time [2]. The experimental and clinical findings on ESHP have strongly advocated the potential of this technology for improving the cardiac protection during preservation time, facilitating transplantation of the sub-optimal, marginal donor hearts, and expanding the donor pool. However, the cardiac function reportedly declines gradually during ESHP [3-6]. The clinical device for normothermic ESHP currently perfuses the heart only in empty-beating non-working mode, and evaluation of the donor heart viability during the perfusion is based on metabolic findings (perfusate lactate concentration in particular) [1]. Thus, the deteriorating of the cardiac function observed in experimental ESHP has raised some doubts about the efficacy of the current clinical ESHP protocols for preservation, and assessment of the donor heart [7]. The responsible mechanisms for the functional decline of the ex-situ perfused heart are yet to be clarified however as we have shown previously, inefficient energy metabolism and extracorporeal perfusion-related inflammation may

play an important role [3, 8]. On the other hand, the extracorporeal circulation-induced tissue stress and damage is a highly neglected topic in the setting of ESHP [8].

Oxidative stress resulting from the imbalance between generation of reactive oxygen and nitrogen species (RONS) and antioxidative defence of the cells, is induced via several pathways and mechanisms in clinical extracorporeal life support systems (e.g. cardiopulmonary bypass) [9, 10]. Besides their role in myocardial pathophysiological conditions [11, 12], RONS are a by-prorduct of vital, physiological cellular functions, (mainly production of energy in mitochondria) [13, 14]. Due to its intense energy requirements and production, to maintain an efficient myocardial function the mitochondrial energy production and antioxidative capacities must be in a tight balance [14]. Such a balance may be difficult to achieve in significant workloads if the antioxidant capacity of myocardial cells is overwhelmed or decreased, or RONS production is highly enhanced [15].

ESHP system shares many aspects and components with clinical extracorporeal circulation systems including artificial surfaces, filters, and oxygenators [2]. Thus in the present study, we aimed to, to assess the oxidative stress and related responses in porcine hearts perfused ex-situ, and to compare it in a whole blood-based, or leukocyte-depleted perfusate, in either empty-beating non-working mode-perfused hearts (NWM), or the hearts ex- situ-perfused in a semi-physiologic working mode (WM) for an extended period of 12 hours.

6.3. Methods:

Female domestic pigs (41-55 kg, n=14) were used as organ and blood donors. The institutional animal care committee of the University of Alberta approved the experimental protocol as compliant with the animal research ethics and Canadian Council on Animal Care Guidelines and federal and provincial regulations/legislation.

6.3.1. Surgical procedure:

The hearts were procured and prepared, as described in detail before (1, 2). Briefly, the pigs were premedicated with an intramuscular dose of ketamine (20 mg/kg) and atropine (0.05 mg/kg). Orotracheal intubation was established, and general anesthesia was maintained with isoflurane (1-2%). The heart was exposed through a standard median sternotomy. After infusion of 1000 mL of Ringer's lactate solution, and intravenous delivery of heparin (1000 U/kg bolus) 850 ml of whole blood was retrieved through a two-stage venous cannula placed into the right atrium. The blood used and was used to prime the ESHP circuit. The pigs were then euthanized by exsanguination. After a cross-clamp was placed across the ascending aorta, the hearts were arrested with 500 ml of reverse 4:1 blood cardioplegia (modified Del Nido) and were excised, weighed, immediately mounted on the custom ESHP apparatus and perfused for 12 hours.

6.3.2. Ex-situ heart perfusion apparatus and protocol

The ESHP apparatus was constructed using a custom built venous reservoir-embedded organ chamber holding a silicon support membrane with integrated aortic (Ao), left atrial (LA) and pulmonary artery (PA) cannulas, two centrifugal pumps (ROTAFLOW, MAQUET, USA), an arterial line filter (LivaNova, UK), and a membrane oxygenator (D 905, LivaNova, UK) (Figure 6.1). A custom-designed computer system was used allowing

consistent control of pump speed to achieve and maintain desired LA pressures and Ao pressure (40 mmHg). Perfusion was initiated in a non-working (Langendorff) mode (NWM, Left atrial pressure = 0 mmHg) in all the perfused hearts. The coronary sinus blood flow and left atrial flow were measured using a TS410 *Tubing Flow Module and* two flow meters (Transonic, US). The physiologic working mode (WM) was achieved by increasing the left atrial pump revolutions per minute to maintain desired left atrial pressure (6 mmHg).

A combination (1:1) of whole donor blood (or leukocyte-depleted blood) and modified Krebs-Henseleit buffer containing 8% albumin used for the perfusate [5]. Glucose (500 milligrams/hour), insulin (2 units/hour), and dobutamine (4 micrograms/minute) were infused during the perfusion.

Blood gas analysis was performed on the perfusate samples collected from aortic root, and venous line (pulmonary artery), (ABL 800 blood gas analyser, Radiometer, US). A pH of 7.35-7.45, partial pressure of arterial oxygen (paO_2) of 100-150 mmHg, and partial pressure of arterial carbon dioxide ($paCO_2$) of 35-45 mmHg was maintained during the perfusion. At hours 0 (T0, start of perfusion), 5 (T5, mid-perfusion), and 11 (T11, late perfusion) after the start of the perfusion, perfusate samples were collected from the aortic root and were stored in -80 ˚C for further analysis. At the termination of ESHP, samples were taken from the anterolateral wall of the left ventricle and were flash frozen in liquid nitrogen and stored at -80˚C for later assessments.

6.3.3. Study groups:

ESHP was initiated in NWM with isolated aortic root perfusion in both groups. The perfusate was warmed from room temperature to 38˚C over a 1-hour period.

6.3.3.1. Working mode (loaded) perfusion group (n=5)

After warming up (1-hour), hearts were transitioned to WM (left atrial pressure= 6 mmHg), AAI paced at 100 beats/minute, and were maintained in a loaded state over the remainder of the preservation period.

Note: In the comparison between working mode perfusion with whole-blood-based versus leucocyte-reduced perfusates, we added another working mode experiment to our cohort in order to have balanced comparison groups with the total of 6 ESHPs per group.

6.3.3.2. Non-working mode (unloaded) perfusion group (n=5)

Hearts were maintained in an unloaded state throughout the preservation period (12 hours) except for brief transitions into working mode to facilitate assessments of myocardial function and metabolism at T1, T5, and T11.

6.3.3.3. *In vivo* control group (n=4)

Samples from healthy *in vivo* porcine left ventricles were obtained to serve as normal heart control samples for subsequent analyses.

6.3.3.4. Leucocyte reduced perfusate (WM-Lred) group (n=6)

The whole blood from the donor pig was leukocyte depleted before priming the circuit using bedside leukocyte filters. After warming up (1-hour), hearts were transitioned to WM (left atrial pressure= 6 mmHg), and were maintained in a loaded state over the remainder of the preservation period.

6.3.4. Assessment of myocardial metabolism

6.3.4.1. Myocardial oxygen consumption and glucose utilization

were measured as described before [3].

6.3.4.2. Perfusate lactate

Concentrations of venous and arterial perfusate were measured with blood gas analysis (ABL 800 blood gas analyser, Radiometer, US).

6.3.4.3. Activity of adenosine mono phosphate (AMP)-activated protein kinase (AMPK)

The activation state of AMPK, as one of the main regulators of energy metabolism was assessed in LV with immunoblot analysis of relative AMPK Thr172 phosphorylation [16].

6.3.4.4. Activity of creatine kinase (CK)

The enzymatic activity of CK known as metabolic reserve of the myocardium, also negatively affected by oxidative stress [17] was assessed in the LV using a colorimetric assay kit (Abcam Inc, Cambridge, MA) according to the manufacturer's instruction.

6.3.5. Assessment of oxidative stress markers/responsive pathways

6.3.5.1. Protein carbonylation

Protein carbonylation, an indicator of protein oxidation [18], was measured in LV tissue using an ELISA kit (Abcam Inc, Cambridge, MA) according to manufacturer's instructions.

6.3.5.2. Malondialdehyde adduct

Malondealdehyde (MDA) adduct, a marker of lipid peroxidation and oxidative stress [19] was measured in LV tissue with immunoblot analysis.

6.3.5.3. Protein cysteine sulfonation

The post-translational modification of cysteine residue of proteins (protein cysteine sulfonation) as a marker of oxidative stress and injury [20], was assessed in LV samples with immunoblot analysis.

6.3.5.4. Tissue glutathione content

The total and reduced glutathione content, as a marker of anti-oxidant reserve of the myocardium and a marker of cell damage and leakage through the cell membrane [21, 22], was measured in LV tissue using a colorimetric assay kit (Thermo Fisher, Waltham, MA).

6.3.5.5. Pentose phosphate pathway (PPP) activity

The activity of PPP, as the oxidative stress-responsive anabolic pathway of glucose producing nicotinamide adenine dinucleotide phosphate (NADPH) [23], was assessed in LV tissues measuring the activity of glucose-6 phosphate dehydrogenase (G6PH), the rate-limiting enzyme of PPP using a colorimetric assay kit (BioVision, San Francisco, USA).

6.3.5.6. Hexosamine biosynthesis pathway (HBP) activity

The activity of HBP as an accessory pathway of glucose involved in oxidative stress-induced modifictions [23] was assesses with immunoblot analysis of downstream O-linked N-acetylglucosamine (O-GlcNAc)-modified proteins.

6.3.5.7. Activation of protein kinase B (Akt)

Activation of Akt was assessed with immunoblot analysis of relative phosphorylation of Akt at Serine-473 (Ser473) and Threonine-308 (Thr308) [24] in LV tissues.

6.3.6. Assessment of circulating markers of inflammation and oxidative stress

6.3.6.1. Pro-inflammatory cytokines

The perfusate concentrations of pro-inflammatory cytokines TNF-α, and IL-8 were measured using commercially available enzyme-linked immunosorbent assay (ELISA)

kits (R&D systems, US) in accordance with the manufacturer's instructions. The values were compared between the starting point (T0) and T5 and T11 within each perfusion group, and also between the perfusion groups.

6.3.6.2. Oxidized low-density lipoprotein

The perfusate oxidized low-density lipoprotein (Ox-LDL) as a widely studied marker of oxidative stress [25] was assessed using an ELISA kit (My biosource, US) according to manufacturer's instructions.

6.3.7. Assessment of myocardial function

Myocardial functional parameters were assessed after starting of ESHP at hour-1 (T1, early-perfusion), hour-5 (T5, mid-perfusion), and hour-11 (T11, late perfusion) in a physiologic working mode (left atrial pressure= 6 mmHg). The cardiac index (CI) was determined by measuring the blood flow through the left atrial line divided by the heart weight ($mL.minute^{-1}.gram^{-1}$).

To assess maximum rate of pressure change (dP/dt maximum) and minimum rate of pressure change (dP/dt minimum) in the left ventricle (LV), a 5F pigtail catheter was placed in the LV via the subclavian artery. The LV stroke work (SW) was calculated as described before [3].

6.3.8. Myocardial injury

6.3.8.1. Myocardial edema

Edema formation was measured by the weight gained by the heart over the 12-hour ESHP and was reported as a percentage of initial heart weight as described previously [2].

6.3.8.2. Troponin-I

The perfusate cardiac troponin-I (cTn-I) was determined at T1, T5, and T11 using a porcine ELISA Kit (Life Diagnostics, US) and was adjusted with heart weight.

6.3.8.3. Cytokine and oxidative stress response element gene expression

RNA was extracted from LV tissue of the perfused hearts and *in vivo* samples using TRIzol reagent (Life technologies). cDNA expression of the following genes was measured as described previously elsewhere using Taqman RT-PCR (Termo Fisher Scientic & Applied Biosystems); Tnfα, IL-1β, IL-6, and Nuclear factor erythroid 2-related factor 2 (NRF2). Expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and β-actin were used as internal control.

6.3.9. Statistical analysis

The normally- distributed data (assessed with the Shapiro-Wilk test and evaluation of the histogram plot of the data distributions) were reported as the mean ± the standard error of the mean. The non-normally- distributed continuous data were transformed to normality using the “two-step transformation to normality” as described before (12) and were reported as median [Interquartile Range (IQR)]. The data were compared between the groups using the independent samples t-test, or the analysis of variance (one way-ANOVA), followed by the Fisher’s Least Significant Difference (LSD) or Games-Howell post hoc tests where appropriate. The Welch correction was applied when the assumption of equal variances was not met. The overtime trend of changes within each group was assessed using a linear regression model. A 2-sided p-value < 0.05 was

considered statistically significant. The analysis was performed with the IBM SPSS statistics software (Version 21.0).

6.4. Results:

No difference was detected in donor animal weight or heart weight, myocardial ischemic time, defibrillation requirements for reanimation, and perfusion duration between the experimental groups. Similarly, the perfusate characteristics were not statistically different between the experimental groups (data not shown).

6.4.1. Myocardial metabolism

Myocardial oxygen consumption (MVO_2), did not show a statistically meaningful change over the perfusion time in WM (T1: 3.55 [3.53,4.89], T5: 2.86 [2.50,3.68], T11: 3.55 [2.31,4.17] mL/min/100g, p=0.303), but it showed a trend for overtime declining in NWM (T1: 4.45 [3.96,5.40], T5: 2.36 [1.62,3.69], T11: 2.67 [1.58,4.30] mL/min/100g p=0.082). There was not a significant difference in MVO_2 values between WM and NWM at T1 (p=0.398). T5 (p=0.851), or T11 (p=0.149) (Figure 6.2A).

Concentration of glucose was similar between WM and NWM during the whole perfusion (data not shown). Glucose utilization did not change significantly during the perfusion in either WM or NWM (p=0.220 and p=0.354 respectively) and it was higher in WM in mid-perfusion (T5-T7, p=0.026) and late perfusion (T9-T11, p=0.004) assessments during the perfusion but it was similar between WM and NWM in early perfusion (T1-T3, p=0.128) period.

Venoarterial lactate difference did not change during the perfusion in either of the two perfusion groups (WM, p=0.153 and NWM, p=0.755), and there was not a statistically

meaningful difference between WM and NWM at T1 (p=0.78), T5 (p=0.372), or T11 (p=0.337) (Figure 6.2B).

The LV active, phosphorylated AMPK (compared to total AMPK) was similar between healthy *in vivo* LV samples and WM (p=0.22), but significantly lower in NWM compared to both *in vivo* and WM (p<0.001 and p=0.001 respectively), (Figure 6.3A). Similarly, whilst the enzymatic activity of creatine kinase, was significantly lower in NWM compared to *in vivo* and WM (NWM: 2.14 [1.40,3.17], *in vivo*: 4.11 [3.44,4.36], p=0.007 and WM: 3.96 [3.19,4.22], p= 0.040 nmol/min/mL). There was not a difference between WM and *in vivo* (p=0.347), (Figure 6.3B).

6.4.2. Oxidative stress markers/responsive pathways in left ventricular tissue

Protein carbonylation in LV tissue, was significantly lower in *in vivo* compared to *ex-situ* perfused hearts (nmol carbonyl/mg protein, *in vivo*: 0.72 [0.30,0.86], WM: 3.68 [3.51,6.45], p=0.022 and NWM: 5.42 [1.93,6.78], p=0.002,) (Figure 6.4A). LV MDA was also lower in *in vivo* compared to *ex-situ* perfused hearts (p=0.005) (Figure 6.4B). There was not a significant difference in the protein carbonylation or MDA between WM and NWM (p=0.232 and p=0.658 respectively). LV cystein sulfonation was significantly lower in *in vivo* compared to WM (p=0.023) and NWM (p=0.019), without a meaningful difference between WM and NWM (p=0.784) (Figure 6.4C).

The LV tissue content of total glutathione was significantly lower in ex situ perfused hearts (µmol/L total glutathione *in vivo*: 82.10 [80.72,87.37], compared with WM: 31.40 [28.57,39.37], p= 0.001, and compared with NWM: 39.90 [34.45,44.62], p=0.017). Similarly the LV reduced glutathione was lower in the perfused hearts compared to *in vivo* (µmol/L reduced glutathione *in vivo*: 72.65 [61.78, 74.28], compared with WM: 27.65

[25.35,33.52], p=0.001 and compared with NWM: 35.65 [29.25,38.25], p=0.017). There was not a difference in total glutathione content between WM and NWM (p= 0.060), however the reduced glutathione was lower in WM compared to NWM (p=0.047), (Figure 6.5A&B).

The enzymatic activity of G6PD, as the rate-limiting enzyme of PPP was significantly induced in *ex-situ* perfused hearts compared to *in vivo* (nmol/min/mL *in vivo*: 23.66 [20.26,26.13] compared with WM= 32.20 [27.39,34.45], p=0.018, and compared with NWM= 29.26 [27.87,35.77], p=0.026) but it was similar between WM and NWM (p=0.942), (Figure 6.6A). The LV O-GlcNAc-modified protein concentration was higher in general in both WM and NWM compared to *in vivo* (p=0.002 and p<0.001 respectively), and it was also higher in NWM compared to WM (p=0.002), (Figure 6.6B).

Akt phosphorylation (activation) at Threonine308 was induced in both of the ex-situ-perfused groups compared to *in vivo* (WM p<0.001 and NWM p=0.005) and was higher in WM compared to NWM (p=0.026). Similarly, Akt phosphorylation at Serine473 was higher in both WM and NWM compared to *in vivo* (WM p<0.001 and NWM p=0.006). Serine473-phosphorylated Akt was not different between WM and NWM (p=0.053) (Figure 6.7A&B).

6.4.3. Circulating markers of inflammation and oxidative stress

Relative to the baseline (T0) values, the perfusate levels of TNFα significantly increased during ESHP in both groups (ng/mL WM, T0: 8.50 [3.50,43.00], T5: 6450 [1575,7750], T11: 5000 [1375,5825], p=0.034 and NWM T0: 0 [0,37.75], T5: 5650 [3375,8200], T11: 3750 [2675,7250], p=0.003). There was not a statistically meaningful difference in TNFα values between WM and NWM at T0 (p=0.168), T5 (p=0.943), or T11 (p=0.851). (Figure

6.8A). The perfusate concentration of IL-8, while non-detectable at T0, significantly increased by T5 in both groups (μg/mL WM, T5: 14250 [11787,16250], T11: 16250 [15250,19300], p<0.001 and NWM T5: 11500 [10500,17000], T11: 18500 [15750,24125], p<0.001) (Figure 6.8B). There was not a difference in perfusate concentration of the IL-8 between WM and NWM at T5 (p=0.597), or T11 (p=0.897).

The perfusate Ox-LDL, while was non detectable at T0, significantly and continuously increased during perfusion in both groups (ng/mL WM, T5: 7.73 [6.24,9.26], T11: 20.00 [15.11,21.73], p<0.001 and NWM T5: 5.30 [5.08,7.45], T11: 14.60 [12.91,18.03], p<0.001). There was not a significant difference in perfusate concentration of Ox-LDL between WM and NWM in T5 or T11 (p=0.225 and p=0.204 respectively) (Figure 6.8C).

6.4.4. Myocardial function

During 12 hours of ESHP, although the myocardial function in general declined regardless of the perfusion mode, the hearts perfused in WM had a better functional preservation. CI, while did not change in WM (p=0.083), significantly declined in NWM hearts (p=0.048). LVSW declined during the perfusion in both WM and NWM (WM p=0.027, NWM p=0.012), (Table 6.1). Max dp/dt, did not significantly change in either of the groups (WM, p=0.126 and NWM, p=0.509). Min dp/dt, did not change overtime in WM (p=0.199) however, it significantly declined in NWM (p=0.022). CI overtime preservation (T5/T1 and T11/T1) was significantly higher in WM compared to NWM at T5 (p<0.006) and T11 (p<0.002). Similarly, preservation of SW was also better in WM at both T5 (p=0.048) and T11 p=0.013). Max dp/dt was preserved similarly in WM and NWM at T5 (0.188) and T11 (p=0.558), min dp/dt was preserved significantly better in WM at T5 (p=0.013), and was similar between WM and NWM at T11 (p= 0.206), (Table 6.2).

6.4.5. Tissue injury

The hearts in both WM and NWM gained a significant weight during the perfusion, reflecting formation of tissue edema (WM, start weight: 263.00 [229.00,276.00], end weight: 320.00 [288.00,339.00] p=0.003, NWM, start weight: 267 [230.50,350.00], end weight: 333.00 [270.50,426.00] 21.9±4 p=0.021). The percentage of the edema was not different between WM and NWM (% WM: 21.95 [20.00,28.86], NWM: 18.91 [13.76,31.56], p=0.231), (Figure 6.9 A).

The concentration of cTnI in the perfusate, while non-detectible at T0, significantly increased during perfusion in both WM and NWM, (ng/mL WM, T5: 31.50 [28.50,39.00], T11: 105.20 [87.00,118.10], p<0.001 and NWM T5: 33.50 [26.25,41.75] T11: 134.73 [114.57,150.00], p<0.001). The cTnI at T5 values was not statistically different between WM and NWM (p=0.654), however cTnI at T11 was significantly higher in NWM (p=0.034) (Figure 6.9 B).

6.4.6. Effects of ex-situ perfusion of heart with leukocyte-depleted blood-based perfusate

There was not a significant difference in *in vivo* leukocyte count between Lred-WM, and WM (with whole blood-based perfusate), (p=0.669). The leukocyte-depleted perfusate had significantly lower leukocyte count at the start of the perfusion (T0), (*in vivo* leukocyte: 12.37 [11.19-13.63]* 10^9*L^{-1}, T0: 0.15 [0.03-0.53]* 10^9*L^{-1}). The WM perfusion with leukocyte-depleted perfusate did not improve myocardial functional preservation when compared with whole blood-based perfusate. Very interestingly, the pro-

inflammatory cytokines, markers of oxidative stress, and activity of oxidative stress-responsive anabolic pathways of glucose metabolism, were similar between the two strategies (Table 6.3 and Figure 6.10). Perfusate cTN-I during perfusion was similar at T0, T5, and T11 between WM and WM-Lred (p=0.65, p=0.56, and p=0.082 respectively), and tissue edema were also similar between WM and WM-Lred (p= 0.429).

In assessment of the expression of inflammatory cytokine genes and oxidative stress-responsive element NRF2 gene, interestingly, the expression of IL-1β was significantly lower in the both WM (p=0.045) and WM-Lred (p=0.002) compared to *in vivo* however, the values were similar between WM and WM-Lred groups (p=0.991). Similarly, IL-6 gene expression was also lower in WM (p=0.004) and WM-Lred (p=0.028) compared to *in* vivo and values were not different between WM and NWM (p=0.99). The gene expression of TNF-α was not statistically different between the groups (p=0.805). On the other hand, the expression of NRF-2 gene, was lower in WM compared to *in vivo* (p=0.002), while it was similar between WM-Lred and *in vivo* (p=0.21). In the meanwhile, there was no significant difference between WM and WM-Lred either (p=0.35), (Figure 6.11).

6.5. Discussion:

6.5.1. Oxidative stress and inflammation develops during ESHP, while the cardiac antioxidant reserve and myocardial function decline

The time-dependent decline of the cardiac function has been frequently observed in experimental ESHP, and has raised question about the potentials of ESHP for superior preservation of the donated heart compared to cold storage [3-6]. While the development of oxidative stress and/or inflammation, and related cardiac dysfunction in the context of

clinical extracorporeal circulation (such as CPB and ECMO) are well established [26, 27], there is a significant gap in the literature about these phenomena in the setting of ESHP.

The systematic oxidative stress developed during clinical extracorporeal circulation may be associated with *ischemia reperfusion injury (IRI),* sequestration of natural antioxidants and the microelements necessary for their activity by the circuit components, activation of the immune, and contact systems due to contact of the blood elements with artificial, non-endothelialized surfaces of the circuit, and hemolysis [8, 9, 28, 29]. Thus, the oxidative stress may develop in a continuous fashion during the perfusion [30]. In the present study, alongside the functional decline, the markers of oxidative stress/damage were also induced in the LV tissue of the ex-situ perfused hearts. Malondialdehyde, a terminal product of lipid peroxidation, and products of oxidative modification of proteins (carbonylated and sulfonated proteins) were all significantly higher in ex-situ perfused hearts compared to healthy *in vivo* LV samples regardless of the perfusion mode (WM and NWM). These end products are not solely the markers of oxidative stress, but also play a role in the oxidative stress-induced cardiac dysfunction and failure. In a model of isolated rat myocyte, Folden *et al.* showed that MDA can directly suppress contractile function of the heart via p38 mitogen-activated protein kinase (MAPK) phosphorylation (activation).The related deleterious effects were reduced by antioxidative compounds [19]. In a study by Ichihara *et al.* on a hamster model of cardiomyopathy, while carbonylated protein content of LV showed a negative correlation with ATP content of LV, it had a correlation with plasma cTn-I [31]. Carbonylated proteins that are the major products of protein oxidation, can react with lysine residues of other proteins as well, eventually leading to the formation of protein aggregates. Such protein aggregates may

not be degradable by normal cellular mechanisms thus, may exacerbate accumulation of oxidation-modified proteins and related cellular dysfunction [18]. High levels of reactive oxygen species, H_2O_2 in particular, cause hyperoxidation of cysteine residues of proteins leading to irreversible sulfination and/or sulfonation, which is considered as an oxidative damage-related modification, rather than having a signalling role. Sulfonation of cysteine residues may be involved in dysfunction of the mitochondrial complex-I and cell damage and death in the myocardium subjected to oxidative stress [20, 32, 33]. Besides the significant increase in the markers of oxidative stress and damage in the LV tissue of the ex-situ perfused hearts, the LV tissue content of total and reduced glutathione were significantly lower in ex-situ perfused hearts compared to *in vivo*. The myocardial glutathione depletion, which also reflects the deficiency in its circulating/systemic concentrations, has been suggested to have a strong association with myocardial functional and structural abnormalities in various pathologic cardiac conditions. Similarly, depletion of myocardial glutathione may be associated with impaired recovery of function in isolated perfused hearts after being exposed to global ischemia [34, 35].

In agreement with our previous observations [8], in the present study the levels of pro-inflammatory cytokines TNF-α and IL-8 significantly increased during perfusion, reaching to quite high concentrations in a few hours after the start of the perfusion. The pathologic conditions accompanied by increased circulating pro-Inflammatory cytokines (e.g. septic shock) are associated with cardiac dysfunction and failure. Increased levels of pro-inflammatory can be induced by RONS, and can also further induce oxidative stress and damage in a maladaptive cycle [36]. Thus, in the setting of ESHP, increased concentrations of circulating pro-inflammatory cytokines may cause/exacerbate oxidative

stress and contribute to the functional decline of the heart. Similarly, the perfusate level of Ox-LDL, which is generated mainly within the vascular wall, significantly increased throughout the perfusion in both WM and NWM, suggesting the ongoing oxidative stress the myocardial tissue is exposed toward. Ox-LDL, can directly induce negative inotropic and lusitropic effects on heart, independent of the risk factors such as age, blood pressure, and cholesterol levels [25, 37].

6.5.2. Oxidative stress-responsive anabolic pathways are induced during ESHP

Efficiency of energy metabolism is essential for a healthy functioning heart. Numerous vital processes in the cell may be altered by oxidative stress and inflammation, including energy metabolism. The effects of oxidative stress on energy metabolism, is well-investigated in cancer. Interestingly, the recent evidence suggests that similar modifications occuring in cancer may occur in non-neoplastic conditions such as heart failure [38]. We have shown previously that the actvity of pyruvate kinase (PK), which catalizes the final step of glycolysis was lower in ex-situ-perfused pig hearts compared to *in vivo*. At the same time, expression of M2 isoform of PK (PKM2) which acts as a gear to divert glycolysis to the anabolic pathways was higher in ex-situ-perfused hearts [3].

The anabolic pathways of glucose such as PPP and HBP, are important pathways in regulation of glucose metabolism in cardiomyocytes and are involved in antioxidant defence and/or oxidative stress-induced modifications [23]. Here, we observed a higher activity of PPP and HBP in *ex-situ* perfused hearts compared to *in vivo*. The PPP activity was similar between WM and NWM but the HBP activity was significantly higher in NWM compared to WM. The activity of PPP increases in response to oxidative stress, to maintain the redox status of the cell by generation NADPH which retains the pool of

reduced anti-oxidants such as reduced glutathione. Paradoxically, hyperactivation of PPP may lead to production of superoxide and thus, exacerbate oxidative stress and related damage [39-41].

The HBP although is important for maintenace of cardiovascular function, but its activity can be induced by oxidative stress through inhibition of the rate-limiting enzyme of glycolysis pathway, glyceraldehyde-3-phosphate dehydrogenase leading to increased utilization of glucose, acetyl-coA, and glutamine by HBP. Increased O-linked GlcNac modification of proteins may alter the function of proteins, and cause mitochondrial impairment, cell death and cardiac mechanical dysfunction [41-43]. Thus, hyperactivation of PPP and HBP in the context of persistent oxidative stress may contribute to the inefficiency of energy-yielding metabolism and the functional decline of the *ex-situ* perfused hearts.

6.5.3. Ex-situ perfusion of the heart in semi-physiological workload does not exacerbate oxidative stress and related responses

In the present study, numerous markers of inflammation and oxidative stress were induced in both LV tissue and perfusate during ESHP. However, oxidative stress-related markers and modifications developed with comparable values in NWM and WM. This was an interesting observation since the hearts perfused in the WM for majority of the time during ESHP, endured semi-physiological workloads during this process. Thus, when compared to empty-beating NWM hearts, the hearts perfused in the WM had higher energy demand and energy production dynamic in the mitochondria. In the meanwhile, the LV values of glutathione were lower in the perfused hearts when compared to *in vivo*, suggesting decreased antioxidative potential of the perfused hearts. In this situation,

higher energy demands and production in WM could have lead to higher production of ROS and thus oxidative stress related modifications/markers in WM compared to NWM, particularly with the lower reduced glutathione levels in the WM. This observation suggested that in ESHP 1) the development of oxidative stress is a general consequence of extracorporeal perfusion regardless of the workload and 2) *ex-situ* perfusion of the heart in the semi-physiologic workloads is not associated with exacerbation of the oxidative stress.

6.5.4. The effectors of myocardial metabolic reserve and survival alter in a load-dependent manner during ESHP

In the present study, the activity of AMPK and CK were similar between WM hearts and healthy *in vivo* controls while were significantly lower in NWM hearts. AMPK and CK have a key role in regulation of metabolism and ATP availability in the myocardium, and contribute to cardiomyocyte function and viability [16, 44]. AMPK activation also induces antioxidant defence through activation of several antioxidant genes including superoxide dismutase gene during oxidative stress, and maintaining cellular NADPH [45, 46]. Oxidative stress is known to impair the activity of CK however, the WM hearts preserved the activity of CK in the presence of oxidative stress, while NWM hearts maintained lower CK activity compared to *in vivo* and WM. The activation of AMPK and CK are among the important mediators of the cardioprotection induced by the ventricular wall stretch and contraction [47-49]. Thus, the semi-physiologic ventricular wall stretch and heart chamber pressures during ESHP in WM hearts, may lead to preservation of AMPK and CK activity, and exert cardioprotective effects during ex-situ perfusion.

In the presence of oxidative stress, protein kinase B (Akt) activation may be induced as a part of cell's protective mechanisms [50]. Here, phosphorylation of Ser473 and Thr308 (both required for full activation of Akt) was significantly induced in the perfused hearts in comparison to *in vivo*. Activation of Akt is critical for the basic function of cardiomyocytes and exerts cardioprotective effects. Upon activation, Akt migrates to different sites in the cell (e.g. plasma membrane, mitochondria, and nucleus), and through regulation of various key effectors, it can improve metabolism, inotropism, and survival in short term, but long term activation may lead to cardiac dysfunction and failure [24, 51]. Here, while Thr308 phosphorylation of Akt was higher in WM compared to NWM, Ser473 phosphorylation was not statistically different between WM and NWM. Phosphorylation of Akt at Thr308 is mainly regulated by phosphoinisitide-dependent kinase-1 (PDK1) enzyme, however, phosphorylation at Ser473 is mostly dependent on the mammalian target of rapamycin complex2 (mTORC2). It has been reported that lower Ser437 phosphorylation in the favor of enhanced Thr308 phosphorylation may have cardioprotective effects [52, 53]. This is also in accordance with our observations in the present study that myocardial workload during ESHP may affect induction of the stress-responsive pathways including Akt. On the other hand, Akt activation is known to blunt AMPK activation. While Akt activation has an inhibitory effect on AMPK activation, oxidative stress induces AMPK and Akt activation simultaneously, balancing the metabolism and redox status of the cell [54]. Interestingly in the present study, despite higher activation of Akt in WM hearts, they preserved AMPK activation in the presence of oxidative stress while the hearts ex-situ perfused in NWM had lower AMPK activation compared to *in vivo* controls and WM.

Due to the sub-physiologic workload and wall stretch in NWM hearts, the induction and maintenance of AMPK activation may not occur efficiently in NWM-perfused hearts particularly when exposed to stimuli such as oxidative stress. This phenomenon may be associated with the more severe functional decline in NWM compared to WM hearts as was observed here.

6.5.5. Perfusion in WM is associated with superior functional preservation and lower myocardial damage

Despite similar perfusate and LV values of the markers of oxidative stress and inflammation, and comparable edema formation in WM and NWM, and in accordance with our previous observations, the hearts perfused in the semi-physiological WM preserved their function significantly better over 12 hours of normothermic ESHP. The mechanisms for this phenomenon may have not been clarified yet however, the superior functional preservation in WM may be associated with maintained AMPK and CK activity status in WM which may have protective effects against stresses such as oxidative stress and inflammation.

Similarly, cTn-I leak was lower in WM compared to NWM hearts in extended perfusion times. According to studies by our group and others, the release of cTn-I may not always reflect the ultimate cell necrosis and death but may be related to reversible myocardial stress. Thus, the hearts perfused in WM may not only provides a tool for functional assessment of the heart during ESHP, may offer better cardiac protection against stress and stimuli during ESHP.

6.5.6. Leukocyte depletion of the blood-based perfusate does not improve myocardial functional preservation and does not attenuate inflammation and oxidative stress during ESHP

Clinical ESHP id performed with leukocyte-depleted donor blood to minimize/prevent inflammatory responses during ESHP [1]. Interestingly, in the present study, depletion of leukocyte in the perfusate (to less that 5% of the original values) did not made a significant improvement in the inflammatory and oxidative stress during perfusion, and also it did not improve the functional preservation of the heart, or edema formation during ESHP. This observation suggests that during ESHP, there may be sources other than leukocytes that significantly contribute to inflammation in this setting. Although some non-immune tissues including myocardial cells have been suggested to act like analogue of immune cells, and produce pro-inflammatory mediators including cytokines. However, here we did not detect any increased expression of cytokine genes in the perfused hearts compared to *in vivo*. This observation, may suggest that while the production of cytokines by myocardial cells may not particularly increase during ESHP, yet, 1) they may release their pro-inflammatory cytokine stores to the perfusate [56,57], and/or 2) red blood cells may release their cytokine stores as a result of extracorporeal circulation-related mechanical stress and hemolysis [57]. Such alterations may have a significant contribution to development of inflammatory and oxidative stress responses during ESHP, and warrant more studies.

6.6. Study limitations:

The ischemic times in this study are negligible and do not reflect current clinical practice as our main aim was to investigate ESHP-related oxidative stress and related alterations in metabolism that could have been impacted by a significant ischemia-reperfusion injury. Therefore, in this study, the hearts were not exposed to longer ischemic times prior to initiation of ESHP. Due to unstability of RNOS, we did not assessed the RNOS directly in the perfusate and myocardial tissue and instead measured to different markers of oxidative stress.

6.7. Conclusion

Myocardial function deteriorates gradually during ESHP while oxidative stress-related proteins and lipid modifications occur in both perfusate and LV tissue, suggesting that the heart is exposed to persistent oxidative stress during ESHP that may have a key role in the functional decline. Only the hearts perfused in WM preserve the activation status of some of the enzymes with a key role in myocardial energy metabolism and reserve and cardioprotection and ESHP in semi-physiological workloads improves the functional preservation of the during the perfusion. For improving preservation of the function and viability of the ex-situ perfused heart, optimising the clinical protocols of ESHP including 1) perfusion in more physiological workloads, and 2) addressing persistent oxidative stress, and related alterations to energy-yielding metabolism is inevitable.

6.8. Funding

This work was supported by the Canadian Institutes for Health Research/Canadian National Transplant Research Program and the University Hospital Foundation.

6.9. Conflict of interest

DF and JN are founders of Tevosol, Inc., Edmonton, AB, Canada (https://www.tevosol.com/)

6.10. References:

1. Ardehali A, Esmailian F, Deng M, Soltesz E, Hsich E, Naka Y, Mancini D, Camacho M, Zucker M, Leprince P *et al*: **Ex-vivo perfusion of donor hearts for human heart transplantation (PROCEED II): a prospective, open-label, multicentre, randomised non-inferiority trial**. *Lancet (London, England)* 2015, **385**(9987):2577-2584.
2. Hatami S, White CW, Ondrus M, Qi X, Buchko M, Himmat S, Lin L, Cameron K, Nobes D, Chung HJ *et al*: **Normothermic ex situ heart perfusion in working mode: assessment of cardiac function and metabolism**. *Journal of visualized experiments : JoVE* 2019(143).
3. Hatami S, White C, Shan S, Haromy A, Qi X, Ondrus M, Kinnear A, Himmat S, Michelakis E, Nagendran J *et al*: **Myocardial functional decline during prolonged ex situ heart perfusion**. *The Annals of thoracic surgery* 2019, **108**:499-507.
4. White CW, Ambrose E, Muller A, Li Y, Le H, Thliveris J, Arora RC, Lee TW, Dixon IM, Tian G *et al*: **Avoidance of Profound Hypothermia During Initial Reperfusion Improves the Functional Recovery of Hearts Donated After Circulatory Death**. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2016, **16**(3):773-782.
5. White CW, Hasanally D, Mundt P, Li Y, Klein J, Xiang B, Müller A, Ambrose E, Ravandi A, Arora RC *et al*: **A wholeblood–based perfusate provides superior preservation of myocardial function during ex vivo heart perfusion**. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* 2014, **S1053-2498**(14):01356-01354.
6. Ferdinandy P, Panas D, Schulz R: **Peroxynitrite contributes to spontaneous loss of cardiac efficiency in isolated working rat hearts**. *The American journal of physiology* 1999, **276**(6):H1861-1867.
7. Hatami S, Freed DH: **Machine perfusion of donor heart: state of the art**. *Current Transplantation Reports* 2019, **6**(3):242-250.
8. Hatami S, White C, Qi X, Buchko M, Ondrus M, Kinnear A, Himmat S, Sergi C, Nagendran J, Freed D: **Immunity and stress responses are induced during ex-situ heart perfusion**. *Circ Heart Fail* 2020, **13**(6):e006552.
9. McDonald CI, Fraser JF, Coombes JS, Fung YL: **Oxidative stress during extracorporeal circulation**. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* 2014, **46**(6):937-943.
10. Zakkar M, Guida G, Suleiman MS, Angelini GD: **Cardiopulmonary bypass and oxidative stress**. *Oxidative medicine and cellular longevity* 2015, **2015**:189863.
11. Curtis JM, Hahn WS, Long EK, Burrill JS, Arriaga EA, Bernlohr DA: **Protein carbonylation and metabolic control systems**. *Trends in endocrinology and metabolism: TEM* 2012, **23**(8):399-406.
12. Suzuki YJ, Hao JJ: **Results supporting the concept of the oxidant-mediated protein amino acid conversion, a naturally occurring protein engineering process, in human cells**. *F1000Research* 2017, **6**:594.
13. Bertero E, Maack C: **Calcium signaling and reactive oxygen species in mitochondria**. *Circulation research* 2018, **122**(10):1460-1478.
14. Nickel A, Kohlhaas M, Maack C: **Mitochondrial reactive oxygen species production and elimination**. *Journal of molecular and cellular cardiology* 2014, **73**:26-33.
15. Santos CX, Anilkumar N, Zhang M, Brewer AC, Shah AM: **Redox signaling in cardiac myocytes**. *Free radical biology & medicine* 2011, **50**(7):777-793.
16. Dyck JR, Lopaschuk GD: **AMPK alterations in cardiac physiology and pathology: enemy or ally?** *The Journal of physiology* 2006, **574**(Pt 1):95-112.

17. Nain S, Ling B, Bandy B, Alcorn J, Wojnarowicz C, Laarveld B, Olkowski AA: **The role of oxidative stress in the development of congestive heart failure in a chicken genotype selected for rapid growth**. *Avian pathology : journal of the WVPA* 2008, **37**(4):367-373.
18. Cecarini V, Gee J, Fioretti E, Amici M, Angeletti M, Eleuteri AM, Keller JN: **Protein oxidation and cellular homeostasis: Emphasis on metabolism**. *Biochimica et biophysica acta* 2007, **1773**(2):93-104.
19. Folden DV, Gupta A, Sharma AC, Li SY, Saari JT, Ren J: **Malondialdehyde inhibits cardiac contractile function in ventricular myocytes via a p38 mitogen-activated protein kinase-dependent mechanism**. *British journal of pharmacology* 2003, **139**(7):1310-1316.
20. Murray CI, Van Eyk JE: **Chasing cysteine oxidative modifications: proteomic tools for characterizing cysteine redox status**. *Circulation Cardiovascular genetics* 2012, **5**(5):591.
21. Singh A, Lee KJ, Lee CY, Goldfarb RD, Tsan MF: **Relation between myocardial glutathione content and extent of ischemia-reperfusion injury**. *Circulation* 1989, **80**(6):1795-1804.
22. Lucchinetti E, Lou PH, Hatami S, Qi X, Clanachan AS, Freed DH, Zaugg M: **Enhanced myocardial protection in cardiac donation after circulatory death using Intralipid((R)) postconditioning in a porcine model**. *Canadian journal of anaesthesia = Journal canadien d'anesthesie* 2019.
23. Doenst T, Nguyen Tien D, Abel ED: **Cardiac metabolism in heart failure**. *Circulation research* 2013, **113**(6):709-724.
24. Abeyrathna P, Su Y: **The critical role of Akt in cardiovascular function**. *Vascular pharmacology* 2015, **74**:38-48.
25. Rietzschel ER, Langlois M, De Buyzere ML, Segers P, De Bacquer D, Bekaert S, Cooman L, Van Oostveldt P, Verdonck P, De Backer GG *et al*: **Oxidized low-density lipoprotein cholesterol is associated with decreases in cardiac function independent of vascular alterations**. *Hypertension (Dallas, Tex : 1979)* 2008, **52**(3):535-541.
26. Baehner T, Boehm O, Probst C, Poetzsch B, Hoeft A, Baumgarten G, Knuefermann P: **[Cardiopulmonary bypass in cardiac surgery]**. *Der Anaesthesist* 2012, **61**(10):846-856.
27. Pyles LA, Gustafson RA, Fortney J, Einzig S: **Extracorporeal membrane oxygenation induced cardiac dysfunction in newborn lambs**. *Journal of cardiovascular translational research* 2010, **3**(6):625-634.
28. Chen Q, Yu W, Shi J, Shen J, Hu Y, Gong J, Li J, Li N: **The effect of extracorporeal membrane oxygenation therapy on systemic oxidative stress injury in a porcine model**. *Artificial organs* 2014, **38**(5):426-431.
29. Millar JE, Fanning JP, McDonald CI, McAuley DF, Fraser JF: **The inflammatory response to extracorporeal membrane oxygenation (ECMO): a review of the pathophysiology**. *Critical care (London, England)* 2016, **20**(1):387.
30. Clermont G, Vergely C, Jazayeri S, Lahet JJ, Goudeau JJ, Lecour S, David M, Rochette L, Girard C: **Systemic free radical activation is a major event involved in myocardial oxidative stress related to cardiopulmonary bypass**. *Anesthesiology* 2002, **96**(1):80-87.
31. Ichihara S, Suzuki Y, Chang J, Kuzuya K, Inoue C, Kitamura Y, Oikawa S: **Involvement of oxidative modification of proteins related to ATP synthesis in the left ventricles of hamsters with cardiomyopathy**. *Scientific reports* 2017, **7**(1):9243.
32. Kang PT, Chen CL, Lin P, Zhang L, Zweier JL, Chen YR: **Mitochondrial complex I in the post-ischemic heart: reperfusion-mediated oxidative injury and protein cysteine sulfonation**. *Journal of molecular and cellular cardiology* 2018, **121**:190-204.
33. Fernandez-Caggiano M, Schroder E, Cho HJ, Burgoyne J, Barallobre-Barreiro J, Mayr M, Eaton P: **Oxidant-induced interprotein disulfide formation in cardiac protein DJ-1 occurs via an interaction with peroxiredoxin 2**. *The Journal of biological chemistry* 2016, **291**(19):10399-10410.

34. Damy T, Kirsch M, Khouzami L, Caramelle P, Le Corvoisier P, Roudot-Thoraval F, Dubois-Rande JL, Hittinger L, Pavoine C, Pecker F: **Glutathione deficiency in cardiac patients is related to the functional status and structural cardiac abnormalities**. *PloS one* 2009, **4**(3):e4871.
35. Werns SW, Fantone JC, Ventura A, Lucchesi BR: **Myocardial glutathione depletion impairs recovery of isolated blood-perfused hearts after global ischaemia**. *Journal of molecular and cellular cardiology* 1992, **24**(11):1215-1220.
36. Li Y, Ge S, Peng Y, Chen X: **Inflammation and cardiac dysfunction during sepsis, muscular dystrophy, and myocarditis**. *Burns & trauma* 2013, **1**(3):109-121.
37. Ho E, Karimi Galougahi K, Liu CC, Bhindi R, Figtree GA: **Biological markers of oxidative stress: Applications to cardiovascular research and practice**. *Redox biology* 2013, **1**:483-491.
38. Rees ML, Subramaniam J, Li Y, Hamilton DJ, Frazier OH, Taegtmeyer H: **A PKM2 signature in the failing heart**. *Biochemical and biophysical research communications* 2015, **459**(3):430-436.
39. Jain M, Brenner DA, Cui L, Lim CC, Wang B, Pimentel DR, Koh S, Sawyer DB, Leopold JA, Handy DE *et al*: **Glucose-6-phosphate dehydrogenase modulates cytosolic redox status and contractile phenotype in adult cardiomyocytes**. *Circulation research* 2003, **93**(2):e9-16.
40. Vimercati C, Qanud K, Mitacchione G, Sosnowska D, Ungvari Z, Sarnari R, Mania D, Patel N, Hintze TH, Gupte SA *et al*: **Beneficial effects of acute inhibition of the oxidative pentose phosphate pathway in the failing heart**. *American journal of physiology Heart and circulatory physiology* 2014, **306**(5):H709-717.
41. Bairwa SC, Parajuli N, Dyck JR: **The role of AMPK in cardiomyocyte health and survival**. *Biochimica et biophysica acta* 2016, **1862**(12):2199-2210.
42. McNulty PH: **Hexosamine biosynthetic pathway flux and cardiomyopathy in type 2 diabetes mellitus. Focus on "Impact of type 2 diabetes and aging on cardiomyocyte function and O-linked N-acetylglucosamine levels in the heart"**. *American journal of physiology Cell physiology* 2007, **292**(4):C1243-1244.
43. Rajamani U, Essop MF: **Hyperglycemia-mediated activation of the hexosamine biosynthetic pathway results in myocardial apoptosis**. *American journal of physiology Cell physiology* 2010, **299**(1):C139-147.
44. Weiss RG, Gerstenblith G, Bottomley PA: **ATP flux through creatine kinase in the normal, stressed, and failing human heart**. *Proceedings of the National Academy of Sciences of the United States of America* 2005, **102**(3):808-813.
45. Jeon SM: **Regulation and function of AMPK in physiology and diseases**. *Experimental & molecular medicine* 2016, **48**(7):e245.
46. Rabinovitch RC, Samborska B, Faubert B, Ma EH, Gravel SP, Andrzejewski S, Raissi TC, Pause A, St-Pierre J, Jones RG: **AMPK maintains cellular metabolic homeostasis through regulation of mitochondrial reactive oxygen species**. *Cell reports* 2017, **21**(1):1-9.
47. Hao J, Kim HS, Choi W, Ha TS, Ahn HY, Kim CH: **Mechanical stretch-induced protection against myocardial ischemia-reperfusion injury involves AMP-activated protein kinase**. *The Korean journal of physiology & pharmacology : official journal of the Korean Physiological Society and the Korean Society of Pharmacology* 2010, **14**(1):1-9.
48. Chanda D, Luiken JJ, Glatz JF: **Signaling pathways involved in cardiac energy metabolism**. *FEBS letters* 2016, **590**(15):2364-2374.
49. Bittl JA, Ingwall JS: **The energetics of myocardial stretch. Creatine kinase flux and oxygen consumption in the noncontracting rat heart**. *Circulation research* 1986, **58**(3):378-383.
50. Zhang Y, Fang Liu F, Bi X, Wang S, Wu X, Jiang F: **The antioxidant compound tert-butylhydroquinone activates Akt in myocardium, suppresses apoptosis and ameliorates pressure overload-induced cardiac dysfunction**. *Scientific reports* 2015, **5**:13005.

51. Catalucci D, Condorelli G: **Effects of Akt on cardiac myocytes**. *Circulation research* 2006, **99**(4):339-341.
52. Di R, Wu X, Chang Z, Zhao X, Feng Q, Lu S, Luan Q, Hemmings BA, Li X, Yang Z: **S6K inhibition renders cardiac protection against myocardial infarction through PDK1 phosphorylation of Akt**. *The Biochemical journal* 2012, **441**(1):199-207.
53. Zhao X, Lu S, Nie J, Hu X, Luo W, Wu X, Liu H, Feng Q, Chang Z, Liu Y *et al*: **Phosphoinositide-dependent kinase 1 and mTORC2 synergistically maintain postnatal heart growth and heart function in mice**. *Molecular and cellular biology* 2014, **34**(11):1966-1975.
54. Horie T, Ono K, Nagao K, Nishi H, Kinoshita M, Kawamura T, Wada H, Shimatsu A, Kita T, Hasegawa K: **Oxidative stress induces GLUT4 translocation by activation of PI3-K/Akt and dual AMPK kinase in cardiac myocytes**. *Journal of cellular physiology* 2008, **215**(3):733-742.
55. Wan S, DeSmet J-M, Barvais L, Goldstein M, Vincent J-L, LeClerc J-L: **Myocardium is a major source of proinflammatory cytokines in patients undergoing cardiopulmonary bypass**. *The Journal of thoracic and cardiovascular surgery* 1996, **112**(3):806-811.
56. Aoyagi T, Matsui T: **The cardiomyocyte as a source of cytokines in cardiac injury**. *Journal of cell science & therapy* 2011, **2012**(S5).
57. Karsten E, Breen E, Herbert BR: **Red blood cells are dynamic reservoirs of cytokines**. *Scientific reports* 2018, **8**(1):3101.

6.11. Tables and figures:

Table 6.1. Left ventricular function parameters during *ex-situ* heart perfusion in working mode and non-working mode groups

Parameter		T1	T5	T11	*p-value*
***CI* (mL/min/g)**	**WM**	7.72±2.22	6.28±1.36	5.62±1.21	0.060
	NWM	7.02±3.03	3.83±1.97	2.61±1.46	0.008
***SW* (mmHg*mL)**	**WM**	1000 [823,1945]	1233 [849,1258]	815 [601,1017]	0.215
	NWM	1061 [939,1466]	455 [3.13,802]	357 [201,645]	0.001
***dP/dT max* (mmHg/s)**	**WM**	1623±532	1039±311	1113±222	0.065
	NWM	1278±278	1121±279	1102±589	0.495
***dP/dT min* (mmHg/s)**	**WM**	-1155 [-606,-1318]	-1044 [-616,-1224]	-630 [530,813]	0.115
	NWM	--921 [-718,-1012]	-595 [-500,-763]	-444 [-276,-723]	0.027
***ME* (%)**	**WM**	5.95 [5.26,6.42]	5.66 [4.76,10.68]	11.69 [10.49,24.15]	0.003
	NWM	6.20 [3.23,6.97]	4.96 [2.12,7.66]	5.86 [4.85,22.45]	0.869

Data are reported as median [IQR] or mean±SD. CI, cardiac index; dP/dT max, maximum rate of pressure change; dP/dT min, minimum rate of pressure change; NWM, non-working mode perfusion; SW, stroke work

Table 6.2. Preservation of left ventricular function over perfusion time, relative to baseline values assessed at T1, compared between WM and NWM groups

preserved function	group	T5	T11
***CI* %**	**WM**	81.04 [75.85,91.17]	72.16 [68.13,80.97]
	NWM	51.35 [42.50, 64.63]	40.00 [25.83, 52.53]
	p-value	0.006	0.002
***SW* %**	**WM**	82.00 [65.20,130.74]	63.48 [52.01, 84.98]
	NWM	40.19 [29.11, 66.34]	34.86 [20.98, 45.17]
	p-value	0.048	0.013
***dP/dT max* %**	**WM**	69.60 [47.42,90.48]	68.34 [58.57, 87.42]
	NWM	91.20 [71.08,104.46]	73.01 [48.57,127.72]
	p-value	0.188	0.558
***dP/dT min* %**	**WM**	96.02 [82.87,108.18]	79.74 [62.25, 90.79]
	NWM	72.74 [62.01, 8023]	60.90 [32.16, 78.68]
	p-value	0.013	0.181
***ME* %**	**WM**	100.00 [91.67,163.10]	240.00 [160.71,400,00]
	NWM	100.00 [61.67,107.17]	133.33 [71.43, 520.00]
	p-value		

Data are reported as median [IQR]. CI, cardiac index; dP/dT max, maximum rate of pressure change; dP/dT min, minimum rate of pressure change; NWM, non-working mode perfusion; PRSW, preload recruitable stroke work; SE, standard error of the mean; SW, stroke work; WM, working mode perfusion.

Table 6.3. Preservation of left ventricular function over perfusion time, relative to baseline values assessed at T1, compared between WM with whole blood-based perfusate and WM with leukocyte-depleted perfusate

preserved function	group	T5	T11
CI %	**WM**	80.2 [72,87.5]	75.16 [67.13,85.97]
	WM-Lred	71.35 [57.50, 77.63]	63 [44.83, 78.50]
	p-value	0.310	0.699
SW %	**WM**	78.00 [66.20,110.74]	73.48 [59.01, 83.98]
	WM-Lred	61.19 [40.11, 77.34]	53.86 [32.98, 73.17]
	p-value	0.394	0.699

Data are reported as median [IQR]. CI, cardiac index; SW, stroke work; WM, working mode perfusion with whole blood-based perfusate, WM-Lred, working mode perfusion with leukocyte-depleted perfusate .

Figure 6.1. Schematic diagram of the ex-situ heart perfusion circuit.

A, organ chamber and supporting silicone membrane; B, venous reservoir; C, arterial line filter; D, left atrial pump; E, Aortic pump; F, oxygenator; G, heat exchanger; H, medical gas mixer; I, infusion line; J, pressure sensor; K, Flow sensor; H,; Ao, aorta; LA, left atrium; PA, pulmonary artery.

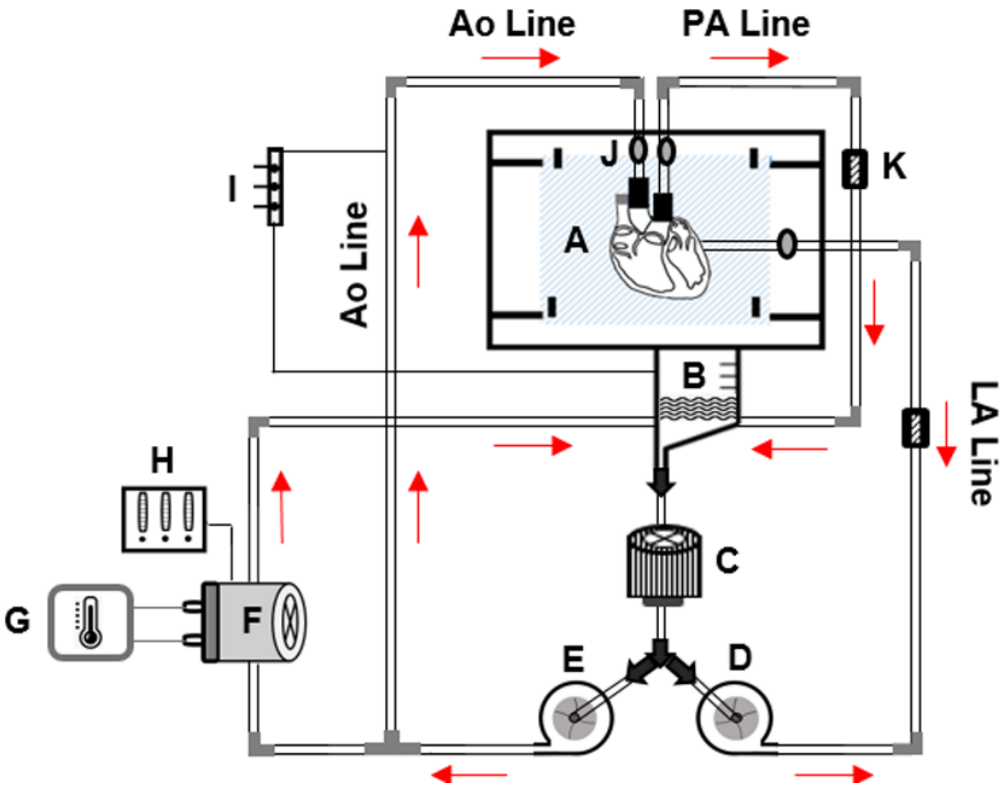


Figure 6.2. A: Myocardial oxygen consumption B: Lactate difference between arterial and venous perfusate

Data are shown as median [IQR]. MVO_2, myocardial oxygen consumption; NWM, non-working mode perfusion; T(x); x: hours after the start of the perfusion; WM, working mode perfusion; *, significant overtime change within group

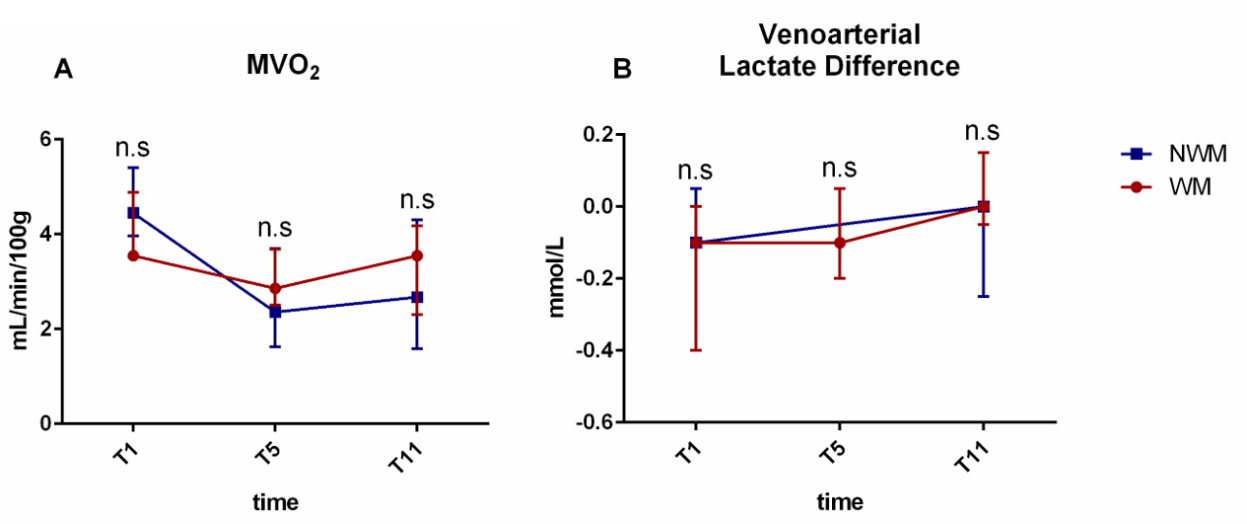


Figure 6.3. Enzymatic activity of AMPK and Creatine kinase A: Immunoblotting of cell lysate prepared from LV samples, using the indicated antibodies.

B: Phosphorylated, relative to total AMPK in left ventricular tissue of ESHP hearts compared *to in vivo*. C: Enzymatic activity of creatine kinase in left ventricular tissue of ESHP hearts compared *to in vivo*

AMPK, adenosine monophosphate kinase; INV, *in vivo*; n.s, no significant difference between the groups; NWM, non-working mode perfusion; p-AMPK, phosphorylated, active adenosine monophosphate kinase; WM, working mode perfusion

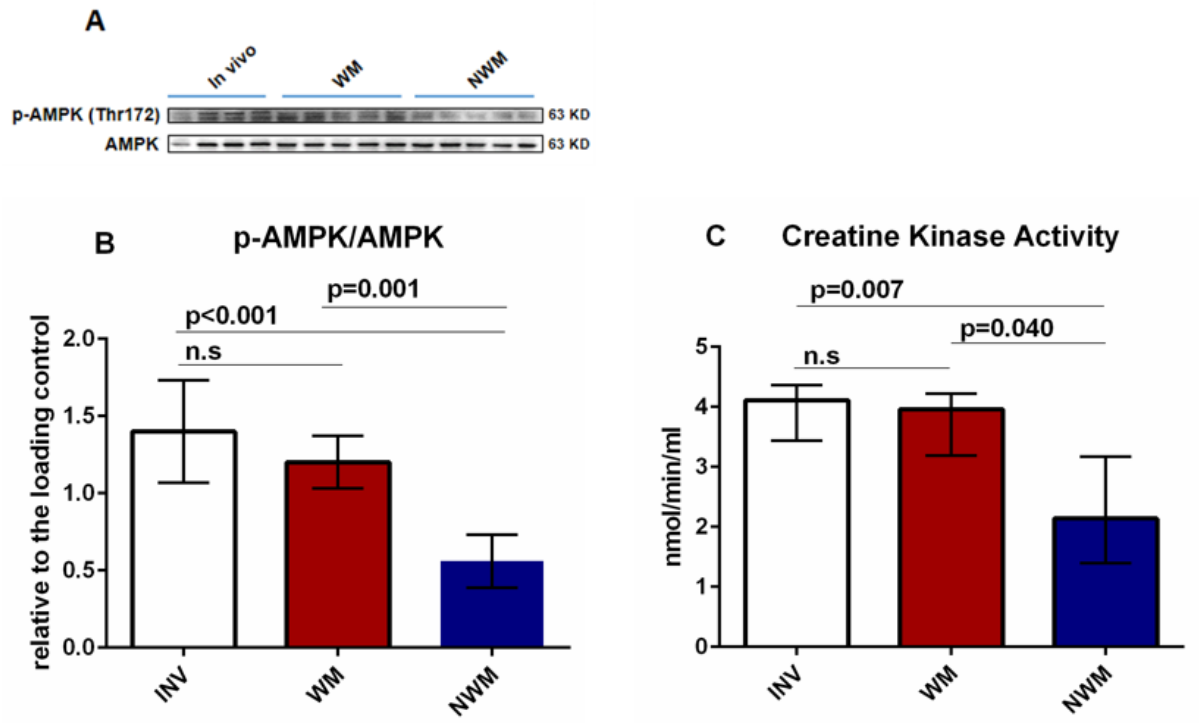


Figure 6.4. Markers of oxidative stress in the LV tissue.

A: Carbonylated proteins in left ventricular tissue of *ex-situ* perfused hearts compared to *in vivo* B: Immunoblotting of cell lysate prepared from LV samples, using the indicated antibodies. C: Malondialdehyde adducts in left ventricular tissue of *ex-situ* perfused hearts compared to *in vivo.* D: cysteine sulfonated proteins in left ventricular tissue of *ex-situ* perfused hearts compared to *in vivo*

INV, *in vivo*; n.s, no significant difference between the groups; MDA, malondialdehyde; NWM, non-working mode perfusion; WM, working mode perfusion

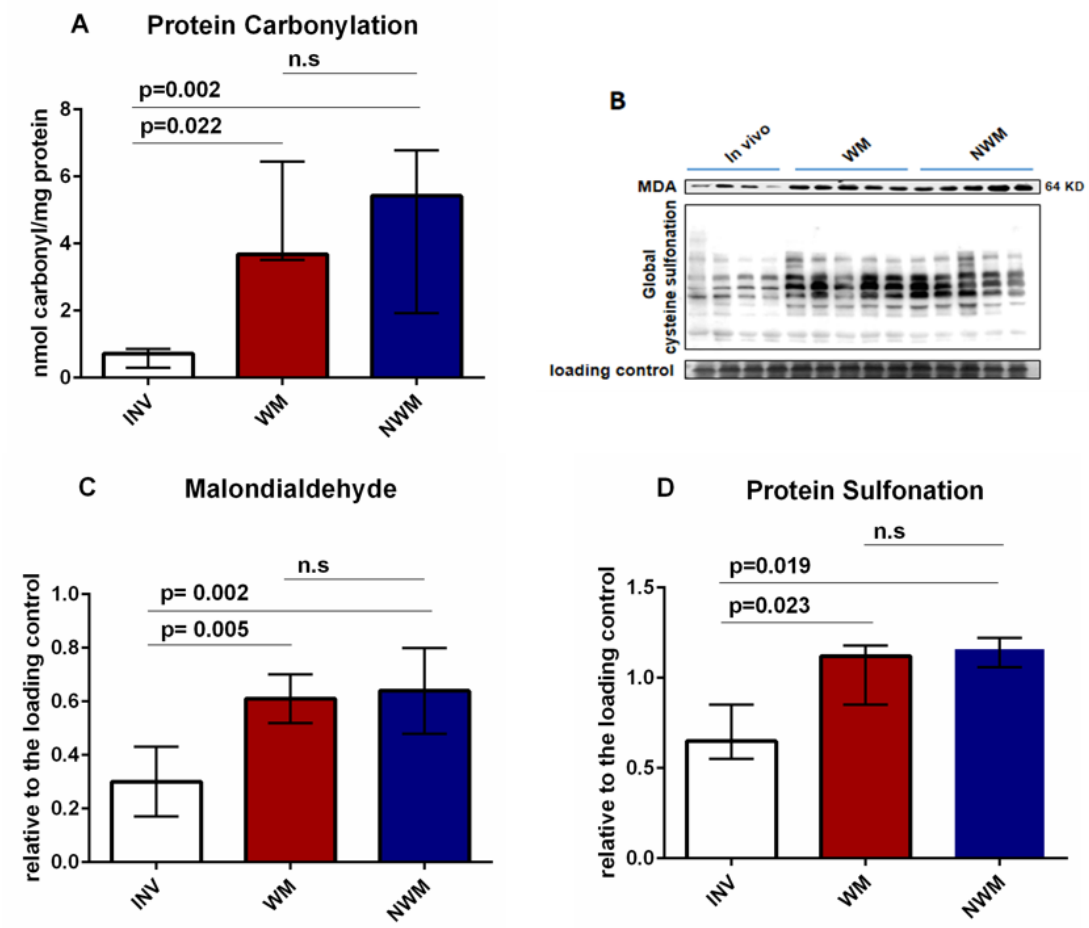


Figure 6.5. Glutathione content of the

LV tissue A: Total glutathione in left ventricular tissue of *ex-situ* perfused hearts compared to *in vivo*. B: Reduced glutathione in left ventricular tissue of *ex-situ* perfused hearts compared to *in vivo*.

INV, *in vivo*; n.s, no significant difference between the groups; NWM, non-working mode perfusion; WM, working mode perfusion

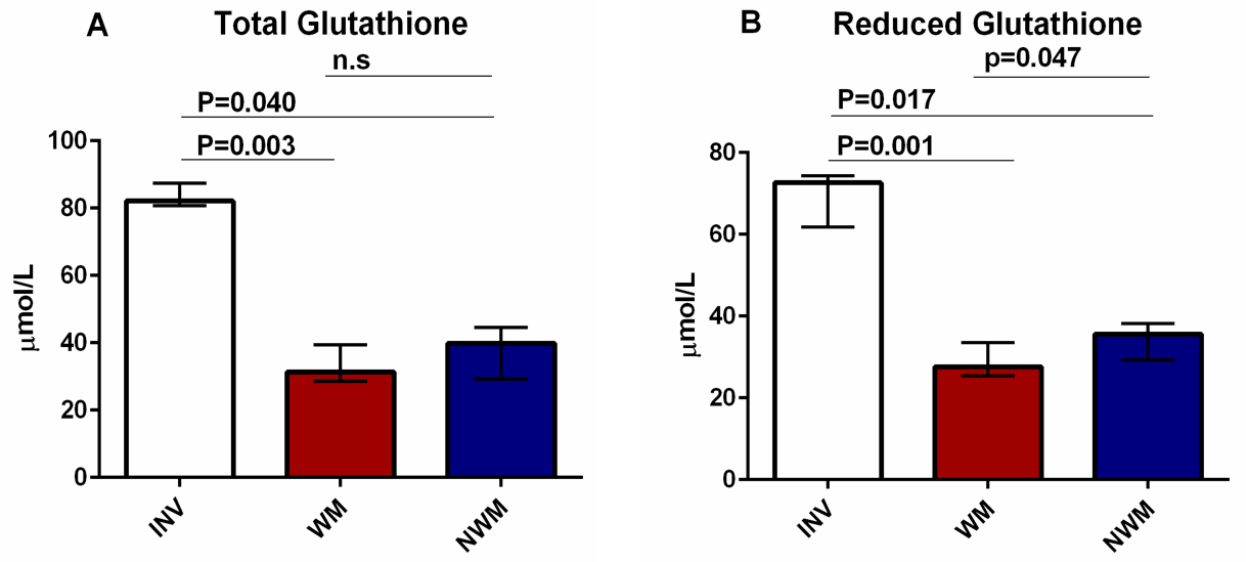


Figure 6.6. Induction of the anabolic pathways of glucose

A: Enzymatic activity of glucose 6-phosphate dehydrogenase in left ventricular tissue of *ex-situ* perfused hearts compared to *in vivo*. B: Immunoblotting of cell lysate prepared from LV samples, using O-linked N-acetylglucosamine primary antibody. C: O-linked N-acetylglucosamine- modified proteins in left ventricular tissue of *ex-situ* perfused hearts compared to *in vivo*.

G6PD, glucose 6-phosphate dehydrogenase; INV, *in vivo*; n.s, no significant difference between the groups; NWM, non-working mode perfusion; O-linkedGlcNAcylation, O-linked N-acetylglucosamine- modification; WM, working mode perfusion

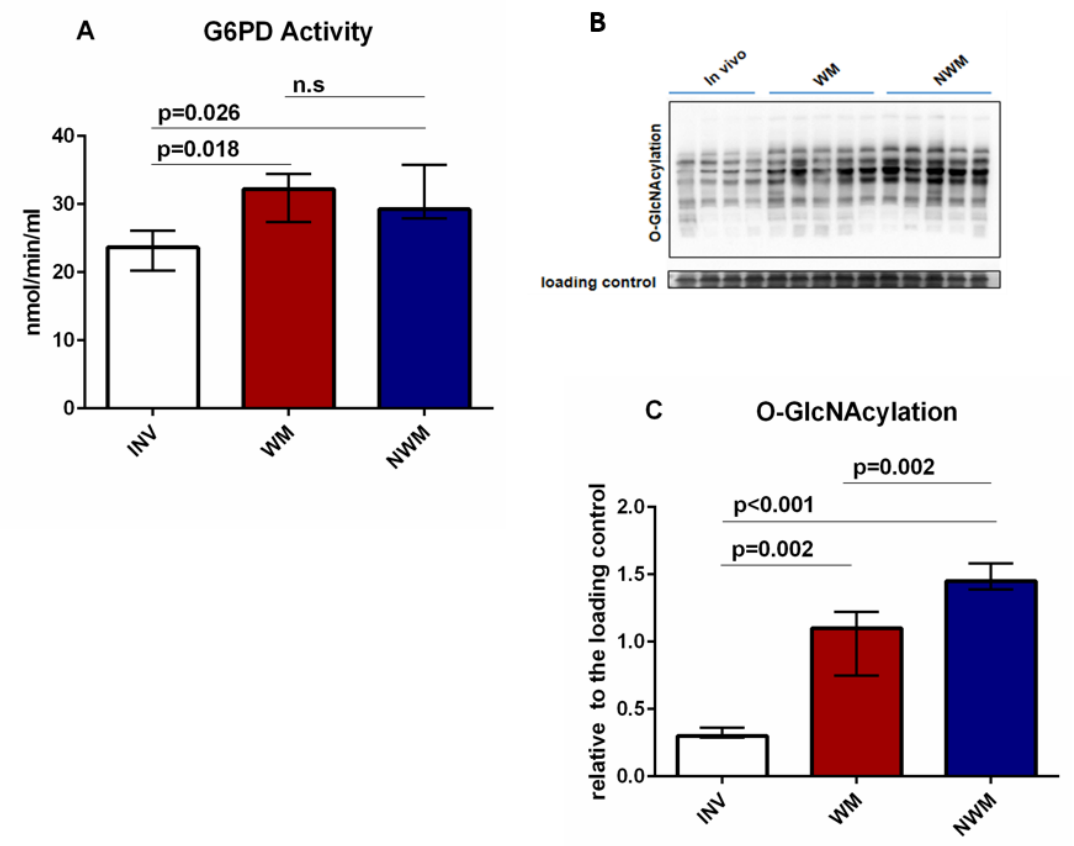


Figure 6.7. Activation of protein kinase B (Akt)

A: Immunoblotting of cell lysate prepared from LV samples, using primary antibodies against total, threonine 308, and serine 437 phosphorylated Akt. B: Threonine 308 phosphorylated Akt relative to total Akt in left ventricular tissue of *ex-situ* perfused hearts compared to *in vivo* C: Serine 437 phosphorylated Akt relative to total Akt in left ventricular tissue of *ex-situ* perfused hearts compared to *in vivo*

INV, *in vivo*; n.s, no significant difference between the groups; NWM, non-working mode perfusion; p-Akt, phosphorylated Akt; WM, working mode perfusion

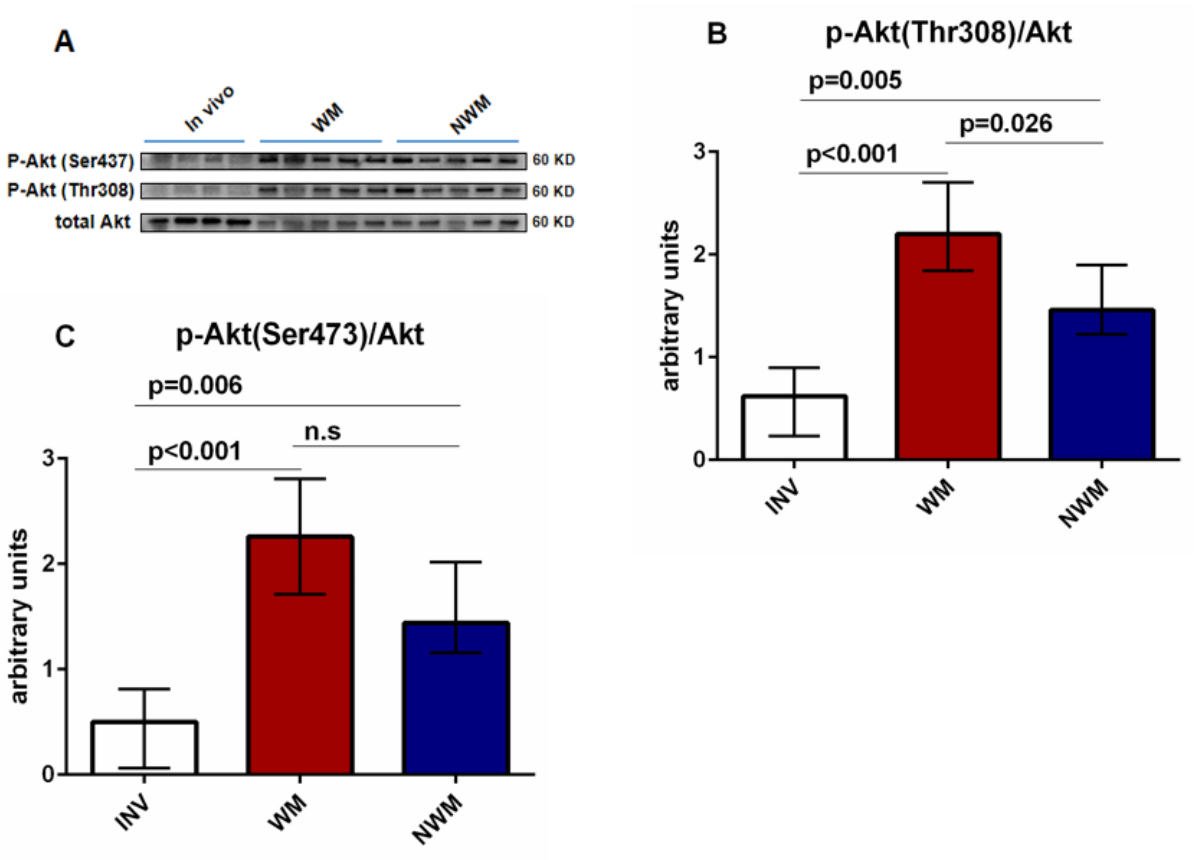


Figure 6.8. Perfusate markers of inflammation and oxidative stress

A: Perfusate concentrations of tumor necrosis factor-alpha during *ex-situ* heart perfusion. B: Perfusate concentrations of interleukin 8 during *ex-situ* heart perfusion. C: Perfusate concentrations of oxidized low density lipoproteins during *ex-situ* heart perfusion.

IL-8, interleukin 8; NWM, non-working mode perfusion; TNF-α, tumor necrosis factor-alpha ;T(x); x: hours after the start of the perfusion; WM, working mode perfusion; *, significant overtime change within group

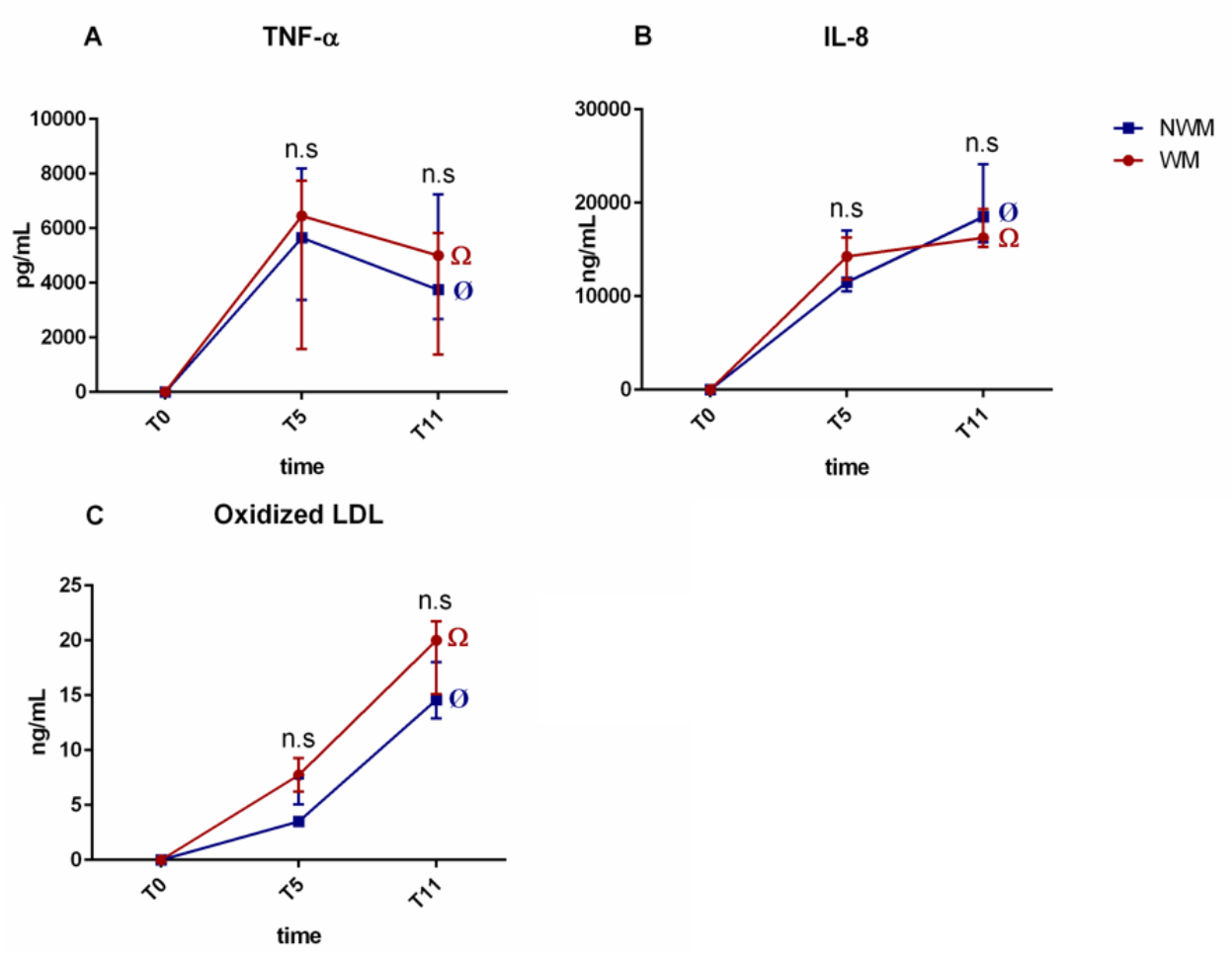


Figure 6.9. Myocardial tissue damage

A: Myocardial edema developed during *ex-situ* heart perfusion. B: Perfusate concentrations of cardiac troponin-I during *ex-situ* heart perfusion.

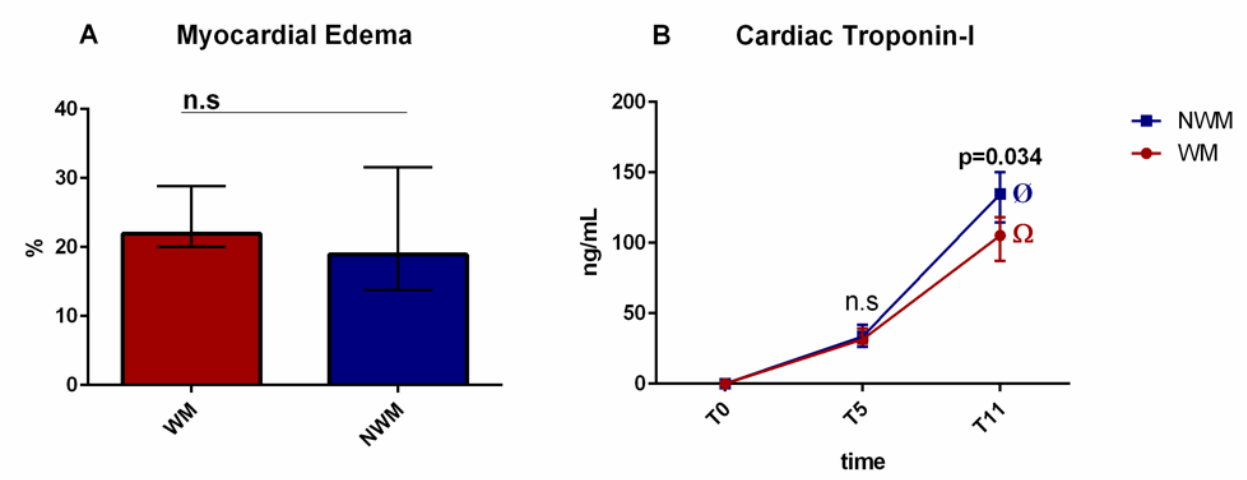


Figure 6.10. Markers of inflammation and oxidative stress between perfusion with whole blood-based perfusate, and leukocyte-depleted perfusate

A: Perfusate concentrations of interleukine 1β, tumor necrosis factor-alpha, and oxidized low density lipoprotein during *ex-situ* heart perfusion. B: Left ventricular malondialdeyde and protein sulfonation. C: Left ventricular pentose phosphate (glucose 6-phosphate enzyme) activity and hexosamine biosynthesis pathway (N-acetyl glucosamination) activity

G6PD, glucose 6-phosphate dehydrogenase enzyme activity; IL-1β, interleukin 1β; INV, in vivo; MDA, malondialdehyde; O-GlcNAc, N-acetyl glucoseamination; Ox-LDL, oxidized low density lipoprotein; TNF-α, tumor necrosis factor-alpha;T(x); x: hours after the start of the perfusion; WM, working mode perfusion with whole blood-based perfusate; WM-Lred, working mode perfusion with leukocyte-depleted perfusate

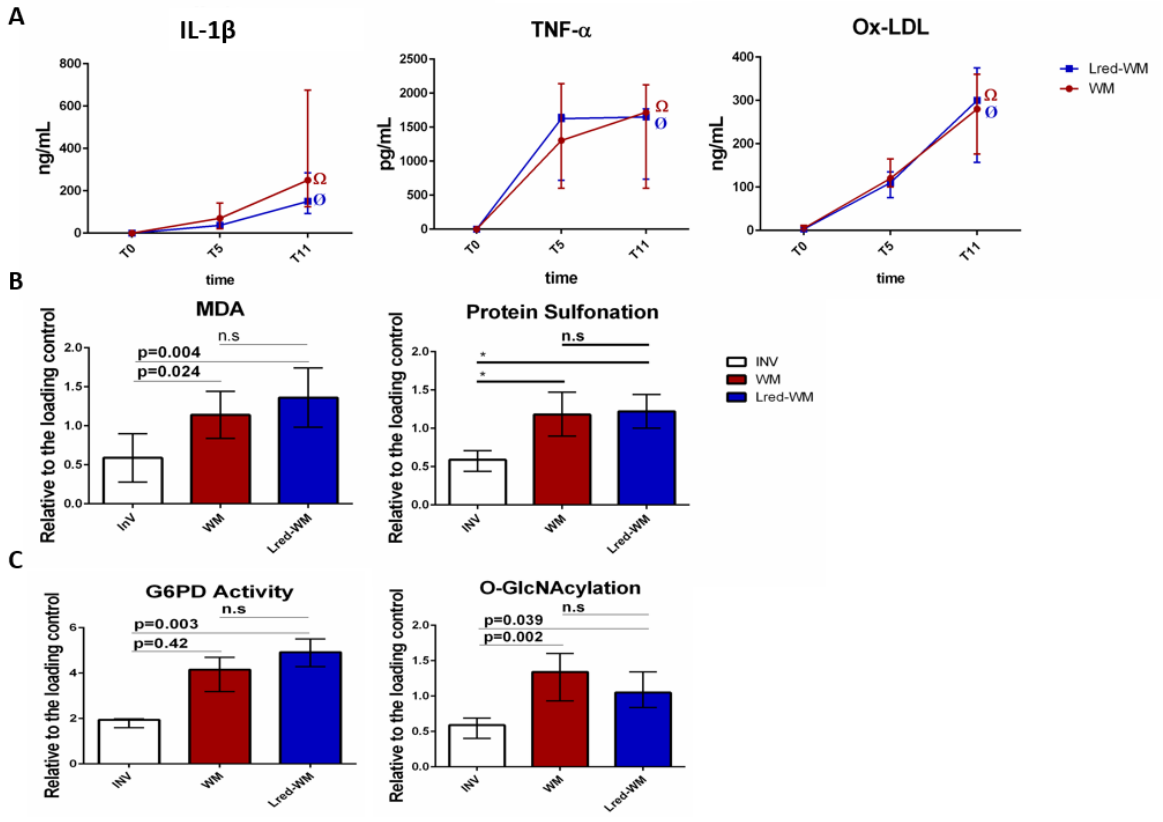
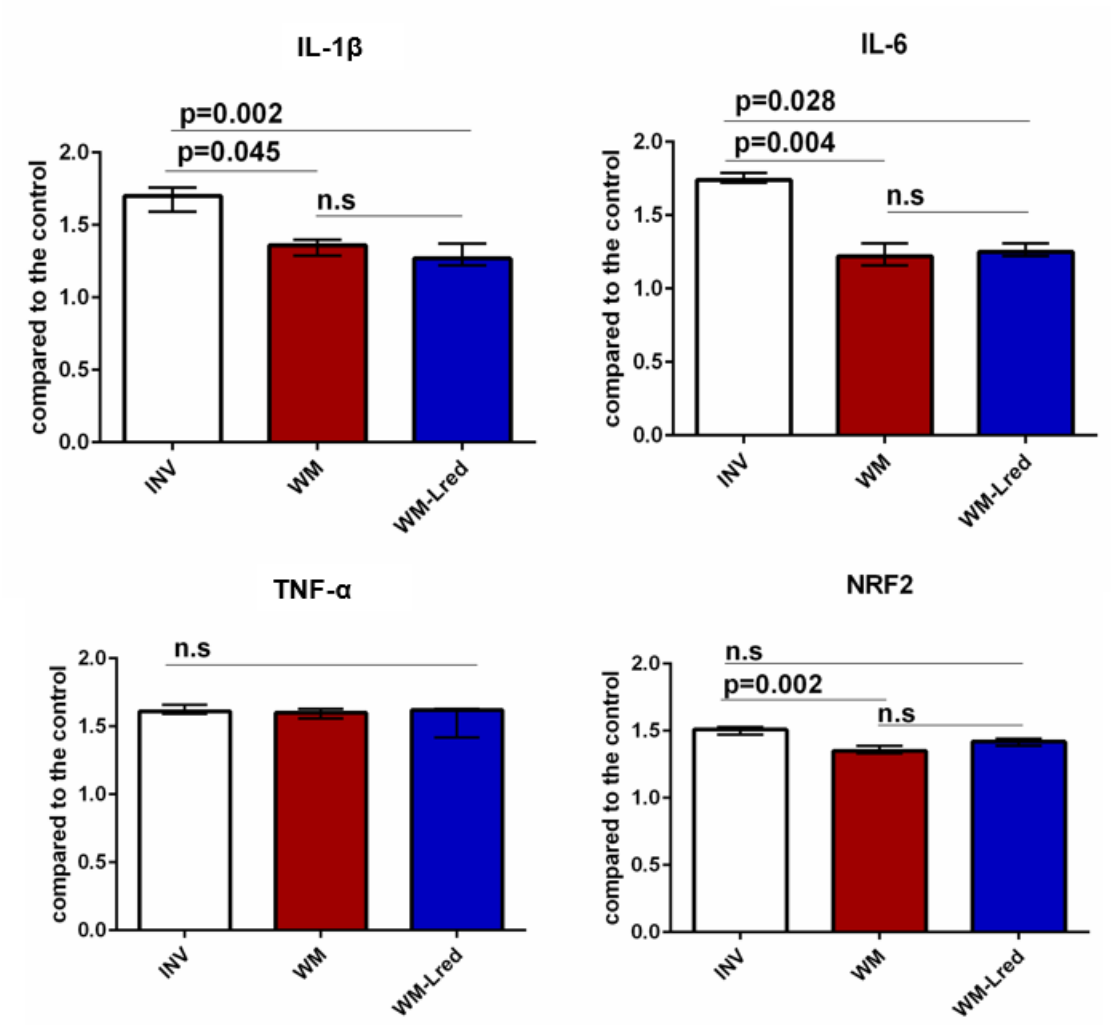


Figure 6.11. Expression of pro-inflammatory cytokines genes and oxidative stress-responsive element NRF2 in myocardial tissue of the hearts perfused with whole blood-based perfusate, and leukocyte-depleted perfusate



IL-1β, interleukin 1β; IL-6, interleukin 6; NRF2, nuclear factor erythroid 2-related factor 2; TNF-α, tumor necrosis factor-alpha ; WM, working mode perfusion with whole blood-based perfusate; WM-Lred, WM perfusion with leukocyte-depleted perfusate

Chapter 7: Summary and future directions

7.1. Preservation methods for the donor heart

Despite the considerable progress made in the medical treatment of heart failure, and mechanical circulatory assist devices, heart transplantation remains the gold standard treatment for end-stage refractory heart conditions [1]. However, the number of patients with heart failure has been increasing [2], reflected to a higher number of patients on the waiting list for a heart transplant, creating a shortage for heart transplants. The shortage of the donor heart has rendered heart transplantation a less reliable treatment modality, despite being the gold standard of the treatment [3].

Since the first human heart transplantation in 1967 by Christian Barnard, the whole process has evolved significantly, leading to excellent outcomes. However, the standard of care for organ preservation remains to be cold static storage (CSS), [1]. Cold storage, although is a very simple and quite inexpensive method of preservation, it comes with significant limitations as well. Cold storage, exposes the heart to a period of cold ischemia which cuts the safe preservation times to a maximum of 4-6 hours in an ideal donor heart. It has also been demonstrated that prolonged cold ischemia is associated with an increased risk of primary graft dysfunction and failure which is the most frequent cause of death during the first 30 days after heart transplantation [4-6]. Meanwhile, due to the static condition of organ in CSS, assessment of the function, metabolism and in general viability of the donated heart would not be possible [1].

Considering the current shortage of donor hearts, the attentions are inclined toward expanding the pool for available donor hearts by including the marginal, sub-optimal

donor hearts. Such donor hearts may include the heart from non-ideal donors, or from the donors with circulatory criteria-determined death (DCD) [1, 3]. These extended-criteria donor hearts are already vulnerable/damaged, due to the donor characteristics (e.g. older age, smoking, diseases), or DCD. Thus, CSS would not be suitable for preservation of these hearts because 1) the cold ischemia endured during CSS will damage the already-sub-optimal hearts, and 2) it will not provide a platform to assess the eligibility of the hearts which have endured a damage/insult in peri-procurement period (DCD hearts) [3, 6].

Ex-situ heart perfusion (ESHP) is an emerging technology, which provides a platform for extracorporeal preservation of the donor heart, while it is being perfused adequately, in a dynamic, beating state [7]. Thus, not only it spares the donor heart from the CSS-related ischemia, but also the viability of the donor heart can be evaluated in this platform, and may potentially improve the condition of the donor heart with therapeutic interventions during the *ex-situ* perfusion [1, 8].

The ESHP method of preservation, has been described in both hypothermic and normothermic setting. Interestingly, hypothermic ESHP has bee applied during the early years of heart transplantation for organ preservation [9, 10] but soon it was discarded by the introduction of SCS method in combination with cardioplegic arrest, which was a simpler and less expensive method and was considered adequate for preservation of the donor heart at the time. However, soon it became obvious that CSS is a less-than-optimal method of preservation [11]. Hypothermic *ex-situ* perfusion has been performed in many different setting regarding the devices and perfusate compositions so far [5, 12-15], but regardless, The necessary setting for hypothermic ESHP is less sophisticated and cost-

demanding when compared to normothermic ESHP [1]. The technology of hypothermic ESHP, has shown promising results including the successful first human heart transplant using Steen's perfusion system in Sweden [16]. It is also possible to perform some viability and functional assessment during preservation time using methods such as diffusion tensor magnetic resonance imaging, myocardial pH tracing, or by placing an inflatable flexible balloon inside LV to measure its contractility [6, 15]. However, this technology has not been applied widely due to the concerns about diastolic dysfunction that may occur as a result of significant myocardial edema formation during hypothermic ESHP, and the impossibility of a reliable functional assessment with more widely and universally used myocardial function parameters, and less invasive methods under hypothermic conditions [3, 16, 17].

Normothermic ESHP, on the other hand, is a better replication of the *in vivo* conditions, and both function and metabolism of the heart may be assessed in this setting prior to transplantation [8, 18]. Yet, due to the high metabolic demands of the heart in semi-physiological conditions, and its general sensitivity against stresses such as ischemia, management of the heart in a normothermic ESHP may be more challenging [1]. Regardless, there has been a strong body of evidence suggesting that normothermic ESHP is a superior method for preservation of the donor hearts, facilitating transplantation of the hearts from extended-criteria donors, contributing to the donor pool size [7, 19, 20].

7.2. Functional decline of the heart during ESHP

Despite the promising potentials of ESHP, experimental studies including studies performed by our team, as well as clinical case reports [20, 21] have shown that myocardial function declines significantly in a time–dependent fashion during ESHP [21].

Similarly, in the present project series, we observed that myocardial function deteriorated gradually during *ex-situ* perfusion. There has been a considerable lack of evidence regarding the underlying mechanisms responsible for this phenomenon. Part of this lack of evidence is related to the fact that the1) ESHP, while employed for many years as a platform to study the heart [22], the concept of using this setting as a method for preservation of the donated heart is fairly new, and 2) currently, the clinical device for ESHP does not offer a platform for functional assessment of the heart [1]. These observations raise reg flags for efficiency of the currnt clinical protocols for assessment and protection of the heart in the setting of normothermic ESHP. In this thesis, we explored some of the potential mechanisms for this phenomenon.

Among the most commonly-identified etiologies of cardiac dysfunction and failure are cell death and metabolic inefficiency. In this thesis, we observed a negligible increase in the rate of cell death in *ex-situ* perfused hearts (12 hours), compared to *in vivo* samples. Thus, the development of myocardial dysfunction during ESHP could have a metabolic origin. The heart is a highly energy-demanding organ, thus efficient support of the energy-yielding metabolites in essential during normothermic ESHP. We demonstrated that the concentration of the free fatty acids (FFAs), as the main source of energy in cardiomyocytes, is rapidly depleted in the whole blood-based perfusate [23].

Current ESHP protocols rely mainly on exogenous glucose as an energy substrate [7]; however, experimental data suggest that glucose may not participate efficiently in energy-yielding catabolism during ESHP [24, 25]. Here, we also demonstrated that alongside the decreasing myocardial oxygen consumption overtime in ESHP, the activity of some of the key enzymes of the glycolysis pathway (pyruvate kinase, PK) also decreases. This was

accompanied by an alteration in expression of the isoforms of the PK, in a similar way with cancer cells adopting to the oxidative environment with altering energy-yielding metabolism of glucose [23, 26]. We demonstrated that catabolism of glucose, is diverted to anabolic pathways, pentose phosphate pathway (PPP), and hexosamine biosynthesis pathway (HBP) [26]. These observations strongly suggested that metabolic alteration/inefficiency plays an important role in the decline of cardiac function during ESHP.

7.3. Extracorporeal perfusion-related alteration and their effect of the heart during *ex-situ* perfusion, a neglected topic

Extracorporeal mechanical circulation systems are routinely utilized to support the body circulation during cardiac surgery (cardiopulmonary bypass, CPB), and to support blood ventilation in patients with critical heart or lung conditions (extracorporeal membrane oxygenation, ECMO). The mechanical circulation systems are associated with induction of systemic inflammatory response (SIRS) and systemic oxidative stress in exposed patients, negatively affecting the outcomes [27, 28]. Ischemia reperfusion injury (IRI), sequestration of natural antioxidants and the microelements necessary for their activity by the circuit components, and activation of the immune system and contact system due to contact of the blood elements with artificial, non-endothelialized surfaces of the circuit, and hemolysis and release of free heme into the circulation are among the most important underlying factors [27-29]. Oxidative stress refers to the loss of the balance between the production of reactive oxygen and nitrogen species (RONS), and antioxidant defences. It causes damage to the cell structures/organelles, mitochondrial dysfunction, disrupts energy-yielding metabolism, and results in myocardial cell dysfunction [30, 31] and may

occur to be an ongoing, persistent phenomenon occurring during the extracorporeal circulation, overwhelming some other important etiologies such as IRI [32]. In this thesis, we showed that various markers of inflammation and oxidative stress are induced in perfusate and myocardial tissue of pig hearts during a 12-hour period of normothermic ESHP. This observation was accompanied with the induction of oxidative stress-responsive elements/ pathways including protein kinase B (Akt), PPP and HBP. Moreover, we detected a significant reduction in myocardial tissue content of glutathione know as the major antioxidant of the cell. Our observations strongly suggest that similar to widely used clinical extracorporeal life support systems, inflammation and oxidative stress develop significantly during normothermic ESHP regardless of the ventricular workload, and thus may have a significant contribution to functional decline and other challenging issues observed during ESHP such as the formation of edema.

7.4. Edema in *ex-situ* perfused heart

Edema, has been demonstrated to be a significant limiting factor in hypothermic ESHP. However, it has been also frequently reported in normothermic ESHP studies. We also observed a significant edema formed in the ex-situ-perfused hearts in all of the projects in this thesis. The edema formed during ESHP was similar between different perfusion modalities.

Although edema formation has been significantly lower with blood-based perfusates, it still raises the concerns as edema can impair diastolic function/relaxation, and may also lead to capillary collapse and impairment of myocardial perfusion during normothermic ESHP [17, 33]. Interestingly edema formation has been frequently reported, even with the perfusates holding adequate colloid properties (blood and/or colloid-added crystalloids)

[23]. Induction of inflammatory responses and oxidative stress triggered by exposure to the extracorporeal circuit may be an important reason for edema formation, especially with our observation of increasing perfusate oxidized low-density lipoprotein (ox-LDL) produced by the vascular wall during normothermic ESHP. The experimental observations suggest that in the hearts perfused with blood-based perfusates, only approximately 10% of the functional decline is attributable to edema formation [17]. Yet it may not only be a cause for functional decline, but may point to stress and stimuli severely affecting vascular health and their barrier function.

7.5. Efficiency of the *ex-situ* heart perfusion devices

Over time, there has been a considerable heterogeneity in the design of the devices for ESHP. Yet, in the majority of experimental ESHP studies, the heart has been suspended from the aorta [34]. This is a position considerably different from the *in-vivo* position in the mediastinum wherein the myocardial mass is supported by the pericardium and the diaphragm which balance the hydrostatic, inertial, and gravitational forces, prevent excessive dilatation and wall stress, and modulate the weight borne by the heart [35]. Similarly in the clinical ESHP, the heart is also perfused in a semi-suspended state [7, 17].

Here we showed that ESHP in a supported, semi-anatomical position improved the functional preservation and recovery of the heart, and attenuated cardiac troponin I (cTn-I) leak during an extended period of normothermic perfusion and. This experience emphasizes the importance of imitation of near-anatomical and physiological state for the heart during ESHP.

Experimentally, both empty-beating non-working mode (NWM), and semi-physiologic working mode (WM) has been used for perfusion of the heart with the latter also providing the opportunity to assess cardiac function. The current device for clinical normothermic ESHP (Transmedics Organ Care System device, OCS) only performs the perfusion in NWM, and relies mainly on the metabolic findings to predict eligibility of the donor heart for a safe transplantation. However, the adequacy of metabolic findings (mainly perfusate lactate values) for predicting the functional status of the heart has been challenged several times by our group and others [1]. According to our observations in previous works by our group and in this thesis, not only the perfusate lactate does not show a strong correlation with the functional status of the heart during ESHP, but also the functional quality of the heart changes gradually during the perfusion [8, 23]. These observations strongly advocated the necessity for evaluation of cardiac function during ESHP rather than relying only on the metabolic findings to increase the odds of a safe cardiac transplantation.

In this thesis, we also introduced a custom-built ESHP apparatus and operational software designed in our lab by Dr. D.Freed, which is capable of perfusing the heart in both NWM and WM, with minimal need for supervision/operator. In this device, the desired left atrial (LA) pressures may be ordered to the software, and it will adjust the pumps to achieve and maintain the desired LA pressures to be able to perfuse the hearts in different workloads. Moreover, the software is able to calculate and archive the functional parameters through the information collected from the flow and pressure sensors in the circuit [18]. Taken together, optimization of the clinical ESHP protocol and device to perfuse the heart in a semi-physiological load, and semi-anatomical position

may be a critical step for improving the cardioprotection and functional recovery of the *ex-situ* perfused hearts.

7.6. Left ventricular workload during ex-situ perfusion, a cardioprotective factor

During ESHP, the cardiac function declines gradually regardless of the left ventricular workloads. However, in the previous works by our group and in the projects related to this thesis, we have always observed that the hearts perfused in the semi-physiologic workloads (WM) for the majority of the perfusion period, preserved their functional status significantly better compared to the heats perfused in empty-beating NWM. Also, the WM-perfused hearts released significantly lower amounts of cTn-I during an extended 12-hour ESHP. In this thesis, we demonstrated that while the myocardial tissue phosphorylated (active) adenosine monophosphate kinase (p-AMPK) was significantly lower in the NWM-perfused hearts, the representative values were similar between WM-perfused hearts and *in vivo* healthy control hearts. Similarly, the enzymatic activity of the creatine kinase (CK), was significantly lower in NWM compared to *in vivo* and WM. Both AMPK and CK activity status play an important role in maintaining the metabolic reserve of the myocardium, and AMPK activity has protective effects against conditions such and oxidative stress. The activation of AMPK and CK are among the important mediators of the cardioprotection induced by the ventricular wall stretch and contraction [36-38]. Thus, the semi-physiologic ventricular wall stretch and heart chamber pressures during ESHP in WM hearts, may lead to preservation of AMPK and CK activity, and exert cardioprotective effects during *ex-situ* perfusion [39]. Thus the addition of the WM perfusion modality to the clinical devices for ESHP, not only improves assessment of the heart, but may exert cardioprotective effects during *ex-situ* perfusion as well.

7.7. Future directions for improving ESHP protocols

With the results reported in this thesis, it can be concluded that studies to detect an optimized metabolic support for the heart, and also detection of the main sources of the inflammatory cytokines and reactive oxygen and nitrogen in the setting of ESHP seem to be inevitable. Since there has been a great heterogeneity in the studies evaluating the effects of anti-inflammatory and antioxidative agents in the setting of ECC (including study designs and type of the agent used), efficiently targeting the inflammation and oxidative stress with reliable agents during ESHP requires more studies. The anti-inflammatory and antioxidative therapies that may have cardioprotective effect during ESHP may include addition of glutathione, different vitamins and minerals necessary for the activity of anti-inflammatory/antioxidative systems to the perfusate.

Improving the biomaterial used in the circuit that would cause less hemolysis, less deposition of perfusate content on the circuit surfaces, and lower activation effect on the contact and immune systems would not only benefit cardioprotection during ESHP, but will also improve the reliability of the cardiac parameters of function and metabolism gathered during ESHP.

7.8. Future perspectives of ESHP

With the improving protocols for ESHP and feasibility of longer safe preservation times for the donor heart with this technology, a geographically distant heart donation may be possible using a portable ESHP apparatus that will provide the opportunity of a heart transplantation from a potential donor with a significant geographical distance from a potential recipient. Such an apparatus, would definitely improve with the addition of

portable imaging assessment systems such as portable echocardiography/angiography device synchronized with the operational software [18].

Potentially, the setting of ESHP may provide a platform to pursue the treatment modalities that may not be easily possible in an organ-specific manner *in vivo*, including gene therapy, or administration of therapeutic nanoparticles [40], rejection-mitigating interventions using small interfering RNA [41]. In summary, ESHP technique may offer a unique platform for reconditioning and treatment of the injured hearts, or to improve the statistics and outcomes of heart transplantation, however, studies are warranted to improve the current protocols of ESHP.

7.9. References

1. Hatami S, Freed DH: **Machine perfusion of donor heart: state of the art**. *Current Transplantation Reports* 2019, **6**(3):242-250.
2. Savarese G, Lund LH: **Global public health burden of heart failure**. *Cardiac failure review* 2017, **3**(1):7-11.
3. White CW, Messer SJ, Large SR, Conway J, Kim DH, Kutsogiannis DJ, Nagendran J, Freed DH: **Transplantation of hearts donated after circulatory death**. *Frontiers in cardiovascular medicine* 2018, **5**:8.
4. Monteagudo Vela M, Garcia Saez D, Simon AR: **Current approaches in retrieval and heart preservation**. *Annals of cardiothoracic surgery* 2018, **7**(1):67-74.
5. Choong JW, Ou R, Lim YW, Rosenfeldt FL: **Cold crystalloid perfusion provides cardiac preservation superior to cold storage for donation after circulatory death**. *Transplantation* 2016, **100**(3):546-553.
6. 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**. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* 2008, **34**(2):318-325.
7. Ardehali A, Esmailian F, Deng M, Soltesz E, Hsich E, Naka Y, Mancini D, Camacho M, Zucker M, Leprince P *et al*: **Ex-vivo perfusion of donor hearts for human heart transplantation (PROCEED II): a prospective, open-label, multicentre, randomised non-inferiority trial**. *Lancet (London, England)* 2015, **385**(9987):2577-2584.
8. White CW, Ambrose E, Muller A, Li Y, Le H, Hiebert B, Arora R, Lee TW, Dixon I, Tian G *et al*: **Assessment of donor heart viability during ex vivo heart perfusion**. *Canadian journal of physiology and pharmacology* 2015, **93**(10):893-901.
9. Barnard CN: **The operation. A human cardiac transplant: an interim report of a successful operation performed at Groote Schuur Hospital, Cape Town**. *South African medical journal = Suid-Afrikaanse tydskrif vir geneeskunde* 1967, **41**(48):1271-1274.
10. Wicomb WN, Cooper DK, Novitzky D, Barnard CN: **Cardiac transplantation following storage of the donor heart by a portable hypothermic perfusion system**. *The Annals of thoracic surgery* 1984, **37**(3):243-248.
11. Minasian SM, Galagudza MM, Dmitriev YV, Karpov AA, Vlasov TD: **Preservation of the donor heart: from basic science to clinical studies**. *Interactive cardiovascular and thoracic surgery* 2015, **20**(4):510-519.
12. Michel SG, La Muraglia GM, 2nd, Madariaga ML, Titus JS, Selig MK, Farkash EA, Allan JS, Anderson LM, Madsen JC: **Twelve-hour hypothermic machine perfusion for donor heart preservation leads to improved ultrastructural characteristics compared to conventional cold storage**. *Annals of transplantation : quarterly of the Polish Transplantation Society* 2015, **20**:461-468.
13. Rosenfeldt F, Ou R, Salamonsen R, Marasco S, Zimmet A, Byrne J, Cosic F, Saxena P, Esmore D: **A novel combination technique of cold crystalloid perfusion but not cold storage facilitates transplantation of canine hearts donated after circulatory death**. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* 2016, **35**(11):1358-1364.
14. Tolboom H, Olejnickova V, Reser D, Rosser B, Wilhelm MJ, Gassmann M, Bogdanova A, Falk V: **Moderate hypothermia during ex vivo machine perfusion promotes recovery of hearts**

donated after cardiocirculatory deathdagger. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* 2016, **49**(1):25-31.
15. Van Caenegem O, Beauloye C, Bertrand L, Horman S, Lepropre S, Sparavier G, Vercruysse J, Bethuyne N, Poncelet AJ, Gianello P *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 : official journal of the European Association for Cardio-thoracic Surgery* 2016, **49**(5):1348-1353.
16. Van Raemdonck D, Rega F, Rex S, Neyrinck A: **Machine perfusion of thoracic organs**. *Journal of thoracic disease* 2018, **10**(Suppl 8):S910-s923.
17. Messer S, Ardehali A, Tsui S: **Normothermic donor heart perfusion: current clinical experience and the future**. *Transplant international : official journal of the European Society for Organ Transplantation* 2015, **28**(6):634-642.
18. Hatami S, White CW, Ondrus M, Qi X, Buchko M, Himmat S, Lin L, Cameron K, Nobes D, Chung HJ *et al*: **Normothermic ex situ heart perfusion in working mode: assessment of cardiac function and metabolism**. *Journal of visualized experiments : JoVE* 2019(143).
19. Iyer A, Gao L, Doyle A, Rao P, Cropper JR, Soto C, Dinale A, Kumarasinghe G, Jabbour A, Hicks M *et al*: **Normothermic ex vivo perfusion provides superior organ preservation and enables viability assessment of hearts from DCD donors**. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2015, **15**(2):371-380.
20. Messer S, Page A, Colah S, Axell R, Parizkova B, Tsui S, Large S: **Human heart transplantation from donation after circulatory-determined death donors using normothermic regional perfusion and cold storage**. *The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation* 2018, **37**(7):865-869.
21. Sandha JK, White CW, Muller A, Avery E, Thliveris J, Dixon IMC, Arora RC, Tian G, Hryshko LV, Nagendran J *et al*: **Steroids limit myocardial edema during ex vivo perfusion of hearts donated after circulatory death**. *The Annals of thoracic surgery* 2018, **105**(6):1763-1770.
22. Olejnickova V, Novakova M, Provaznik I: **Isolated heart models: cardiovascular system studies and technological advances**. *Medical & biological engineering & computing* 2015, **53**(7):669-678.
23. Hatami S, White C, Shan S, Haromy A, Qi X, Ondrus M, Kinnear A, Himmat S, Michelakis E, Nagendran J *et al*: **Myocardial functional decline during prolonged ex situ heart perfusion**. *The Annals of thoracic surgery* 2019, **108**:499-507.
24. Cobert ML, Peltz M, West LM, Merritt ME, Jessen ME: **Glucose is an ineffective substrate for preservation of machine perfused donor hearts**. *The Journal of surgical research* 2012, **173**(2):198-205.
25. Peltz M, He TT, Adams GAt, Koshy S, Burgess SC, Chao RY, Meyer DM, Jessen ME: **Perfusion preservation maintains myocardial ATP levels and reduces apoptosis in an ex vivo rat heart transplantation model**. *Surgery* 2005, **138**(4):795-805.
26. Hatami S, White C, Qi X, Buchko M, Ondrus M, Kinnear A, Himmat S, Sergi C, Nagendran J, Freed D: **Immunity and stress responses are induced during ex-situ heart perfusion**. *Circ Heart Fail* 2020, **13**(6):e006552.
27. McDonald CI, Fraser JF, Coombes JS, Fung YL: **Oxidative stress during extracorporeal circulation**. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* 2014, **46**(6):937-943.
28. Millar JE, Fanning JP, McDonald CI, McAuley DF, Fraser JF: **The inflammatory response to extracorporeal membrane oxygenation (ECMO): a review of the pathophysiology**. *Critical care (London, England)* 2016, **20**(1):387.

29. Chen Q, Yu W, Shi J, Shen J, Hu Y, Gong J, Li J, Li N: **The effect of extracorporeal membrane oxygenation therapy on systemic oxidative stress injury in a porcine model**. *Artificial organs* 2014, **38**(5):426-431.
30. Neri M, Riezzo I, Pomara C, Schiavone S, Turillazzi E: **Oxidative-Nitrosative Stress and Myocardial Dysfunctions in Sepsis: Evidence from the Literature and Postmortem Observations**. *Mediators of inflammation* 2016, **2016**:3423450.
31. Ungvari Z, Gupte SA, Recchia FA, Batkai S, Pacher P: **Role of oxidative-nitrosative stress and downstream pathways in various forms of cardiomyopathy and heart failure**. *Current vascular pharmacology* 2005, **3**(3):221-229.
32. Clermont G, Vergely C, Jazayeri S, Lahet JJ, Goudeau JJ, Lecour S, David M, Rochette L, Girard C: **Systemic free radical activation is a major event involved in myocardial oxidative stress related to cardiopulmonary bypass**. *Anesthesiology* 2002, **96**(1):80-87.
33. Jahania MS, Sanchez JA, Narayan P, Lasley RD, Mentzer RM, Jr.: **Heart preservation for transplantation: principles and strategies**. *The Annals of thoracic surgery* 1999, **68**(5):1983-1987.
34. Hatami S, Qi X, White CW, Buchko M, Bozsu S, Himmat S, Nagendran J, Freed DH: **Preservation and recovery of myocardial function during normothermic ex situ heart perfusion: does the position of the heart during perfusion matter?** *The Journal of Heart and Lung Transplantation* 2020, **39**(4):S145-S146.
35. Rehman I, Rehman A: **Anatomy, Thorax, Pericardium**. Treasure Island (FL): StatPearls Publishing StatPearls Publishing LLC.; 2019.
36. Hao J, Kim HS, Choi W, Ha TS, Ahn HY, Kim CH: **Mechanical stretch-induced protection against myocardial ischemia-reperfusion injury involves AMP-activated protein kinase**. *The Korean journal of physiology & pharmacology : official journal of the Korean Physiological Society and the Korean Society of Pharmacology* 2010, **14**(1):1-9.
37. Chanda D, Luiken JJ, Glatz JF: **Signaling pathways involved in cardiac energy metabolism**. *FEBS letters* 2016, **590**(15):2364-2374.
38. Bittl JA, Ingwall JS: **The energetics of myocardial stretch. Creatine kinase flux and oxygen consumption in the noncontracting rat heart**. *Circulation research* 1986, **58**(3):378-383.
39. Hatami S, Qi X, White C, Buchko M, Bozso S, Himmat S, Nagendran J, Freed D: **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.
40. Bishawi M, Roan J-N, Milano CA, Daneshmand MA, Schroder JN, Chiang Y, Lee FH, Brown ZD, Nevo A, Watson MJ *et al*: **A normothermic ex vivo organ perfusion delivery method for cardiac transplantation gene therapy**. *Scientific reports* 2019, **9**(1):8029.
41. Cui J, Qin L, Zhang J, Abrahimi P, Li H, Li G, Tietjen GT, Tellides G, Pober JS, Mark Saltzman W: **Ex vivo pretreatment of human vessels with siRNA nanoparticles provides protein silencing in endothelial cells**. *Nature Communications* 2017, **8**(1):191.

Bibiography

Abeyrathna, P., & Su, Y. (2015). The critical role of Akt in cardiovascular function. *Vascul Pharmacol, 74*, 38-48. doi: 10.1016/j.vph.2015.05.008

Aboelnazar, N. S., Himmat, S., Hatami, S., White, C. W., Burhani, M. S., Dromparis, P., . . . Nagendran, J. (2018). Negative pressure ventilation decreases inflammation and lung edema during normothermic ex-vivo lung perfusion. *J Heart Lung Transplant, 37*(4), 520-530. doi: 10.1016/j.healun.2017.09.007

Abraham, W. T., & Smith, S. A. (2013). Devices in the management of advanced, chronic heart failure. *Nat Rev Cardiol, 10*(2), 98-110.

Al-Fares, A., Pettenuzzo, T., & Del Sorbo, L. (2019). Extracorporeal life support and systemic inflammation. *7*(Suppl 1), 46. doi: 10.1186/s40635-019-0249-y

Alexiou, C., Sheppard, S., Tang, A., Rengarajan, A., Smith, D., Haw, M., & Gibbs, R. (2006). Leukocytes-depleting filters preferentially remove activated leukocytes and reduce the expression of surface adhesion molecules during the simulated extracorporeal circulation of human blood. *Asaio j, 52*(4), 438-444. doi: 10.1097/01.mat.0000225894.61493.2f

Ali- Hasan- Al- Saegh, S., Mirhosseini, S. J., Tahernejad, M., Mahdavi, P., Shahidzadeh, A., Karimi-Bondarabadi, A. A., . . . Sabashnikov, A. (2016). Impact of antioxidant supplementations on cardio-renal protection in cardiac surgery: an updated and comprehensive meta-analysis and systematic review. *Cardiovasc Ther, 34*(5), 360-370. doi: 10.1111/1755-5922.12207

Alvarez-Ayuso, L., Gómez-Heras, S. G., Jorge, E., Guardiola, J. M., Torralba, A., Granado, F., . . . García-Poblete, E. (2010). Vitamin E action on oxidative state, endothelial function and morphology in long-term myocardial preservation. *Histol Histopathol, 25*(5), 577-587. doi: 10.14670/hh-25.577

Amberger, A., Schneeberger, S., Hernegger, G., Brandacher, G., Obrist, P., Lackner, P., . . . Mark, W. (2002). Gene expression profiling of prolonged cold ischemia and reperfusion in murine heart transplants. *Transplantation, 74*(10), 1441-1449. doi: 10.1097/01.tp.0000036051.53949.44

Amella, C., Cappello, F., Kahl, P., Fritsch, H., Lozanoff, S., & Sergi, C. (2008). Spatial and temporal dynamics of innervation during the development of fetal human pancreas. *Neuroscience, 154*(4), 1477-1487. doi: 10.1016/j.neuroscience.2008.04.050

An, J., Shi, F., Liu, S., Ma, J., & Ma, Q. (2017). Preoperative statins as modifiers of cardiac and inflammatory outcomes following coronary artery bypass graft surgery: a meta-analysis. *Interact Cardiovasc Thorac Surg, 25*(6), 958-965. doi: 10.1093/icvts/ivx172

Anic, D., Gasparovic, H., Ivancan, V., & Batinic, D. (2004). Effects of corticosteroids on inflammatory response following cardiopulmonary bypass. *Croat Med J, 45*(2), 158-161.

Anthony, C., Michel, J., Christofi, M., Wilson, S. H., Granger, E., Cropper, J., . . . Macdonald, P. (2014). Ex vivo coronary angiographic evaluation of a beating donor heart. *Circulation, 130*(25), e341-343. doi: 10.1161/circulationaha.114.010289

Aoyagi, T., & Matsui, T. (2011). The Cardiomyocyte as a Source of Cytokines in Cardiac Injury. *J Cell Sci Ther, 2012*(S5). doi: 10.4172/2157-7013.s5-003

Ardehali, A., Esmailian, F., Deng, M., Soltesz, E., Hsich, E., Naka, Y., . . . Kobashigawa, J. (2015). Ex-vivo perfusion of donor hearts for human heart transplantation (PROCEED II): a prospective, open-label, multicentre, randomised non-inferiority trial. *Lancet, 385*(9987), 2577-2584. doi: 10.1016/s0140-6736(15)60261-6

Aronson, D., & Krum, H. (2012). Novel therapies in acute and chronic heart failure. *Pharmacol Ther, 135*(1), 1-17.

Atherton, J. J. (2012). Chronic heart failure: we are fighting the battle, but are we winning the war? *Scientifica (Cairo), 2012*, 279731.

Aupperle, H., Garbade, J., Ullmann, C., Schneider, K., Krautz, C., Dhein, S., . . . Schoon, H. A. (2007). Comparing the ultrastructural effects of two different cardiac preparation- and perfusion-techniques in a porcine model of extracorporal long-term preservation. *Eur J Cardiothorac Surg, 31*(2), 214-221.

Avery, C. L., Loehr, L. R., Baggett, C., Chang, P. P., Kucharska-Newton, A. M., Matsushita, K., . . . Heiss, G. (2012). The population burden of heart failure attributable to modifiable risk factors: the ARIC (Atherosclerosis Risk in Communities) study. *J Am Coll Cardiol, 60*(17), 1640-1646.

Avlas, O., Fallach, R., Shainberg, A., Porat, E., & Hochhauser, E. (2011). Toll-like receptor 4 stimulation initiates an inflammatory response that decreases cardiomyocyte contractility. *Antioxid Redox Signal, 15*(7), 1895-1909. doi: 10.1089/ars.2010.3728

Baddeley, A. J., Gundersen, H. J. G., & Cruz-Orive, L. M. (1986). Estimation of surface area from vertical sections. *Journal of Microscopy, 142*(3), 259-276. doi: 10.1111/j.1365-2818.1986.tb04282.x

Baehner, T., Boehm, O., Probst, C., Poetzsch, B., Hoeft, A., Baumgarten, G., & Knuefermann, P. (2012). [Cardiopulmonary bypass in cardiac surgery]. *Anaesthesist, 61*(10), 846-856. doi: 10.1007/s00101-012-2050-0

Bairwa, S. C., Parajuli, N., & Dyck, J. R. (2016). The role of AMPK in cardiomyocyte health and survival. *Biochim Biophys Acta, 1862*(12), 2199-2210. doi: 10.1016/j.bbadis.2016.07.001

Barnard, C. N. (1967). The operation. A human cardiac transplant: an interim report of a successful operation performed at Groote Schuur Hospital, Cape Town. *S Afr Med J, 41*(48), 1271-1274.

Benham, A. M., van Lith, M., Sitia, R., & Braakman, I. (2013). Ero1-PDI interactions, the response to redox flux and the implications for disulfide bond formation in the mammalian endoplasmic reticulum. *Philos Trans R Soc Lond B Biol Sci, 368*(1617), 20110403. doi: 10.1098/rstb.2011.0403

Bertero, E., & Maack, C. (2018). Calcium signaling and reactive oxygen species in mitochondria. *Circ Res, 122*(10), 1460-1478. doi: 10.1161/circresaha.118.310082

Biermann, J., Neumann, T., Angermann, C. E., Erbel, R., Maisch, B., Pittrow, D., . . . Neumann, A. (2012). Economic burden of patients with various etiologies of chronic systolic heart failure analyzed by resource use and costs. *Int J Cardiol, 156*(3), 323-325.

Bishawi, M., Roan, J.-N., Milano, C. A., Daneshmand, M. A., Schroder, J. N., Chiang, Y., . . . Bowles, D. E. (2019). A normothermic ex vivo organ perfusion delivery method for cardiac transplantation gene therapy. *Sci Rep, 9*(1), 8029. doi: 10.1038/s41598-019-43737-y

Bittl, J. A., & Ingwall, J. S. (1986). The energetics of myocardial stretch. Creatine kinase flux and oxygen consumption in the noncontracting rat heart. *Circ Res, 58*(3), 378-383.

Boodram, S., & Evans, E. (2008). Use of leukocyte-depleting filters during cardiac surgery with cardiopulmonary bypass: a review. *J Extra Corpor Technol, 40*(1), 27-42.

Borlaug, B. A., & Paulus, W. J. (2011). Heart failure with preserved ejection fraction: pathophysiology, diagnosis, and treatment. *Eur Heart J, 32*(6), 670-679.

Bourbon, A., Vionnet, M., Leprince, P., Vaissier, E., Copeland, J., McDonagh, P., . . . Gandjbakhch, I. (2004). The effect of methylprednisolone treatment on the cardiopulmonary bypass-induced systemic inflammatory response. *Eur J Cardiothorac Surg, 26*(5), 932-938. doi: 10.1016/j.ejcts.2004.07.044

Brant, S. M., Cobert, M. L., West, L. M., Shelton, J. M., Jessen, M. E., & Peltz, M. (2014). Characterizing cardiac donation after circulatory death: implications for perfusion preservation. *Ann Thorac Surg, 98*(6), 2107-2113; discussion 2113-2104. doi: 10.1016/j.athoracsur.2014.05.091

Brown, R. A., Leung, E., Kankaanranta, H., Moilanen, E., & Page, C. P. (2012). Effects of heparin and related drugs on neutrophil function. *Pulm Pharmacol Ther, 25*(2), 185-192. doi: 10.1016/j.pupt.2012.01.006

Buckberg, G. D., Brazier, J. R., Nelson, R. L., Goldstein, S. M., McConnell, D. H., & Cooper, N. (1977). Studies of the effects of hypothermia on regional myocardial blood flow and metabolism during

cardiopulmonary bypass. I. The adequately perfused beating, fibrillating, and arrested heart. *J Thorac Cardiovasc Surg, 73*(1), 87-94.

Bui, A. L., Horwich, T. B., & Fonarow, G. C. (2011). Epidemiology and risk profile of heart failure. *Nat Rev Cardiol, 8*(1), 30-41.

Butler, J., Khadim, G., Paul, K. M., Davis, S. F., Kronenberg, M. W., Chomsky, D. B., . . . Wilson, J. R. (2004). Selection of patients for heart transplantation in the current era of heart failure therapy. *J Am Coll Cardiol, 43*(5), 787-793.

Canter, M. O., Daniels, J., & Bridges, B. C. (2018). Adjunctive therapies during extracorporeal membrane oxygenation to enhance multiple organ support in critically ill children. *Frontiers in pediatrics, 6*, 78. http://europepmc.org/abstract/MED/29670870 https://doi.org/10.3389/fped.2018.00078 https://europepmc.org/articles/PMC5893897, https://europepmc.org/articles/PMC5893897?pdf=render doi:10.3389/fped.2018.00078

Cao, S. S., & Kaufman, R. J. (2014). Endoplasmic reticulum stress and oxidative stress in cell fate decision and human disease. *Antioxid Redox Signal, 21*(3), 396-413. doi: 10.1089/ars.2014.5851

Caputo, M., Mokhtari, A., Miceli, A., Ghorbel, M. T., Angelini, G. D., Parry, A. J., & Suleiman, S. M. (2014). Controlled reoxygenation during cardiopulmonary bypass decreases markers of organ damage, inflammation, and oxidative stress in single-ventricle patients undergoing pediatric heart surgery. *J Thorac Cardiovasc Surg, 148*(3), 792-801.e798; discussion 800-791. doi: 10.1016/j.jtcvs.2014.06.001

Caputo, M., Mokhtari, A., Rogers, C. A., Panayiotou, N., Chen, Q., Ghorbel, M. T., . . . Parry, A. J. (2009). The effects of normoxic versus hyperoxic cardiopulmonary bypass on oxidative stress and inflammatory response in cyanotic pediatric patients undergoing open cardiac surgery: a randomized controlled trial. *J Thorac Cardiovasc Surg, 138*(1), 206-214. doi: 10.1016/j.jtcvs.2008.12.028

Cashen, K., Reeder, R., Dalton, H. J., Berg, R. A., Shanley, T. P., Newth, C. J. L., . . . Meert, K. L. (2018). Hyperoxia and hypocapnia during pediatric extracorporeal membrane oxygenation: associations with complications, mortality, and functional status among survivors. *Pediatr Crit Care Med, 19*(3), 245-253. doi: 10.1097/pcc.0000000000001439

Catalucci, D., & Condorelli, G. (2006). Effects of Akt on cardiac myocytes. *Circ Res, 99*(4), 339-341. doi: 10.1161/01.RES.0000239409.90634.a9

Cecarini, V., Gee, J., Fioretti, E., Amici, M., Angeletti, M., Eleuteri, A. M., & Keller, J. N. (2007). Protein oxidation and cellular homeostasis: Emphasis on metabolism. *Biochim Biophys Acta, 1773*(2), 93-104. doi: 10.1016/j.bbamcr.2006.08.039

Celik, J. B., Gormus, N., Okesli, S., Gormus, Z. I., & Solak, H. (2004). Methylprednisolone prevents inflammatory reaction occurring during cardiopulmonary bypass: effects on TNF-alpha, IL-6, IL-8, IL-10. *Perfusion, 19*(3), 185-191. doi: 10.1191/0267659104pf733oa

Chanda, D., Luiken, J. J., & Glatz, J. F. (2016). Signaling pathways involved in cardiac energy metabolism. *FEBS Lett, 590*(15), 2364-2374. doi: 10.1002/1873-3468.12297

Chaney, M. A. (2002). Corticosteroids and cardiopulmonary bypass : a review of clinical investigations. *Chest, 121*(3), 921-931. doi: 10.1378/chest.121.3.921

Charles, E. J., Hunter Mehaffey, J., Huerter, M. E., Sharma, A. K., Stoler, M. H., Roeser, M. E., . . . Laubach, V. E. (2018). Ex vivo assessment of porcine donation after circulatory death lungs that undergo increasing warm ischemia times. *Transplant Direct, 4*(12), e405. doi: 10.1097/txd.0000000000000845

Chase, P., Arena, R., Guazzi, M., Myers, J., Peberdy, M. A., & Bensimhon, D. (2010). Prognostic usefulness of the functional aerobic reserve in patients with heart failure. *Am Heart J, 160*(5), 922-927.

Chee, Y. R., Watson, R. W., McCarthy, J., Chughtai, J. Z., Nolke, L., & Healy, D. G. (2017). High dose statin prophylaxis in cardiopulmonary bypass related surgery: clinical utility. *J Cardiothorac Surg, 12*(1), 20. doi: 10.1186/s13019-017-0582-8

Chen, E. P., Bittner, H. B., Kendall, S. W., & Van Trigt, P. (1996). Hormonal and hemodynamic changes in a validated animal model of brain death. *Crit Care Med, 24*(8), 1352-1359.

Chen, Q., Yu, W., Shi, J., Shen, J., Hu, Y., Gong, J., . . . Li, N. (2014). The effect of extracorporeal membrane oxygenation therapy on systemic oxidative stress injury in a porcine model. *Artif Organs, 38*(5), 426-431. doi: 10.1111/aor.12198

Chen, Y. F., Tsai, W. C., Lin, C. C., Tsai, L. Y., Lee, C. S., Huang, C. H., . . . Chen, M. L. (2004). Effect of leukocyte depletion on endothelial cell activation and transendothelial migration of leukocytes during cardiopulmonary bypass. *Ann Thorac Surg, 78*(2), 634-642; discussion 642-633. doi: 10.1016/j.athoracsur.2004.02.091

Chenoweth, D. E., Cooper, S. W., Hugli, T. E., Stewart, R. W., Blackstone, E. H., & Kirklin, J. W. (1981). Complement activation during cardiopulmonary bypass: evidence for generation of C3a and C5a anaphylatoxins. *N Engl J Med, 304*(9), 497-503. doi: 10.1056/nejm198102263040901

Chiu, B., Jantuan, E., Shen, F., Chiu, B., & Sergi, C. (2017). Autophagy-inflammasome interplay in heart failure: a systematic review on basics, pathways, and therapeutic perspectives. *Ann Clin Lab Sci, 47*(3), 243-252.

Choong, J. W., Ou, R., Lim, Y. W., & Rosenfeldt, F. L. (2016). Cold crystalloid perfusion provides cardiac preservation superior to cold storage for donation after circulatory death. *Transplantation, 100*(3), 546-553. doi: 10.1097/tp.0000000000000926

Church, J. T., Alghanem, F., Deatrick, K. B., Trahanas, J. M., Phillips, J. P., Hee Song, M., . . . Owens, G. E. (2017). Normothermic ex vivo heart perfusion: effects of live animal blood and plasma cross circulation. *Asaio j, 63*(6), 766-773. doi: 10.1097/mat.0000000000000583

Clermont, G., Vergely, C., Jazayeri, S., Lahet, J. J., Goudeau, J. J., Lecour, S., . . . Girard, C. (2002). Systemic free radical activation is a major event involved in myocardial oxidative stress related to cardiopulmonary bypass. *Anesthesiology, 96*(1), 80-87.

Cobert, M. L., Peltz, M., West, L. M., Merritt, M. E., & Jessen, M. E. (2012). Glucose is an ineffective substrate for preservation of machine perfused donor hearts. *J Surg Res, 173*(2), 198-205. doi: 10.1016/j.jss.2011.05.028

Cohen, O., De La Zerda, D. J., Beygui, R., Hekmat, D., & Laks, H. (2007). Donor brain death mechanisms and outcomes after heart transplantation. *Transplant Proc, 39*(10), 2964-2969.

Collins, M. J., Moainie, S. L., Griffith, B. P., & Poston, R. S. (2008). 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, 34*(2), 318-325. doi: 10.1016/j.ejcts.2008.03.043

Collins, M. J., Ozeki, T., Zhuo, J., Gu, J., Gullapalli, R., Pierson, R. N., . . . Poston, R. S. (2007). Use of diffusion tensor imaging to predict myocardial viability after warm global ischemia: possible avenue for use of non-beating donor hearts. *J Heart Lung Transplant, 26*(4), 376-383. doi: 10.1016/j.healun.2006.12.013

Cook, C., Cole, G., Asaria, P., Jabbour, R., & Francis, D. P. (2013). The annual global economic burden of heart failure. *Int J Cardiol, 171*(3), 368-376.

Correction to: Heart Disease and Stroke Statistics-2019 Update: A Report From the American Heart Association. (2020). *Circulation, 141*(2), e33. doi: 10.1161/cir.0000000000000746

Cowie, M. R. (2010). Improving the management of chronic heart failure. *Practitioner, 254*(1734), 29-32, 23.

Cui, J., Qin, L., Zhang, J., Abrahimi, P., Li, H., Li, G., . . . Mark Saltzman, W. (2017). Ex vivo pretreatment of human vessels with siRNA nanoparticles provides protein silencing in endothelial cells. *Nature Communications, 8*(1), 191. doi: 10.1038/s41467-017-00297-x

Curtis, J. M., Hahn, W. S., Long, E. K., Burrill, J. S., Arriaga, E. A., & Bernlohr, D. A. (2012). Protein carbonylation and metabolic control systems. *Trends Endocrinol Metab, 23*(8), 399-406. doi: 10.1016/j.tem.2012.05.008

Cypel, M., Yeung, J. C., Hirayama, S., Rubacha, M., Fischer, S., Anraku, M., . . . Keshavjee, S. (2008). Technique for prolonged normothermic ex vivo lung perfusion. *The Journal of Heart and Lung Transplantation, 27*(12), 1319-1325. doi: 10.1016/j.healun.2008.09.003

Dabbagh, A., Rajaei, S., Bahadori Monfared, A., Keramatinia, A. A., & Omidi, K. (2012). Cardiopulmonary bypass, inflammation and how to defy it: focus on pharmacological interventions. *Iran J Pharm Res, 11*(3), 705-714.

Damy, T., Kirsch, M., Khouzami, L., Caramelle, P., Le Corvoisier, P., Roudot-Thoraval, F., . . . Pecker, F. (2009). Glutathione deficiency in cardiac patients is related to the functional status and structural cardiac abnormalities. *PLoS One, 4*(3), e4871. doi: 10.1371/journal.pone.0004871

Datzmann, T., & Trager, K. (2018). Extracorporeal membrane oxygenation and cytokine adsorption. *J Thorac Dis, 10*(Suppl 5), S653-s660. doi: 10.21037/jtd.2017.10.128

Davies, M., Hobbs, F., Davis, R., Kenkre, J., Roalfe, A. K., Hare, R., . . . Lancashire, R. J. (2001). Prevalence of left-ventricular systolic dysfunction and heart failure in the Echocardiographic Heart of England Screening study: a population based study. *Lancet, 358*(9280), 439-444.

Day, J. R., & Taylor, K. M. (2005). The systemic inflammatory response syndrome and cardiopulmonary bypass. *Int J Surg, 3*(2), 129-140. doi: 10.1016/j.ijsu.2005.04.002

de Amorim, C. G., Malbouisson, L. M., da Silva, F. C., Jr., Fiorelli, A. I., Murakami, C. K., & Carmona, M. J. (2014). Leukocyte depletion during CPB: effects on inflammation and lung function. *Inflammation, 37*(1), 196-204. doi: 10.1007/s10753-013-9730-z

de Frutos, F., Gea, A., Hernandez-Estefania, R., & Rabago, G. (2015). Prophylactic treatment with coenzyme Q10 in patients undergoing cardiac surgery: could an antioxidant reduce complications? A systematic review and meta-analysis. *Interact Cardiovasc Thorac Surg, 20*(2), 254-259. doi: 10.1093/icvts/ivu334

de Jonge, N., Kirkels, J. H., Klopping, C., Lahpor, J. R., Caliskan, K., Maat, A. P., . . . Balk, A. H. (2008). Guidelines for heart transplantation. *Neth Heart J, 16*(3), 79-87.

De Silva, R. J., Vuylsteke, A., Fritchley, S. J., Trull, A. K., Dunning, J. J., & Wallwork, J. (2006). APT070 inhibits complement activation during in vitro cardiopulmonary bypass. *European Journal of Cardio-Thoracic Surgery, 30*(1), 72-76. doi: 10.1016/j.ejcts.2006.03.012

Demir, T., Ergenoglu, M. U., Demir, H. B., Tanrikulu, N., Sahin, M., Gok, E., . . . Demirsoy, E. (2015). Pretreatment with methylprednisolone improves myocardial protection during on-pump coronary artery bypass surgery. *Heart Surg Forum, 18*(4), E171-177. doi: 10.1532/hsf.1367

Deng, M., Soltesz, E., Hsich, E., Naka, Y., Mancini, D., Esmailian, F., . . . Ardehali, A. (2013). Ex-vivo perfusion of human donor hearts reduces cold ischemia time. *The JournalofHeartandLungTransplantation, 32*(4s), 410.

Deng, M. C., Wiedner, M., Erren, M., Mollhoff, T., Assmann, G., & Scheld, H. H. (1995). Arterial and venous cytokine response to cardiopulmonary bypass for low risk CABG and relation to hemodynamics. *Eur J Cardiothorac Surg, 9*(1), 22-29. doi: 10.1016/s1010-7940(05)80044-2

Detry, O., Le Dinh, H., Noterdaeme, T., De Roover, A., Honore, P., Squifflet, J. P., & Meurisse, M. (2012). Categories of donation after cardiocirculatory death. *Transplant Proc, 44*(5), 1189-1195.

Dhanwate, A. D. (2014). Brainstem death: A comprehensive review in Indian perspective. *Indian J Crit Care Med, 18*(9), 596-605.

Dhital, K. K., Chew, H. C., & Macdonald, P. S. (2017). Donation after circulatory death heart transplantation. *Curr Opin Organ Transplant, 22*(3), 189-197. doi: 10.1097/mot.0000000000000419

Dhital, K. K., Iyer, A., Connellan, M., Chew, H. C., Gao, L., Doyle, A., . . . Macdonald, P. (2015). Adult heart transplantation with distant procurement and ex-vivo preservation of donor hearts after circulatory death: a case series. *Lancet, 385*(9987), 2585-2591. doi: 10.1016/s0140-6736(15)60038-1

Di, R., Wu, X., Chang, Z., Zhao, X., Feng, Q., Lu, S., . . . Yang, Z. (2012). S6K inhibition renders cardiac protection against myocardial infarction through PDK1 phosphorylation of Akt. *Biochem J, 441*(1), 199-207. doi: 10.1042/bj20110033

Doenst, T., Nguyen Tien, D., & Abel, E. D. (2013). Cardiac metabolism in heart failure. *Circ Res, 113*(6), 709-724. doi: 10.1161/CIRCRESAHA.113.300376

Doyle, A. J., & Hunt, B. J. (2018). Current understanding of how extracorporeal membrane oxygenators activate haemostasis and other blood components. *Front Med (Lausanne), 5*, 352. doi: 10.3389/fmed.2018.00352

Drosatos, K., Lymperopoulos, A., Kennel, P. J., Pollak, N., Schulze, P. C., & Goldberg, I. J. (2015). Pathophysiology of sepsis-related cardiac dysfunction: driven by inflammation, energy mismanagement, or both? *Curr Heart Fail Rep, 12*(2), 130-140. doi: 10.1007/s11897-014-0247-z

Dvorak, L., Pirk, J., Cerny, S., & Kovar, J. (2006). The role of leukocyte depleting filters in heart transplantation: early outcomes in prospective, randomized clinical trial. *European Journal of Cardio-Thoracic Surgery, 30*(4), 621-627. doi: 10.1016/j.ejcts.2006.07.022

Dyck, J. R., & Lopaschuk, G. D. (2006). AMPK alterations in cardiac physiology and pathology: enemy or ally? *J Physiol, 574*(Pt 1), 95-112. doi: 10.1113/jphysiol.2006.109389

Edwards, N., Chen, J., Mazzeo, P., Chen, J., & Edwards, N. (2004). Donor Selection and Management of the High-Risk Donor *Cardiac Transplantation* (pp. 19-36): Humana Press.

Eren, N., Cakir, O., Oruc, A., Kaya, Z., & Erdinc, L. (2003). Effects of N-acetylcysteine on pulmonary function in patients undergoing coronary artery bypass surgery with cardiopulmonary bypass. *Perfusion, 18*(6), 345-350. doi: 10.1191/0267659103pf696oa

Faa, A., Iacovidou, N., Xanthos, T., Locci, A., Pampaloni, P., Aroni, F., . . . Fanos, V. (2012). Hypoxia/reoxygenation-induced myocardial lesions in newborn piglets are related to interindividual variability and not to oxygen concentration. *Clinics (Sao Paulo), 67*(5), 503-508. doi: 10.6061/clinics/2012(05)16

Farneti, P. A., Sbrana, S., Spiller, D., Cerillo, A. G., Santarelli, F., Di Dario, D., . . . Glauber, M. (2008). Reduction of blood coagulation and monocyte-platelet interaction following the use of a minimal extracorporeal circulation system (Synergy) in coronary artery bypass grafting (CABG). *Perfusion, 23*(1), 49-56. doi: 10.1177/0267659108091336

Ferdinandy, P., Panas, D., & Schulz, R. (1999). Peroxynitrite contributes to spontaneous loss of cardiac efficiency in isolated working rat hearts. *Am J Physiol, 276*(6), H1861-1867. doi: 10.1152/ajpheart.1999.276.6.H1861

Fernandez-Caggiano, M., Schroder, E., Cho, H. J., Burgoyne, J., Barallobre-Barreiro, J., Mayr, M., & Eaton, P. (2016). Oxidant-induced interprotein disulfide formation in cardiac protein DJ-1 occurs via an interaction with peroxiredoxin 2. *J Biol Chem, 291*(19), 10399-10410. doi: 10.1074/jbc.M115.699850

Fillinger, M. P., Rassias, A. J., Guyre, P. M., Sanders, J. H., Beach, M., Pahl, J., . . . Yeager, M. P. (2002). Glucocorticoid effects on the inflammatory and clinical responses to cardiac surgery. *J Cardiothorac Vasc Anesth, 16*(2), 163-169. doi: 10.1053/jcan.2002.31057

Fischer, U. M., Cox, C. S., Jr., Allen, S. J., Stewart, R. H., Mehlhorn, U., & Laine, G. A. (2003). The antioxidant N-acetylcysteine preserves myocardial function and diminishes oxidative stress after cardioplegic arrest. *J Thorac Cardiovasc Surg, 126*(5), 1483-1488. doi: 10.1016/s0022-5223(03)00792-x

Fitton, T. P., Barreiro, C. J., Bonde, P. N., Wei, C., Gage, F., Rodriguez, R., & Conte, J. V. (2005). Attenuation of DNA damage in canine hearts preserved by continuous hypothermic perfusion. *Ann Thorac Surg, 80*(5), 1812-1820. doi: 10.1016/j.athoracsur.2005.04.081

Folden, D. V., Gupta, A., Sharma, A. C., Li, S. Y., Saari, J. T., & Ren, J. (2003). Malondialdehyde inhibits cardiac contractile function in ventricular myocytes via a p38 mitogen-activated protein kinase-dependent mechanism. *Br J Pharmacol, 139*(7), 1310-1316. doi: 10.1038/sj.bjp.0705384

Fortenberry, J. D., Bhardwaj, V., Niemer, P., Cornish, J. D., Wright, J. A., & Bland, L. (1996). Neutrophil and cytokine activation with neonatal extracorporeal membrane oxygenation. *J Pediatr, 128*(5 Pt 1), 670-678. doi: 10.1016/s0022-3476(96)80133-8

Franke, A., Lante, W., Kurig, E., Zoller, L. G., Weinhold, C., & Markewitz, A. (2006). Hyporesponsiveness of T cell subsets after cardiac surgery: a product of altered cell function or merely a result of absolute cell count changes in peripheral blood? *Eur J Cardiothorac Surg, 30*(1), 64-71. doi: 10.1016/j.ejcts.2006.03.029

Freed, D. H., & White, C. W. (2015). Donor heart preservation: straight up, or on the rocks? *Lancet, 385*(9987), 2552-2554. doi: 10.1016/s0140-6736(15)60614-6

Frering, B., Philip, I., Dehoux, M., Rolland, C., Langlois, J. M., & Desmonts, J. M. (1994). Circulating cytokines in patients undergoing normothermic cardiopulmonary bypass. *J Thorac Cardiovasc Surg, 108*(4), 636-641.

Fujii, Y., Tatsumi, E., Nakamura, F., & Oite, T. (2020). PaO 2 greater than 300 mmHg promotes an inflammatory response during extracorporeal circulation in a rat extracorporeal membrane oxygenation model. *J Thorac Dis, 12*(3), 749-757.

Gao, F., Yao, C. L., Gao, E., Mo, Q. Z., Yan, W. L., McLaughlin, R., . . . Ma, X. L. (2002). Enhancement of glutathione cardioprotection by ascorbic acid in myocardial reperfusion injury. *J Pharmacol Exp Ther, 301*(2), 543-550. doi: 10.1124/jpet.301.2.543

Gao, Z., & Cooper, T. A. (2013). Reexpression of pyruvate kinase M2 in type 1 myofibers correlates with altered glucose metabolism in myotonic dystrophy *Proc Natl Acad Sci U S A, 110*(33).

Garbade, J., Krautz, C., Aupperle, H., Ullmann, C., Lehmann, S., Kempfert, J., . . . Mohr, F. W. (2009). Functional, metabolic, and morphological aspects of continuous, normothermic heart preservation: effects of different preparation and perfusion techniques. *Tissue Eng Part C Methods, 15*(2), 275-283. doi: 10.1089/ten.tec.2008.0475

Garcia Saez, D., Zych, B., Sabashnikov, A., Bowles, C. T., De Robertis, F., Mohite, P. N., . . . Simon, A. R. (2014). Evaluation of the organ care system in heart transplantation with an adverse donor/recipient profile. *Ann Thorac Surg, 98*(6), 2099-2105; discussion 2105-2096. doi: 10.1016/j.athoracsur.2014.06.098

Gemmell, C. H., Ramirez, S. M., Yeo, E. L., & Sefton, M. V. (1995). Platelet activation in whole blood by artificial surfaces: identification of platelet-derived microparticles and activated platelet binding to leukocytes as material-induced activation events. *J Lab Clin Med, 125*(2), 276-287.

Giacinto, O., Satriano, U., Nenna, A., Spadaccio, C., Lusini, M., Mastroianni, C., . . . Chello, M. (2019). Inflammatory response and endothelial dysfunction following cardiopulmonary bypass: pathophysiology and pharmacological targets. *Recent Pat Inflamm Allergy Drug Discov, 13*(2), 158-173. doi: 10.2174/1872213x13666190724112644

Giannelli, S., Jr., McKenna, J. P., Bordiuk, J. M., Miller, L. D., & Jerome, C. R. (1976). Prevention of increased hemoglobin-oxygen affinity in open-heart operations with inosine-phosphate-pyruvate solution. *Ann Thorac Surg, 21*(5), 386-396.

Giomarelli, P., Scolletta, S., Borrelli, E., & Biagioli, B. (2003). Myocardial and lung injury after cardiopulmonary bypass: role of interleukin (IL)-10. *Ann Thorac Surg, 76*(1), 117-123. doi: 10.1016/s0003-4975(03)00194-2

Goila, A. K., & Pawar, M. (2009). The diagnosis of brain death. *Indian J Crit Care Med, 13*(1), 7-11.

Golej, J., Winter, P., Schoffmann, G., Kahlbacher, H., Stoll, E., Boigner, H., & Trittenwein, G. (2003). Impact of extracorporeal membrane oxygenation modality on cytokine release during rescue from infant hypoxia. *Shock, 20*(2), 110-115. doi: 10.1097/01.shk.0000075571.93053.2c

Gorbet, M. B., & Sefton, M. V. (2004). Biomaterial-associated thrombosis: roles of coagulation factors, complement, platelets and leukocytes. *Biomaterials, 25*(26), 5681-5703. doi: 10.1016/j.biomaterials.2004.01.023

Gralinski, M. R., Wiater, B. C., Assenmacher, A. N., & Lucchesi, B. R. (1996). Selective inhibition of the alternative complement pathway by sCR1[desLHR-A] protects the rabbit isolated heart from human complement-mediated damage. *Immunopharmacology, 34*(2-3), 79-88. doi: 10.1016/0162-3109(96)00105-1

Graulich, J., Sonntag, J., Marcinkowski, M., Bauer, K., Kossel, H., Buhrer, C., . . . Versmold, H. T. (2002). Complement activation by in vivo neonatal and in vitro extracorporeal membrane oxygenation. *Mediators Inflamm, 11*(2), 69-73. doi: 10.1080/09629350220131908

Graulich, J., Walzog, B., Marcinkowski, M., Bauer, K., Kössel, H., Fuhrmann, G., . . . Versmold, H. T. (2000). Leukocyte and endothelial activation in a laboratory model of extracorporeal membrane oxygenation (ECMO). *Pediatric Research, 48*(5), 679-684. doi: 10.1203/00006450-200011000-00021

Greenberg, B., Czerska, B., Delgado, R. M., Bourge, R., Zile, M. R., Silver, M., . . . Konstam, M. A. (2008). Effects of continuous aortic flow augmentation in patients with exacerbation of heart failure inadequately responsive to medical therapy: results of the Multicenter Trial of the Orqis Medical Cancion System for the Enhanced Treatment of Heart Failure Unresponsive to Medical Therapy (MOMENTUM). *Circulation, 118*(12), 1241-1249.

Groenendyk, J., Lee, D., Jung, J., Dyck, J. R., Lopaschuk, G. D., Agellon, L. B., & Michalak, M. (2016). Inhibition of the unfolded protein response mechanism prevents cardiac fibrosis. *PLoS One, 11*(7), e0159682. doi: 10.1371/journal.pone.0159682

Guha, K., & McDonagh, T. (2013). Heart failure epidemiology: European perspective. *Curr Cardiol Rev, 9*(2), 123-127.

Guibert, E. E., Petrenko, A. Y., Balaban, C. L., Somov, A. Y., Rodriguez, J. V., & Fuller, B. J. (2011). Organ preservation: current concepts and new strategies for the next decade. *Transfus Med Hemother, 38*(2), 125-142. doi: 10.1159/000327033

Gundersen, H. J. G., & Jensen, E. B. (1987). The efficiency of systematic sampling in stereology and its prediction*. *Journal of Microscopy, 147*(3), 229-263. doi: 10.1111/j.1365-2818.1987.tb02837.x

Haeffner-Cavaillon, N., Roussellier, N., Ponzio, O., Carreno, M. P., Laude, M., Carpentier, A., & Kazatchkine, M. D. (1989). Induction of interleukin-1 production in patients undergoing cardiopulmonary bypass. *J Thorac Cardiovasc Surg, 98*(6), 1100-1106.

Halazun, K. J., Al-Mukhtar, A., Aldouri, A., Willis, S., & Ahmad, N. (2007). Warm ischemia in transplantation: search for a consensus definition. *Transplant Proc, 39*(5), 1329-1331.

Haldeman, G. A., Croft, J. B., Giles, W. H., & Rashidee, A. (1999). Hospitalization of patients with heart failure: National Hospital Discharge Survey, 1985 to 1995. *Am Heart J, 137*(2), 352-360.

Halonen, J., Halonen, P., Jarvinen, O., Taskinen, P., Auvinen, T., Tarkka, M., . . . Hakala, T. (2007). Corticosteroids for the prevention of atrial fibrillation after cardiac surgery: a randomized controlled trial. *Jama, 297*(14), 1562-1567. doi: 10.1001/jama.297.14.1562

Halter, J., Steinberg, J., Fink, G., Lutz, C., Picone, A., Maybury, R., . . . Nieman, G. (2005). Evidence of systemic cytokine release in patients undergoing cardiopulmonary bypass. *J Extra Corpor Technol, 37*(3), 272-277.

Hamamoto, M., Suga, M., Takahashi, Y., Sato, Y., Inamori, S., Yagihara, T., . . . Kitamura, S. (2006). Suppressive effect of phosphodiesterase type 4 inhibition on systemic inflammatory responses after cardiopulmonary bypass. *J Artif Organs, 9*(3), 144-148. doi: 10.1007/s10047-006-0335-2

Hamed, A., Tsui, S., Huber, J., Lin, R., . Poggio, E., & Ardehali4, A. (2009). Serum lactate is a highly sensitive and specific predictor of post cardiac transplant outcomes using the organ care system. *J Heart Lung Transplant, 28*(2), S71.

Hansen, T. N., Haworth, R. A., & Southard, J. H. (2000). Warm and cold ischemia result in different mechanisms of injury to the coronary vasculature during reperfusion of rat hearts. *Transplant Proc, 32*(1), 15-18.

Hao, J., Kim, H. S., Choi, W., Ha, T. S., Ahn, H. Y., & Kim, C. H. (2010). Mechanical stretch-induced protection against myocardial ischemia-reperfusion injury involves AMP-activated protein kinase. *Korean J Physiol Pharmacol, 14*(1), 1-9. doi: 10.4196/kjpp.2010.14.1.1

Hassanein, W. H., Zellos, L., Tyrrell, T. A., Healey, N. A., Crittenden, M. D., Birjiniuk, V., & Khuri, S. F. (1998). Continuous perfusion of donor hearts in the beating state extends preservation time and improves recovery of function. *J Thorac Cardiovasc Surg, 116*(5), 821-830.

Hatami, S., & Freed, D. H. (2019). Machine perfusion of donor heart: state of the art. *Current Transplantation Reports, 6*(3), 242-250. doi: 10.1007/s40472-019-00251-4

Hatami, S., Qi, X., White, C., Buchko, M., Bozso, S., Himmat, S., . . . Freed, D. (2019). 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, 35*(10), S106. doi: 10.1016/j.cjca.2019.07.499

Hatami, S., Qi, X., White, C. W., Buchko, M., Bozsu, S., Himmat, S., . . . Freed, D. H. (2020). Preservation and recovery of myocardial function during normothermic ex situ heart perfusion: does the position of the heart during perfusion matter? *The Journal of Heart and Lung Transplantation, 39*(4), S145-S146. doi: 10.1016/j.healun.2020.01.1070

Hatami, S., Qi, X., White, C. W., Burhani, M., Aboelnazar, N., Himmat, S., . . . Freed, D. H. (2017). Endoplasmic reticulum stress in ex vivo heart prfusion: a comparison between working vs non-working modes. *Canadian Journal of cardiology, 33*(10).

Hatami, S., White, C., Qi, X., Buchko, M., Ondrus, M., Kinnear, A., . . . Freed, D. (2020). Immunity and stress responses are induced during ex-situ heart perfusion. *Circ. Heart Fail, 13*(6), e006552.

Hatami, S., White, C., Shan, S., Haromy, A., Ondrus, M., Kinnear, A., . . . Freed, H. (2017). *Functional decline of the ex vivoperfused heart is not due to cell death*. Paper presented at the Canadian Sosciety for Transplantation, Halifax.

Hatami, S., White, C., Shan, S., Haromy, A., Qi, X., Ondrus, M., . . . Freed, D. (2019). Myocardial functional decline during prolonged ex situ heart perfusion. *Ann Thorac Surg, 108*, 499-507.

Hatami, S., White, C. W., Ondrus, M., Qi, X., Buchko, M., Himmat, S., . . . Freed, D. H. (2019). Normothermic ex situ heart perfusion in working mode: assessment of cardiac function and metabolism. *J Vis Exp*(143). doi: 10.3791/58430

Hayes, M., Langley, M., Langley, M., Schneider, B., Drake, D., Rojas-Peña, A., . . . Haft, J. W. (2019). *Steroid use in ex-vivo normothermic heart perfusion*. Paper presented at the American Society for Artificial Internal Organs, San Francisco, CA, US.

Hayes, R. A., Shekar, K., & Fraser, J. F. (2013). Is hyperoxaemia helping or hurting patients during extracorporeal membrane oxygenation? Review of a complex problem. *Perfusion, 28*(3), 184-193. doi: 10.1177/0267659112473172

Heart Failure Society Of, A. (2006). Evaluation of patients for ventricular dysfunction and heart failure. *J Card Fail, 12*(1), e16-25.

Heidenreich, P. A., Trogdon, J. G., Khavjou, O. A., Butler, J., Dracup, K., Ezekowitz, M. D., . . . Woo, Y. J. (2011). Forecasting the future of cardiovascular disease in the United States: a policy statement from the American Heart Association. *Circulation, 123*(8), 933-944.

Hickman, P. E., Potter, J. M., Aroney, C., Koerbin, G., Southcott, E., Wu, A. H., & Roberts, M. S. (2010). Cardiac troponin may be released by ischemia alone, without necrosis. *Clin Chim Acta, 411*(5-6), 318-323. doi: 10.1016/j.cca.2009.12.009

Hill, A., Clasen, K. C., Wendt, S., Majoros Á, G., Stoppe, C., & Adhikari, N. K. J. (2019). Effects of Vitamin C on Organ Function in Cardiac Surgery Patients: A Systematic Review and Meta-Analysis. *11*(9). doi: 10.3390/nu11092103

Hill, G. E., Alonso, A., Spurzem, J. R., Stammers, A. H., & Robbins, R. A. (1995). Aprotinin and methylprednisolone equally blunt cardiopulmonary bypass–induced inflammation in humans. *J Thorac Cardiovasc Surg, 110*(6), 1658-1662. doi: 10.1016/S0022-5223(95)70027-7

Hinghofer-Szalkay, H. (2011). Gravity, the hydrostatic indifference concept and the cardiovascular system. *Eur J Appl Physiol, 111*(2), 163-174. doi: 10.1007/s00421-010-1646-9

Hirthler, M., Simoni, J., & Dickson, M. (1992). Elevated levels of endotoxin, oxygen-derived free radicals, and cytokines during extracorporeal membrane oxygenation. *J Pediatr Surg, 27*(9), 1199-1202. doi: 10.1016/0022-3468(92)90787-8

Ho, E., Karimi Galougahi, K., Liu, C. C., Bhindi, R., & Figtree, G. A. (2013). Biological markers of oxidative stress: Applications to cardiovascular research and practice. *Redox Biol, 1*, 483-491. doi: 10.1016/j.redox.2013.07.006

Ho, K. M., & Tan, J. A. (2009). Benefits and risks of corticosteroid prophylaxis in adult cardiac surgery: a dose-response meta-analysis. *Circulation, 119*(14), 1853-1866. doi: 10.1161/circulationaha.108.848218

Hobbs, R., & Boyle, A. (2014). Heart Failure. *Disease management project.* Retrieved November 15, 2014, fromhttp://www.clevelandclinicmeded.com/medicalpubs/diseasemanagement/cardiology/heart-failure/

Homeister, J. W., Satoh, P., & Lucchesi, B. R. (1992). Effects of complement activation in the isolated heart. Role of the terminal complement components. *Circ Res, 71*(2), 303-319. doi: 10.1161/01.res.71.2.303

Horie, T., Ono, K., Nagao, K., Nishi, H., Kinoshita, M., Kawamura, T., . . . Hasegawa, K. (2008). Oxidative stress induces GLUT4 translocation by activation of PI3-K/Akt and dual AMPK kinase in cardiac myocytes. *J Cell Physiol, 215*(3), 733-742. doi: 10.1002/jcp.21353

Hornby, K., Ross, H., Keshavjee, S., Rao, V., & Shemie, S. D. (2006). Non-utilization of hearts and lungs after consent for donation: a Canadian multicentre study. *Can J Anaesth, 53*(8), 831-837. doi: 10.1007/bf03022801

Hosgood, S. A., Moore, T., Kleverlaan, T., Adams, T., & Nicholson, M. L. (2017). Haemoadsorption reduces the inflammatory response and improves blood flow during ex vivo renal perfusion in an experimental model. *Journal of Translational Medicine, 15*(1), 216. doi: 10.1186/s12967-017-1314-5

Hsia, T. Y., McQuinn, T. C., Mukherjee, R., Deardorff, R. L., Squires, J. E., Stroud, R. E., . . . Spinale, F. G. (2010). Effects of aprotinin or tranexamic acid on proteolytic/cytokine profiles in infants after cardiac surgery. *Ann Thorac Surg, 89*(6), 1843-1852; discussion 1852. doi: 10.1016/j.athoracsur.2010.02.069

Hunt, S. A., Baker, D. W., Chin, M. H., Cinquegrani, M. P., Feldman, A. M., Francis, G. S., . . . Smith, S. C., Jr. (2001). ACC/AHA guidelines for the evaluation and management of chronic heart failure in the adult: executive summary a report of the American College of Cardiology/American Heart Association task force on practice guidelines (committee to revise the 1995 guidelines for the evaluation and management of heart failure): developed in collaboration with the international society for heart and lung transplantation; endorsed by the Heart Failure Society of America. *Circulation, 104*(24), 2996-3007.

Ichihara, S., Suzuki, Y., Chang, J., Kuzuya, K., Inoue, C., Kitamura, Y., & Oikawa, S. (2017). Involvement of oxidative modification of proteins related to ATP synthesis in the left ventricles of hamsters with cardiomyopathy. *Sci Rep, 7*(1), 9243. doi: 10.1038/s41598-017-08546-1

Inbar, R., & Shoenfeld, Y. (2009). Elevated cardiac troponins: the ultimate marker for myocardial necrosis, but not without a differential diagnosis. *Isr Med Assoc J, 11*(1), 50-53.

Iskender, I., Cosgun, T., Arni, S., Trinkwitz, M., Fehlings, S., Yamada, Y., . . . Inci, I. (2017). Cytokine filtration modulates pulmonary metabolism and edema formation during ex vivo lung perfusion. *J Heart Lung Transplant*. doi: 10.1016/j.healun.2017.05.021

Ito, H., Hamano, K., Gohra, H., Katoh, T., Fujimura, Y., Tsuboi, H., & Esato, K. (1997). Relationship between respiratory distress and cytokine response after cardiopulmonary bypass. *Surg Today, 27*(3), 220-225. doi: 10.1007/bf00941649

Iwano, H., & Little, W. C. (2013). Heart failure: what does ejection fraction have to do with it? *J Cardiol, 62*(1), 1-3.

Iyer, A., Gao, L., Doyle, A., Rao, P., Cropper, J. R., Soto, C., . . . MacDonald, P. S. (2015). Normothermic ex vivo perfusion provides superior organ preservation and enables viability assessment of hearts from DCD donors. *Am J Transplant, 15*(2), 371-380. doi: 10.1111/ajt.12994

Iyer, A., Gao, L., Doyle, A., Rao, P., Jayewardene, D., Wan, B., . . . Macdonald, P. S. (2014). Increasing the tolerance of DCD hearts to warm ischemia by pharmacological postconditioning. *Am J Transplant, 14*(8), 1744-1752. doi: 10.1111/ajt.12782

Jackson, H. T., Oyetunji, T. A., Thomas, A., Oyetunji, A. O., Hamrick, M., Nadler, E. P., . . . Qureshi, F. G. (2014). The impact of leukoreduced red blood cell transfusion on mortality of neonates undergoing extracorporeal membrane oxygenation. *J Surg Res, 192*(1), 6-11. doi: 10.1016/j.jss.2014.06.013

Jacob-Ferreira, A. L., & Schulz, R. (2013). Activation of intracellular matrix metalloproteinase-2 by reactive oxygen-nitrogen species: Consequences and therapeutic strategies in the heart. *Arch Biochem Biophys, 540*(1-2), 82-93. doi: 10.1016/j.abb.2013.09.019

Jahania, M. S., Sanchez, J. A., Narayan, P., Lasley, R. D., & Mentzer, R. M., Jr. (1999). Heart preservation for transplantation: principles and strategies. *Ann Thorac Surg, 68*(5), 1983-1987.

Jain, M., Brenner, D. A., Cui, L., Lim, C. C., Wang, B., Pimentel, D. R., . . . Liao, R. (2003). Glucose-6-phosphate dehydrogenase modulates cytosolic redox status and contractile phenotype in adult cardiomyocytes. *Circ Res, 93*(2), e9-16. doi: 10.1161/01.res.0000083489.83704.76

Jencks, S. F., Williams, M. V., & Coleman, E. A. (2009). Rehospitalizations among patients in the Medicare fee-for-service program. *N Engl J Med, 360*(14), 1418-1428.

Jeon, S. M. (2016). Regulation and function of AMPK in physiology and diseases. *Exp Mol Med, 48*(7), e245. doi: 10.1038/emm.2016.81

Jindrichova, E., Kratochvilova, S., & Kovar, J. (2007). Glucose administration downregulates lipoprotein lipase activity in vivo: a study using repeated intravenous fat tolerance test. *Physiol Res, 56*(2), 175-181.

Jong, P., Vowinckel, E., Liu, P. P., Gong, Y., & Tu, J. V. (2002). Prognosis and determinants of survival in patients newly hospitalized for heart failure: a population-based study. *Arch Intern Med, 162*(15), 1689-1694.

Jouybar, R., Kabgani, H., Kamalipour, H., Shahbazi, S., Allahyari, E., Rasouli, M., . . . Khademi, S. (2012). The perioperative effect of ascorbic acid on the inflammatory response in coronary artery bypass graft surgery; a randomized control trial oronary artery bypass graft surgery. *INTERNATIONAL CARDIOVASCULAR RESEARCH JOURNAL, 6*(1), -.

Kang, P. T., Chen, C. L., Lin, P., Zhang, L., Zweier, J. L., & Chen, Y. R. (2018). Mitochondrial complex I in the post-ischemic heart: reperfusion-mediated oxidative injury and protein cysteine sulfonation. *J Mol Cell Cardiol, 121*, 190-204. doi: 10.1016/j.yjmcc.2018.07.244

Kappler, B., Ledezma, C. A., van Tuijl, S., Meijborg, V., Boukens, B. J., Ergin, B., . . . de Mol, B. A. J. M. (2019). Investigating the physiology of normothermic ex vivo heart perfusion in an isolated slaughterhouse porcine model used for device testing and training. *BMC Cardiovascular Disorders, 19*(1), 254. doi: 10.1186/s12872-019-1242-9

Karaiskos, T. E., Palatianos, G. M., Triantafillou, C. D., Kantidakis, G. H., Astras, G. M., Papadakis, E. G., & Vassili, M. I. (2004). Clinical effectiveness of leukocyte filtration during cardiopulmonary bypass in patients with chronic obstructive pulmonary disease. *Ann Thorac Surg, 78*(4), 1339-1344. doi: 10.1016/j.athoracsur.2004.04.040

Karsten, E., Breen, E., & Herbert, B. R. (2018). Red blood cells are dynamic reservoirs of cytokines. *Sci Rep, 8*(1), 3101. doi: 10.1038/s41598-018-21387-w

Kelder, J. C., Cramer, M. J., van Wijngaarden, J., van Tooren, R., Mosterd, A., Moons, K. G., . . . Hoes, A. W. (2011). The diagnostic value of physical examination and additional testing in primary care patients with suspected heart failure. *Circulation, 124*(25), 2865-2873.

Keyser, A., Hilker, M. K., Diez, C., Philipp, A., Foltan, M., & Schmid, C. (2011). Prospective randomized clinical study of arterial pumps used for routine on pump coronary bypass grafting. *Artif Organs, 35*(5), 534-542. doi: 10.1111/j.1525-1594.2010.01120.x

Khairallah, M., Labarthe, F., Bouchard, B., Danialou, G., Petrof, B. J., & Des Rosiers, C. (2004). Profiling substrate fluxes in the isolated working mouse heart using 13C-labeled substrates: focusing on the origin and fate of pyruvate and citrate carbons. *Am J Physiol Heart Circ Physiol, 286*(4), H1461-1470. doi: 10.1152/ajpheart.00942.2003

Khan, M. M., Yang, W. L., & Wang, P. (2015). Endoplasmic reticulum stress in sepsis. *Shock, 44*(4), 294-304. doi: 10.1097/shk.0000000000000425

Kilger, E., Weis, F., Briegel, J., Frey, L., Goetz, A. E., Reuter, D., . . . Peter, K. (2003). Stress doses of hydrocortisone reduce severe systemic inflammatory response syndrome and improve early outcome in a risk group of patients after cardiac surgery. *Crit Care Med, 31*(4), 1068-1074. doi: 10.1097/01.ccm.0000059646.89546.98

Kinkhabwala, M. P., & Mancini, D. (2013). Patient selection for cardiac transplant in 2012. *Expert review of cardiovascular therapy, 11*(2), 179-191.

Klein, A. S., Messersmith, E. E., Ratner, L. E., Kochik, R., Baliga, P. K., & Ojo, A. O. (2010). Organ donation and utilization in the United States, 1999-2008. *Am J Transplant, 10*(4 Pt 2), 973-986. doi: 10.1111/j.1600-6143.2009.03008.x

Kolattukudy, P. E., & Niu, J. (2012). Inflammation, endoplasmic reticulum stress, autophagy, and the monocyte chemoattractant protein-1/CCR2 pathway. *Circ Res, 110*(1), 174-189. doi: 10.1161/circresaha.111.243212

Kopp, R., Mottaghy, K., & Kirschfink, M. (2002). Mechanism of complement activation during extracorporeal blood-biomaterial interaction: effects of heparin coated and uncoated surfaces. *Asaio j, 48*(6), 598-605. doi: 10.1097/00002480-200211000-00005

Kozak, L. J., DeFrances, C. J., & Hall, M. J. (2006). National hospital discharge survey: 2004 annual summary with detailed diagnosis and procedure data. *Vital Health Stat 13*(162), 1-209.

Kubota, T., Miyagishima, M., Alvarez, R. J., Kormos, R., Rosenblum, W. D., Demetris, A. J., . . . McNamara, D. M. (2000). Expression of proinflammatory cytokines in the failing human heart: comparison of recent-onset and end-stage congestive heart failure. *J Heart Lung Transplant, 19*(9), 819-824.

Kumar, P., Morton, J. S., Shah, A., Do, V., Sergi, C., Serrano-Lomelin, J., . . . Hornberger, L. K. (2020). Intrauterine exposure to chronic hypoxia in the rat leads to progressive diastolic function and increased aortic stiffness from early postnatal developmental stages. *8*(1), e14327. doi: 10.14814/phy2.14327

Kumar, S., & Maulik, S. (2017). Role of cytokines in heart failure. *PHARMACOL REP, 2*(1).

Kunisch, E., Fuhrmann, R., Roth, A., Winter, R., Lungershausen, W., & Kinne, R. W. (2004). Macrophage specificity of three anti-CD68 monoclonal antibodies (KP1, EBM11, and PGM1) widely used for immunohistochemistry and flow cytometry. *Ann Rheum Dis, 63*(7), 774-784. doi: 10.1136/ard.2003.013029

Kuzmiak-Glancy, S., Jaimes, R., 3rd, Wengrowski, A. M., & Kay, M. W. (2015). Oxygen demand of perfused heart preparations: how electromechanical function and inadequate oxygenation affect physiology and optical measurements. *Exp Physiol, 100*(6), 603-616. doi: 10.1113/ep085042

Latchana, N., Peck, J. R., Whitson, B., & Black, S. M. (2014). Preservation solutions for cardiac and pulmonary donor grafts: a review of the current literature. *J Thorac Dis, 6*(8), 1143-1149. doi: 10.3978/j.issn.2072-1439.2014.05.14

Laumet, G., Ma, J., Robison, A. J., Kumari, S., Heijnen, C. J., & Kavelaars, A. (2019). T cells as an emerging target for chronic pain therapy. *Front Mol Neurosci, 12*, 216. doi: 10.3389/fnmol.2019.00216

Lee, W. C., Chavez, Y. E., Baker, T., & Luce, B. R. (2004). Economic burden of heart failure: a summary of recent literature. *Heart Lung, 33*(6), 362-371.

Lemler, M. S., Bies, R. D., Frid, M. G., Sastravaha, A., Zisman, L. S., Bohlmeyer, T., . . . Stenmark, K. R. (2000). Myocyte cytoskeletal disorganization and right heart failure in hypoxia-induced neonatal pulmonary hypertension. *Am J Physiol Heart Circ Physiol, 279*(3), H1365-1376. doi: 10.1152/ajpheart.2000.279.3.H1365

Li, H., Zuo, J., & Tang, W. (2018). Phosphodiesterase-4 Inhibitors for the Treatment of Inflammatory Diseases. *Front Pharmacol, 9*, 1048. doi: 10.3389/fphar.2018.01048

Li, J. S., Jaggers, J., & Anderson, P. A. W. (2006). The use of TP10, soluble complement receptor 1, in cardiopulmonary bypass. *Expert review of cardiovascular therapy, 4*(5), 649-654. doi: 10.1586/14779072.4.5.649

Li, Y., Ge, S., Peng, Y., & Chen, X. (2013). Inflammation and cardiac dysfunction during sepsis, muscular dystrophy, and myocarditis. *Burns Trauma, 1*(3), 109-121. doi: 10.4103/2321-3868.123072

Liakopoulos, O. J., Schmitto, J. D., Kazmaier, S., Brauer, A., Quintel, M., Schoendube, F. A., & Dorge, H. (2007). Cardiopulmonary and systemic effects of methylprednisolone in patients undergoing cardiac surgery. *Ann Thorac Surg, 84*(1), 110-118; discussion 118-119. doi: 10.1016/j.athoracsur.2007.01.003

Liang, H. Y., Cauduro, S. A., Pellikka, P. A., Bailey, K. R., Grossardt, B. R., Yang, E. H., . . . Abraham, T. P. (2006). Comparison of usefulness of echocardiographic Doppler variables to left ventricular end-diastolic pressure in predicting future heart failure events. *Am J Cardiol, 97*(6), 866-871.

Liao, R., Podesser, B. K., & Lim, C. C. (2012). The continuing evolution of the Langendorff and ejecting murine heart: new advances in cardiac phenotyping. *Am J Physiol Heart Circ Physiol, 303*(2), H156-167.

Lips, D. J., van der Nagel, T., Steendijk, P., Palmen, M., Janssen, B. J., van Dantzig, J. M., . . . Doevendans, P. A. (2004). Left ventricular pressure-volume measurements in mice: comparison of closed-chest versus open-chest approach. *Basic Res Cardiol, 99*(5), 351-359. doi: 10.1007/s00395-004-0476-5

Liu, C. W., Yang, F., Cheng, S. Z., Liu, Y., Wan, L. H., & Cong, H. L. (2017). Rosuvastatin postconditioning protects isolated hearts against ischemia-reperfusion injury: The role of radical oxygen species, PI3K-Akt-GSK-3beta pathway, and mitochondrial permeability transition pore. *Cardiovasc Ther, 35*(1), 3-9. doi: 10.1111/1755-5922.12225

Liu, J. H., Shen, J. M., Li, L., & Chang, Y. T. (2005). [Effects of fentanyl on cytokines and MDA during cardiopulmonary bypass in patients undergoing valve replacement]. *Zhong Nan Da Xue Xue Bao Yi Xue Ban, 30*(1), 80-83.

Liu, M., & Dudley, S. C., Jr. (2015). Role for the unfolded protein response in heart disease and cardiac arrhythmias. *Int J Mol Sci, 17*(1). doi: 10.3390/ijms17010052

Lloyd-Jones, D., Adams, R. J., Brown, T. M., Carnethon, M., Dai, S., De Simone, G., . . . Wylie-Rosett, J. (2010). Executive summary: heart disease and stroke statistics--2010 update: a report from the American Heart Association. *Circulation, 121*(7), 948-954.

Longnus, S. L., Mathys, V., Dornbierer, M., Dick, F., Carrel, T. P., & Tevaearai, H. T. (2014). Heart transplantation with donation after circulatory determination of death. *Nat Rev Cardiol, 11*(6), 354-363.

Lopaschuk, G. D., Ussher, J. R., Folmes, C. D., Jaswal, J. S., & Stanley, W. C. (2010). Myocardial fatty acid metabolism in health and disease. *Physiol Rev, 90*(1), 207-258. doi: 10.1152/physrev.00015.2009

Lucchinetti, E., Lou, P. H., Hatami, S., Qi, X., Clanachan, A. S., Freed, D. H., & Zaugg, M. (2019). Enhanced myocardial protection in cardiac donation after circulatory death using Intralipid((R)) postconditioning in a porcine model. *Can J Anaesth*. doi: 10.1007/s12630-019-01322-x

Mahalwar, R., & Khanna, D. (2013). Pleiotropic antioxidant potential of rosuvastatin in preventing cardiovascular disorders. *Eur J Pharmacol, 711*(1-3), 57-62. doi: 10.1016/j.ejphar.2013.04.025

Mahmood, S., Bilal, H., Zaman, M., & Tang, A. (2012). Is a fully heparin-bonded cardiopulmonary bypass circuit superior to a standard cardiopulmonary bypass circuit? *Interact Cardiovasc Thorac Surg, 14*(4), 406-414. doi: 10.1093/icvts/ivr124

Malanga, C. J. (2007). Structure and function of the heart. In S. J. Enna & D. B. Bylund (Eds.), *XPharm: The Comprehensive Pharmacology Reference* (pp. 1-5): Elsevier.

Mallet, R. T., Sun, J., Knott, E. M., Sharma, A. B., & Olivencia-Yurvati, A. H. (2005). Metabolic cardioprotection by pyruvate: recent progress. *Exp Biol Med (Maywood), 230*(7), 435-443.

Manara, A. R., Murphy, P. G., & O'Callaghan, G. (2012). Donation after circulatory death. *Br J Anaesth, 108 Suppl 1*, i108-121.

Mangano, D. T., Rieves, R. D., & Weiss, K. D. (2006). Judging the safety of aprotinin. *N Engl J Med, 355*(21), 2261-2262. doi: 10.1056/NEJMc066520

Mant, J., Doust, J., Roalfe, A., Barton, P., Cowie, M. R., Glasziou, P., . . . Hobbs, F. D. (2009). Systematic review and individual patient data meta-analysis of diagnosis of heart failure, with modelling of implications of different diagnostic strategies in primary care. *Health Technol Assess, 13*(32), 1-207, iii.

Mantovani, A., Dinarello, C. A., Molgora, M., & Garlanda, C. (2019). Interleukin-1 and related cytokines in the regulation of inflammation and immunity. *Immunity, 50*(4), 778-795. doi: 10.1016/j.immuni.2019.03.012

Marasco, S., Kras, A., Schulberg, E., Vale, M., Chan, P., Lee, G. A., & Bailey, M. (2013). Donor brain death time and impact on outcomes in heart transplantation. *Transplant Proc, 45*(1), 33-37.

Mariani, A. W., Pego-Fernandes, P. M., Abdalla, L. G., & Jatene, F. B. (2012). Ex vivo lung reconditioning: a new era for lung transplantation. *J Bras Pneumol, 38*(6), 776-785. doi: 10.1590/s1806-37132012000600015

Martindale, J. J., Fernandez, R., Thuerauf, D., Whittaker, R., Gude, N., Sussman, M. A., & Glembotski, C. C. (2006). Endoplasmic reticulum stress gene induction and protection from ischemia/reperfusion injury in the hearts of transgenic mice with a tamoxifen-regulated form of ATF6. *Circ Res, 98*(9), 1186-1193. doi: 10.1161/01.RES.0000220643.65941.8d

Masson, S., Anand, I., Favero, C., Barlera, S., Vago, T., Bertocchi, F., . . . Latini, R. (2012). Serial measurement of cardiac troponin T using a highly sensitive assay in patients with chronic heart failure: data from 2 large randomized clinical trials. *Circulation, 125*(2), 280-288.

Mc, I. R. B., Timpa, J. G., Kurundkar, A. R., Holt, D. W., Kelly, D. R., Hartman, Y. E., . . . Maheshwari, A. (2010). Plasma concentrations of inflammatory cytokines rise rapidly during ECMO-related SIRS

due to the release of preformed stores in the intestine. *Lab Invest, 90*(1), 128-139. doi: 10.1038/labinvest.2009.119

McBride, W. T., Armstrong, M. A., Gilliland, H., & McMurray, T. J. (1996). The balance of pro and anti-inflammatory cytokines in plasma and bronchoalveolar lavage (BAL) at paediatric cardiac surgery. *Cytokine, 8*(9), 724-729. doi: 10.1006/cyto.1996.0096

McDonagh, T. A., Morrison, C. E., Lawrence, A., Ford, I., Tunstall-Pedoe, H., McMurray, J. J., & Dargie, H. J. (1997). Symptomatic and asymptomatic left-ventricular systolic dysfunction in an urban population. *Lancet, 350*(9081), 829-833.

McDonald, C. I., Fraser, J. F., Coombes, J. S., & Fung, Y. L. (2014). Oxidative stress during extracorporeal circulation. *Eur J Cardiothorac Surg, 46*(6), 937-943. doi: 10.1093/ejcts/ezt637

McGuire, M., & Bradford, A. (1999). Chronic intermittent hypoxia increases haematocrit and causes right ventricular hypertrophy in the rat. *Respir Physiol, 117*(1), 53-58. doi: 10.1016/s0034-5687(99)00047-x

McKee, P. A., Castelli, W. P., McNamara, P. M., & Kannel, W. B. (1971). The natural history of congestive heart failure: the Framingham study. *N Engl J Med, 285*(26), 1441-1446.

McKelvie, R. S., Moe, G. W., Ezekowitz, J. A., Heckman, G. A., Costigan, J., Ducharme, A., . . . Sussex, B. (2013). The 2012 Canadian Cardiovascular Society heart failure management guidelines update: focus on acute and chronic heart failure. *Can J Cardiol, 29*(2), 168-181.

McKeown, D. W., Bonser, R. S., & Kellum, J. A. (2012). Management of the heartbeating brain-dead organ donor. *Br J Anaesth, 108 Suppl 1*, i96-107.

McMurray, J. J., Adamopoulos, S., Anker, S. D., Auricchio, A., Bohm, M., Dickstein, K., . . . Ponikowski, P. (2012). ESC guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC. *Eur J Heart Fail, 14*(8), 803-869.

McNulty, P. H. (2007). Hexosamine biosynthetic pathway flux and cardiomyopathy in type 2 diabetes mellitus. Focus on "Impact of type 2 diabetes and aging on cardiomyocyte function and O-linked N-acetylglucosamine levels in the heart". *Am J Physiol Cell Physiol, 292*(4), C1243-1244. doi: 10.1152/ajpcell.00521.2006

Messer, S., Ardehali, A., & Tsui, S. (2015). Normothermic donor heart perfusion: current clinical experience and the future. *Transpl Int, 28*(6), 634-642. doi: 10.1111/tri.12361

Messer, S., Page, A., Axell, R., Berman, M., Hernandez-Sanchez, J., Colah, S., . . . Large, S. R. (2017). Outcome after heart transplantation from donation after circulatory-determined death donors. *J Heart Lung Transplant, 36*(12), 1311-1318. doi: 10.1016/j.healun.2017.10.021

Messer, S., Page, A., Colah, S., Axell, R., Parizkova, B., Tsui, S., & Large, S. (2018). Human heart transplantation from donation after circulatory-determined death donors using normothermic regional perfusion and cold storage. *J Heart Lung Transplant, 37*(7), 865-869. doi: 10.1016/j.healun.2018.03.017

Messer, S. J., Axell, R. G., Colah, S., White, P. A., Ryan, M., Page, A. A., . . . Large, S. R. (2016). Functional assessment and transplantation of the donor heart after circulatory death. *J Heart Lung Transplant, 35*(12), 1443-1452. doi: 10.1016/j.healun.2016.07.004

Metra, M., Bettari, L., Pagani, F., Lazzarini, V., Lombardi, C., Carubelli, V., . . . Dei Cas, L. (2012). Troponin T levels in patients with acute heart failure: clinical and prognostic significance of their detection and release during hospitalisation. *Clin Res Cardiol, 101*(8), 663-672.

Michel, S. G., La Muraglia, G. M., 2nd, Madariaga, M. L., Titus, J. S., Selig, M. K., Farkash, E. A., . . . Madsen, J. C. (2014). Preservation of donor hearts using hypothermic oxygenated perfusion. *Ann Transplant, 19*, 409-416. doi: 10.12659/aot.890797

Michel, S. G., La Muraglia, G. M., 2nd, Madariaga, M. L., Titus, J. S., Selig, M. K., Farkash, E. A., . . . Madsen, J. C. (2015). Twelve-hour hypothermic machine perfusion for donor heart preservation leads to improved ultrastructural characteristics compared to conventional cold storage. *Ann Transplant, 20*, 461-468. doi: 10.12659/aot.893784

Millar, J. E., Fanning, J. P., McDonald, C. I., McAuley, D. F., & Fraser, J. F. (2016). The inflammatory response to extracorporeal membrane oxygenation (ECMO): a review of the pathophysiology. *Crit Care, 20*(1), 387. doi: 10.1186/s13054-016-1570-4

Minamino, T., Komuro, I., & Kitakaze, M. (2010). Endoplasmic reticulum stress as a therapeutic target in cardiovascular disease. *Circ Res, 107*(9), 1071-1082. doi: 10.1161/circresaha.110.227819

Minasian, S. M., Galagudza, M. M., Dmitriev, Y. V., Karpov, A. A., & Vlasov, T. D. (2015). Preservation of the donor heart: from basic science to clinical studies. *Interact Cardiovasc Thorac Surg, 20*(4), 510-519. doi: 10.1093/icvts/ivu432

Mojcik, C. F., & Levy, J. H. (2001). Aprotinin and the systemic inflammatory response after cardiopulmonary bypass. *Ann Thorac Surg, 71*(2), 745-754. doi: 10.1016/S0003-4975(00)02218-9

Mollnes, T. E., Videm, V., Gotze, O., Harboe, M., & Oppermann, M. (1991). Formation of C5a during cardiopulmonary bypass: inhibition by precoating with heparin. *Ann Thorac Surg, 52*(1), 92-97. doi: 10.1016/0003-4975(91)91426-v

Monteagudo Vela, M., Garcia Saez, D., & Simon, A. R. (2018). Current approaches in retrieval and heart preservation. *Ann Cardiothorac Surg, 7*(1), 67-74. doi: 10.21037/acs.2018.01.06

Morgan, C., Zappitelli, M., & Gill, P. (2009). Statin prophylaxis and inflammatory mediators following cardiopulmonary bypass: a systematic review. *Crit Care, 13*(5), R165. doi: 10.1186/cc8135

Morgan, H. P., O'Reilly, F. J., Wear, M. A., O'Neill, J. R., Fothergill-Gilmore, L. A., Hupp, T., & Walkinshaw, M. D. (2013). M2 pyruvate kinase provides a mechanism for nutrient sensing and regulation of cell proliferation. *Proc Natl Acad Sci U S A, 110*(15), 5881-5886. doi: 10.1073/pnas.1217157110

Morita, S. (2018). Is there a crystal ball for predicting the outcome of cardiomyopathy surgery? Preload recruitable stroke work, may be a possible candidate. *J Cardiol, 71*(4), 325-326. doi: 10.1016/j.jjcc.2017.11.001

Mossanen, J. C., Jansen, T. U., Pracht, J., Liepelt, A., Buendgens, L., Stoppe, C., . . . Tacke, F. (2020). Elevated circulating CD14(++)CD16(+) intermediate monocytes are independently associated with extracardiac complications after cardiac surgery. *Sci Rep, 10*(1), 947. doi: 10.1038/s41598-020-57700-9

Murray, C. I., & Van Eyk, J. E. (2012). Chasing cysteine oxidative modifications: proteomic tools for characterizing cysteine redox status. *Circ Cardiovasc Genet, 5*(5), 591. doi: 10.1161/circgenetics.111.961425

Murthy, S., & Lipman, H. I. (2011). Management of end-stage heart failure. *Prim Care, 38*(2), 265-276, viii.

Nain, S., Ling, B., Bandy, B., Alcorn, J., Wojnarowicz, C., Laarveld, B., & Olkowski, A. A. (2008). The role of oxidative stress in the development of congestive heart failure in a chicken genotype selected for rapid growth. *Avian Pathol, 37*(4), 367-373. doi: 10.1080/03079450802179850

National Institute for Health and Care Excellence. (2016) *OCS Heart system for heart transplant*. London.

Nayyar, S., Magalski, A., Khumri, T. M., Idupulapati, M., Stoner, C. N., Kusnetzky, L. L., . . . Main, M. L. (2006). Contrast administration reduces interobserver variability in determination of left ventricular ejection fraction in patients with left ventricular dysfunction and good baseline endocardial border delineation. *Am J Cardiol, 98*(8), 1110-1114.

Neri, M., Riezzo, I., Pomara, C., Schiavone, S., & Turillazzi, E. (2016). Oxidative-Nitrosative Stress and Myocardial Dysfunctions in Sepsis: Evidence from the Literature and Postmortem Observations. *Mediators Inflamm, 2016*, 3423450. doi: 10.1155/2016/3423450

Neviere, R., Fauvel, H., Chopin, C., Formstecher, P., & Marchetti, P. (2001). Caspase inhibition prevents cardiac dysfunction and heart apoptosis in a rat model of sepsis. *Am J Respir Crit Care Med, 163*(1), 218-225. doi: 10.1164/ajrccm.163.1.2003109

Nickel, A., Kohlhaas, M., & Maack, C. (2014). Mitochondrial reactive oxygen species production and elimination. *J Mol Cell Cardiol, 73*, 26-33. doi: 10.1016/j.yjmcc.2014.03.011

Nieminen, M. S., Brutsaert, D., Dickstein, K., Drexler, H., Follath, F., Harjola, V. P., . . . Tavazzi, L. (2006). EuroHeart Failure Survey II (EHFS II): a survey on hospitalized acute heart failure patients: description of population. *Eur Heart J, 27*(22), 2725-2736.

Noda, K., Tane, S., Haam, S. J., D'Cunha, J., Hayanga, A. J., Luketich, J. D., & Shigemura, N. (2017). Targeting circulating leukocytes and pyroptosis during ex vivo lung perfusion improves lung preservation. *Transplantation, 101*(12), 2841-2849. doi: 10.1097/tp.0000000000001798

Ochiai, K., Zhang, J., Gong, G., Zhang, Y., Liu, J., Ye, Y., . . . From, A. H. (2001). Effects of augmented delivery of pyruvate on myocardial high-energy phosphate metabolism at high workstate. *Am J Physiol Heart Circ Physiol, 281*(4), H1823-1832.

Ohata, T., Sawa, Y., Kadoba, K., Taniguchi, K., Ichikawa, H., Masai, T., . . . Matsuda, H. (1995). Normothermia has beneficial effects in cardiopulmonary bypass attenuating inflammatory reactions. *Asaio j, 41*(3), M288-291. doi: 10.1097/00002480-199507000-00014

Olejnickova, V., Novakova, M., & Provaznik, I. (2015). Isolated heart models: cardiovascular system studies and technological advances. *Med Biol Eng Comput, 53*(7), 669-678. doi: 10.1007/s11517-015-1270-2

Ontaneda, A., & Annich, G. M. (2018). Novel surfaces in extracorporeal membrane oxygenation circuits. *Front Med (Lausanne), 5*, 321. doi: 10.3389/fmed.2018.00321

Orhan, G., Yapici, N., Yuksel, M., Sargin, M., Şenay, Ş., Yalçin, A. S., . . . Aka, S. A. (2006). Effects of N-acetylcysteine on myocardial ischemia–reperfusion injury in bypass surgery. *Heart and Vessels, 21*(1), 42-47. doi: 10.1007/s00380-005-0873-1

Osadchii, O. E., Woodiwiss, A. J., & Norton, G. R. (2006). Contractile responses to selective phosphodiesterase inhibitors following chronic beta-adrenoreceptor activation. *Pflugers Arch, 452*(2), 155-163. doi: 10.1007/s00424-005-0025-6

Ozeki, T., Kwon, M. H., Gu, J., Collins, M. J., Brassil, J. M., Miller, M. B., Jr., . . . Poston, R. S. (2007). Heart preservation using continuous ex vivo perfusion improves viability and functional recovery. *Circ J, 71*(1), 153-159.

Page, A., Messer, S., Axell, R., Naruka, V., Colah, S., Fakelman, S., . . . Large, S. (2017). Does the assessment of DCD donor hearts on the Organ Care System using lactate need redefining? *The Journal of Heart and Lung Transplantation, 36*(4), S16-S17. doi: 10.1016/j.healun.2017.01.030

Page, A., Messer, S., & Large, S. R. (2018). Heart transplantation from donation after circulatory determined death. *Ann Cardiothorac Surg, 7*(1), 75-81. doi: 10.21037/acs.2018.01.08

Panagiotopoulos, I., Palatianos, G., Michalopoulos, A., Chatzigeorgiou, A., Prapas, S., & Kamper, E. F. (2010). Alterations in biomarkers of endothelial function following on-pump coronary artery revascularization. *J Clin Lab Anal, 24*(6), 389-398. doi: 10.1002/jcla.20416

Pappalardo, F., & Montisci, A. (2018). Adjunctive therapies during veno-venous extracorporeal membrane oxygenation. *J Thorac Dis, 10*(Suppl 5), S683-s691. doi: 10.21037/jtd.2017.10.08

Passaroni, A. C., Felicio, M. L., Campos, N., Silva, M. A. M., & Yoshida, W. B. (2018). Hemolysis and inflammatory response to extracorporeal circulation during On-Pump CABG: comparison between roller and centrifugal pump systems. *Braz J Cardiovasc Surg, 33*(1), 64-71. doi: 10.21470/1678-9741-2017-0125

Passmore, M. R., Fung, Y. L., Simonova, G., Foley, S. R., Dunster, K. R., Diab, S. D., . . . Fraser, J. F. (2016). Inflammation and lung injury in an ovine model of extracorporeal membrane oxygenation

support. *Am J Physiol Lung Cell Mol Physiol, 311*(6), L1202-l1212. doi: 10.1152/ajplung.00296.2016

Patarroyo-Aponte, M., & Colvin-Adams, M. (2014). Evaluation of patients with heart failure. *Cardiol Clin, 32*(1), 47-62.

Paterson, I., Mielniczuk, L. M., O'Meara, E., So, A., & White, J. A. (2013). Imaging heart failure: current and future applications. *Can J Cardiol, 29*(3), 317-328.

Pathak, M., Kaira, B. G., Slater, A., & Emsley, J. (2018). Cell receptor and cofactor interactions of the contact activation system and factor XI. *Front Med (Lausanne), 5*, 66. doi: 10.3389/fmed.2018.00066

Paulus, W. J., Tschope, C., Sanderson, J. E., Rusconi, C., Flachskampf, F. A., Rademakers, F. E., . . . Brutsaert, D. L. (2007). How to diagnose diastolic heart failure: a consensus statement on the diagnosis of heart failure with normal left ventricular ejection fraction by the Heart Failure and Echocardiography Associations of the European Society of Cardiology. *Eur Heart J, 28*(20), 2539-2550.

Peltz, M., He, T. T., Adams, G. A. t., Koshy, S., Burgess, S. C., Chao, R. Y., . . . Jessen, M. E. (2005). Perfusion preservation maintains myocardial ATP levels and reduces apoptosis in an ex vivo rat heart transplantation model. *Surgery, 138*(4), 795-805. doi: 10.1016/j.surg.2005.06.040

Perttila, J., Salo, M., Pirttikangas, C. O., Jalonen, J., & Vainio, O. (1994). Effects of cardiopulmonary bypass on lymphocytes and their subset counts with or without use of autotransfusion devices. *J Cardiothorac Vasc Anesth, 8*(5), 532-535. doi: 10.1016/1053-0770(94)90165-1

Philippidis, P., Athanasiou, T., Nadra, I., Ashrafian, H., Haskard, D. O., Landis, R. C., & Taylor, K. M. (2010). Anti-inflammatory haemoglobin scavenging monocytes are induced following coronary artery bypass surgery. *Eur J Cardiothorac Surg, 37*(6), 1360-1366. doi: 10.1016/j.ejcts.2009.12.043

Poston, R. S., Gu, J., Prastein, D., Gage, F., Hoffman, J. W., Kwon, M., . . . Griffith, B. P. (2004). Optimizing donor heart outcome after prolonged storage with endothelial function analysis and continuous perfusion. *Ann Thorac Surg, 78*(4), 1362-1370;; discussion 1362-1370. doi: 10.1016/j.athoracsur.2004.02.143

Prompunt, E., Sanit, J., Barrère-Lemaire, S., Nargeot, J., Noordali, H., Madhani, M., & Kumphune, S. (2018). The cardioprotective effects of secretory leukocyte protease inhibitor against myocardial ischemia/reperfusion injury. *Exp Ther Med, 15*(6), 5231-5242. doi: 10.3892/etm.2018.6097

Puri N, Baram M, & N, C. (2017). *Extracorporeal membrane oxygenation (ECMO), an issue of critical care clinics* Vol. 33. K. Pfaff (Ed.)

Putnam, J. B., Royston, D., Chambers, A. F., Dunbar, S., Lemmer, J. H., Norman, P., . . . Zacharski, L. R. (2003). Evaluating the role of serine protease inhibition in the management of tumor micrometastases. *Oncology (Williston Park), 17*(10 Suppl 10), 9-30; quiz 31-32.

Putzu, A., Capelli, B., Belletti, A., Cassina, T., Ferrari, E., Gallo, M., . . . Landoni, G. (2016). Perioperative statin therapy in cardiac surgery: a meta-analysis of randomized controlled trials. *Critical Care, 20*(1), 395. doi: 10.1186/s13054-016-1560-6

Putzu, A., de Carvalho e Silva, C. M. P. D., de Almeida, J. P., Belletti, A., Cassina, T., Landoni, G., & Hajjar, L. A. (2018). Perioperative statin therapy in cardiac and non-cardiac surgery: a systematic review and meta-analysis of randomized controlled trials. *Annals of Intensive Care, 8*(1), 95. doi: 10.1186/s13613-018-0441-3

Pyles, L. A., Gustafson, R. A., Fortney, J., & Einzig, S. (2010). Extracorporeal membrane oxygenation induced cardiac dysfunction in newborn lambs. *J Cardiovasc Transl Res, 3*(6), 625-634. doi: 10.1007/s12265-010-9215-5

Rabinovitch, R. C., Samborska, B., Faubert, B., Ma, E. H., Gravel, S. P., Andrzejewski, S., . . . Jones, R. G. (2017). AMPK maintains cellular metabolic homeostasis through regulation of mitochondrial reactive oxygen species. *Cell Rep, 21*(1), 1-9. doi: 10.1016/j.celrep.2017.09.026

Raffaeli, G., Ghirardello, S., Passera, S., Mosca, F., & Cavallaro, G. (2018). Oxidative stress and neonatal respiratory extracorporeal membrane oxygenation. *Front Physiol, 9*(1739). doi: 10.3389/fphys.2018.01739

Rajamani, U., & Essop, M. F. (2010). Hyperglycemia-mediated activation of the hexosamine biosynthetic pathway results in myocardial apoptosis. *Am J Physiol Cell Physiol, 299*(1), C139-147. doi: 10.1152/ajpcell.00020.2010

Ranucci, M., & Baryshnikova, E. (2019). Inflammation and coagulation following minimally invasive extracorporeal circulation technologies. *J Thorac Dis, 11*(Suppl 10), S1480-s1488. doi: 10.21037/jtd.2019.01.27

Rees, M. L., Subramaniam, J., Li, Y., Hamilton, D. J., Frazier, O. H., & Taegtmeyer, H. (2015). A PKM2 signature in the failing heart. *Biochem Biophys Res Commun, 459*(3), 430-436. doi: 10.1016/j.bbrc.2015.02.122

Reffelmann, T., & Kloner, R. A. (2009). Phosphodiesterase 5 inhibitors: are they cardioprotective? *Cardiovasc Res, 83*(2), 204-212. doi: 10.1093/cvr/cvp170

Rehman, I., & Rehman, A. (2019). *Anatomy, Thorax, Pericardium*. Treasure Island (FL): StatPearls Publishing LLC.

Ribeiro, R., Gomes, B., Hondjeu, A., Yu, F., Adamson, M., Ribeiro, R., . . . Badiwala, M. (2019). Assessment of cerebral perfusion and activity during normothermic regional perfusion in a porcine model of donation after circulatory death. *Canadian Journal of cardiology, 35*(10), S8-S9. doi: 10.1016/j.cjca.2019.07.064

Ricci, Z. (2018). Hemolysis during pediatric cardiac surgery: an old issue with renewed concerns. *Journal of Laboratory and Precision Medicine, 3*(2).

Rietzschel, E. R., Langlois, M., De Buyzere, M. L., Segers, P., De Bacquer, D., Bekaert, S., . . . Gillebert, T. C. (2008). Oxidized low-density lipoprotein cholesterol is associated with decreases in cardiac function independent of vascular alterations. *Hypertension, 52*(3), 535-541. doi: 10.1161/hypertensionaha.108.114439

Rinder, C. S., Gaal, D., Student, L. A., & Smith, B. R. (1994). Platelet-leukocyte activation and modulation of adhesion receptors in pediatric patients with congenital heart disease undergoing cardiopulmonary bypass. *J Thorac Cardiovasc Surg, 107*(1), 280-288.

Rinder, C. S., Rinder, H. M., Johnson, K., Smith, M., Lee, D. L., Tracey, J., . . . Smith, B. R. (1999). Role of C3 cleavage in monocyte activation during extracorporeal circulation. *Circulation, 100*(5), 553-558. doi: 10.1161/01.cir.100.5.553

Rinder, C. S., Rinder, H. M., Smith, M. J., Fitch, J. C., Tracey, J. B., Chandler, W. L., . . . Smith, B. R. (2006). Antithrombin reduces monocyte and neutrophil CD11b up regulation in addition to blocking platelet activation during extracorporeal circulation. *Transfusion, 46*(7), 1130-1137. doi: 10.1111/j.1537-2995.2006.00861.x

Rivard, A. L., Gallegos, R., Ogden, I. M., & Bianco, R. W. (2009). Perfusion preservation of the donor heart: basic science to pre-clinical. *J Extra Corpor Technol, 41*(3), 140-148.

Rivard, L., Gallegos, R., Ogden, I., & Bianco, R. (2009). Perfusion preservation of the donor heart: basic science to pre-clinical. *J Extra Corpor Technol., 41*(3), 140-148.

Robertson-Malt, S., Afrane, B., & El Barbary, M. (2007). Prophylactic steroids for pediatric open heart surgery. *Cochrane Database Syst Rev*(4), Cd005550. doi: 10.1002/14651858.CD005550.pub2

Robinson, T. F., Factor, S. M., & Sonnenblick, E. H. (1986). The heart as a suction pump. *Sci Am, 254*(6), 84-91. doi: 10.1038/scientificamerican0686-84

Roger, V. L. (2013). Epidemiology of heart failure. *Circ Res, 113*(6), 646-659.

Roger, V. L., Weston, S. A., Redfield, M. M., Hellermann-Homan, J. P., Killian, J., Yawn, B. P., & Jacobsen, S. J. (2004). Trends in heart failure incidence and survival in a community-based population. *Jama, 292*(3), 344-350.

Rohde, L. E., Bertoldi, E. G., Goldraich, L., & Polanczyk, C. A. (2013). Cost-effectiveness of heart failure therapies. *Nat Rev Cardiol, 10*(6), 338-354.

Rosenbaum, D. H., Peltz, M., DiMaio, J. M., Meyer, D. M., Wait, M. A., Merritt, M. E., . . . Jessen, M. E. (2008). Perfusion preservation versus static preservation for cardiac transplantation: effects on myocardial function and metabolism. *J Heart Lung Transplant, 27*(1), 93-99. doi: 10.1016/j.healun.2007.10.006

Rosenfeldt, F., Ou, R., Salamonsen, R., Marasco, S., Zimmet, A., Byrne, J., . . . Esmore, D. (2016). A novel combination technique of cold crystalloid perfusion but not cold storage facilitates transplantation of canine hearts donated after circulatory death. *J Heart Lung Transplant, 35*(11), 1358-1364. doi: 10.1016/j.healun.2016.03.015

Rosenfeldt, F., Ou, R., Woodard, J., Esmore, D., & Marasco, S. (2014). Twelve-hour reanimation of a human heart following donation after circulatory death. *Heart Lung Circ, 23*(1), 88-90. doi: 10.1016/j.hlc.2013.07.011

Ross, L. F., & Frader, J. (2011). Are we ready to expand donation after cardiac death to the newborn population? *J Pediatr, 158*(1), 6-8.

Sagach, V. F., Scrosati, M., Fielding, J., Rossoni, G., Galli, C., & Visioli, F. (2002). The water-soluble vitamin E analogue Trolox protects against ischaemia/reperfusion damage in vitro and ex vivo. A comparison with vitamin E. *Pharmacol Res, 45*(6), 435-439. doi: 10.1006/phrs.2002.0993

Salameh, A., & Dhein, S. (2015). Strategies for pharmacological organoprotection during extracorporeal circulation targeting ischemia-reperfusion injury. *Front Pharmacol, 6*, 296. doi: 10.3389/fphar.2015.00296

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

Sandha, J. K., White, C. W., Muller, A., Avery, E., Thliveris, J., Dixon, I. M. C., . . . Freed, D. H. (2018a). Steroids Limit Myocardial Edema During Ex Vivo Perfusion Of Hearts Donated After Circulatory Death. *Ann Thorac Surg*. doi: 10.1016/j.athoracsur.2018.01.004

Sandha, J. K., White, C. W., Muller, A., Avery, E., Thliveris, J., Dixon, I. M. C., . . . Freed, D. H. (2018b). Steroids limit myocardial edema during ex vivo perfusion of hearts donated after circulatory death. *Ann Thorac Surg, 105*(6), 1763-1770. doi: 10.1016/j.athoracsur.2018.01.004

Sano, R., & Reed, J. C. (2013). ER stress-induced cell death mechanisms. *Biochim Biophys Acta, 1833*(12), 3460-3470. doi: 10.1016/j.bbamcr.2013.06.028

Santos, C. X., Anilkumar, N., Zhang, M., Brewer, A. C., & Shah, A. M. (2011). Redox signaling in cardiac myocytes. *Free Radic Biol Med, 50*(7), 777-793. doi: 10.1016/j.freeradbiomed.2011.01.003

Savarese, G., & Lund, L. H. (2017). Global public health burden of heart failure. *Card Fail Rev, 3*(1), 7-11. doi: 10.15420/cfr.2016:25:2

Sbrana, S., Parri, M. S., De Filippis, R., Gianetti, J., & Clerico, A. (2004). Monitoring of monocyte functional state after extracorporeal circulation: a flow cytometry study. *Cytometry B Clin Cytom, 58*(1), 17-24. doi: 10.1002/cyto.b.10061

Schneeberger, S., Amberger, A., Mandl, J., Hautz, T., Renz, O., Obrist, P., . . . Kuznetsov, A. V. (2010). Cold ischemia contributes to the development of chronic rejection and mitochondrial injury after cardiac transplantation. *Transpl Int, 23*(12), 1282-1292.

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods, 9*(7), 671-675. doi: 10.1038/nmeth.2089

Schraufnagel, D. P., Steffen, R. J., Vargo, P. R., Attia, T., Elgharably, H., Hasan, S. M., . . . Wierup, P. (2018). Devices for ex vivo heart and lung perfusion. *Expert Rev Med Devices, 15*(3), 183-191. doi: 10.1080/17434440.2018.1430568

Schroder, J. N., D'Alessandro, D., Esmailian, F., Boeve, T., Tang, P., Liao, K., . . . Smith, J. W. (2019). 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, 38*(4), S42. doi: 10.1016/j.healun.2019.01.088

Scott, C., & Thompson, J. P. (2012). Drugs acting on the heart: heart failure and coronary insufficiency. *Anesthesia and Intensive Care Medicine, 13*(8), 378-382.

Seewald, M., Coles, J. A., Jr., Sigg, D. C., & Iaizzo, P. A. (2017). Featured Article: Pharmacological postconditioning with delta opioid attenuates myocardial reperfusion injury in isolated porcine hearts. *Exp Biol Med (Maywood), 242*(9), 986-995. doi: 10.1177/1535370216684041

Sevrioukova, I. F. (2011). Apoptosis-inducing factor: structure, function, and redox regulation. *Antioxid Redox Signal, 14*(12), 2545-2579. doi: 10.1089/ars.2010.3445

Sharma, K., & Kass, D. A. (2014). Heart failure with preserved ejection fraction: mechanisms, clinical features, and therapies. *Circ Res, 115*(1), 79-96.

Shibata, T., Yamamoto, F., Suehiro, S., & Kinoshita, H. (1997). Effects of protease inhibitors on postischemic recovery of the heart. *Cardiovascular Drugs and Therapy, 11*(4), 547-556. doi: 10.1023/A:1007723417775

Singh, A., Lee, K. J., Lee, C. Y., Goldfarb, R. D., & Tsan, M. F. (1989). Relation between myocardial glutathione content and extent of ischemia-reperfusion injury. *Circulation, 80*(6), 1795-1804. doi: 10.1161/01.cir.80.6.1795

Smith, D. H., Johnson, E. S., Blough, D. K., Thorp, M. L., Yang, X., Petrik, A. F., & Crispell, K. A. (2012). Predicting costs of care in heart failure patients. *BMC Health Serv Res, 12*, 434.

Soares, R. O. S., Losada, D. M., Jordani, M. C., Evora, P., & Castro, E. S. O. (2019). Ischemia/reperfusion injury revisited: an overview of the latest pharmacological strategies. *Int J Mol Sci, 20*(20). doi: 10.3390/ijms20205034

Soualmia, F., & El Amri, C. (2018). Serine protease inhibitors to treat inflammation: a patent review (2011-2016). *Expert Opin Ther Pat, 28*(2), 93-110. doi: 10.1080/13543776.2018.1406478

Spoelstra-de Man, A. M., Smit, B., Oudemans-van Straaten, H. M., & Smulders, Y. M. (2015). Cardiovascular effects of hyperoxia during and after cardiac surgery. *Anaesthesia, 70*(11), 1307-1319. doi: 10.1111/anae.13218

Stamp, N. L., Shah, A., Vincent, V., Wright, B., Wood, C., Pavey, W., . . . Larbalestier, R. (2015). Successful heart transplant after ten hours out-of-body time using the TransMedics Organ Care System. *Heart Lung Circ, 24*(6), 611-613. doi: 10.1016/j.hlc.2015.01.005

Stanger, O., Aigner, I., Schimetta, W., & Wonisch, W. (2014). Antioxidant supplementation attenuates oxidative stress in patients undergoing coronary artery bypass graft surgery. *Tohoku J Exp Med, 232*(2), 145-154. doi: 10.1620/tjem.232.145

Stassano, P., Di Tommaso, L., Monaco, M., Iesu, S., Brando, G., Buonpane, S., . . . Pepino, P. (2009). Myocardial revascularization by left ventricular assisted beating heart is associated with reduced systemic inflammatory response. *Ann Thorac Surg, 87*(1), 46-52. doi: 10.1016/j.athoracsur.2008.07.098

Steen, S., Paskevicius, A., Liao, Q., & Sjoberg, T. (2016). Safe orthotopic transplantation of hearts harvested 24 hours after brain death and preserved for 24 hours. *Scand Cardiovasc J, 50*(3), 193-200. doi: 10.3109/14017431.2016.1154598

Steinberg, J. B., Kapelanski, D. P., Olson, J. D., & Weiler, J. M. (1993). Cytokine and complement levels in patients undergoing cardiopulmonary bypass. *J Thorac Cardiovasc Surg, 106*(6), 1008-1016.

Steinbrook, R. (2007). Organ donation after cardiac death. *N Engl J Med, 357*(3), 209-213.

Sutton, N. R., Baek, A., & Pinsky, D. J. (2014). Endothelial cells and inflammation. In I. R. Mackay, N. R. Rose, B. Diamond & A. Davidson (Eds.), *Encyclopedia of Medical Immunology: Autoimmune Diseases* (pp. 367-381). New York, NY: Springer New York.

Suzuki, Y. J., & Hao, J. J. (2017). Results supporting the concept of the oxidant-mediated protein amino acid conversion, a naturally occurring protein engineering process, in human cells. *F1000Res, 6*, 594. doi: 10.12688/f1000research.11376.2

Sznycer-Taub, N. R., Lowery, R., Yu, S., Owens, S. T., Hirsch-Romano, J. C., & Owens, G. E. (2016). Hyperoxia Is Associated With Poor Outcomes in Pediatric Cardiac Patients Supported on Venoarterial Extracorporeal Membrane Oxygenation. *Pediatr Crit Care Med, 17*(4), 350-358. doi: 10.1097/pcc.0000000000000655

Tang, J., Tao, K., Zhou, J., Zhang, C., Gong, L., Luo, N., & Du, L. (2013). Long-term leukocyte filtration should be avoided during extracorporeal circulation. *Mediators Inflamm, 2013*, 612848. doi: 10.1155/2013/612848

Tang, J., Tao, K., Zhou, J., Zhang, C., Gong, L., Luo, N., & Du, L. (2013). Long-term leukocyte filtration should be avoided during extracorporeal circulation. *Mediators Inflamm, 2013*, 612848. doi: 10.1155/2013/612848

Tanindi, A., & Cemri, M. (2011). Troponin elevation in conditions other than acute coronary syndromes. *Vasc Health Risk Manag, 7*, 597-603. doi: 10.2147/vhrm.s24509

Taylor, M. J. (2007). Hypothermic blood substitution: special considerations for protection of cells during ex vivo and in vivo preservation. *Transfus Med Hemother, 34*, 226-244.

Taylor, M. J., & Baicu, S. C. (2010). Current state of hypothermic machine perfusion preservation of organs: The clinical perspective. *Cryobiology, 60*(3 Suppl), S20-35. doi: 10.1016/j.cryobiol.2009.10.006

Templeton, G. F., & Burney, L. L. (2017). Using a two-step transformation to address non-normality from a business value of information technology perspective. *Journal of Information Systems, 31*(2), 149-164. doi: 10.2308/isys-51510

Thangappan, K., Cavarocchi, N. C., Baram, M., Thoma, B., & Hirose, H. (2016). Systemic inflammatory response syndrome (SIRS) after extracorporeal membrane oxygenation (ECMO): Incidence, risks and survivals. *Heart Lung, 45*(5), 449-453. doi: 10.1016/j.hrtlng.2016.06.004

Toft, P., Christiansen, K., Tonnesen, E., Nielsen, C. H., & Lillevang, S. (1997). Effect of methylprednisolone on the oxidative burst activity, adhesion molecules and clinical outcome following open heart surgery. *Scand Cardiovasc J, 31*(5), 283-288. doi: 10.3109/14017439709069549

Tolboom, H., Olejnickova, V., Reser, D., Rosser, B., Wilhelm, M. J., Gassmann, M., . . . Falk, V. (2016). Moderate hypothermia during ex vivo machine perfusion promotes recovery of hearts donated after cardiocirculatory deathdagger. *Eur J Cardiothorac Surg, 49*(1), 25-31. doi: 10.1093/ejcts/ezv066

Toyoda, Y., Guy, T. S., & Kashem, A. (2013). Present status and future perspectives of heart transplantation. *Circ J, 77*(5), 1097-1110.

Trahanas, J. M., Witer, L. J., Alghanem, F., Bryner, B. S., Iyengar, A., Hirschl, J. R., . . . Bocks, M. L. (2016). Achieving 12 hour normothermic ex situ heart perfusion: an experience of 40 porcine hearts. *Asaio j, 62*(4), 470-476. doi: 10.1097/mat.0000000000000382

Trittenwein, G., Rotta, A. T., Gunnarsson, B., & Steinhorn, D. M. (1999). Lipid peroxidation during initiation of extracorporeal membrane oxygenation after hypoxia in endotoxemic rabbits. *Perfusion, 14*(1), 49-57. doi: 10.1177/026765919901400108

Ungvari, Z., Gupte, S. A., Recchia, F. A., Batkai, S., & Pacher, P. (2005). Role of oxidative-nitrosative stress and downstream pathways in various forms of cardiomyopathy and heart failure. *Curr Vasc Pharmacol, 3*(3), 221-229.

Ustunsoy, H., Sivrikoz, M. C., Tarakcioglu, M., Bakir, K., Guldur, E., & Celkan, M. A. (2006). The effects of pentoxifylline on the myocardial inflammation and ischemia-reperfusion injury during cardiopulmonary bypass. *J Card Surg, 21*(1), 57-61. doi: 10.1111/j.1540-8191.2006.00169.x

Vallhonrat, H., Swinford, R. D., Ingelfinger, J. R., Williams, W. W., Ryan, D. P., Tolkoff-Rubin, N., . . . Pascual, M. (1999). Rapid activation of the alternative pathway of complement by extracorporeal membrane oxygenation. *Asaio j, 45*(1), 113-114.

Van Avondt, K., Nur, E., & Zeerleder, S. (2019). Mechanisms of haemolysis-induced kidney injury. *Nat Rev Nephrol, 15*(11), 671-692. doi: 10.1038/s41581-019-0181-0

Van Caenegem, O., Beauloye, C., Bertrand, L., Horman, S., Lepropre, S., Sparavier, G., . . . Vanoverschelde, J. L. (2016). 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. *Eur J Cardiothorac Surg, 49*(5), 1348-1353. doi: 10.1093/ejcts/ezv409

van de Veerdonk, F. L., Netea, M. G., Dinarello, C. A., & Joosten, L. A. (2011). Inflammasome activation and IL-1beta and IL-18 processing during infection. *Trends Immunol, 32*(3), 110-116. doi: 10.1016/j.it.2011.01.003

van Kimmenade, R. R., & Januzzi, J. L., Jr. (2012). Emerging biomarkers in heart failure. *Clin Chem, 58*(1), 127-138.

Van Raemdonck, D., Rega, F., Rex, S., & Neyrinck, A. (2018). Machine perfusion of thoracic organs. *J Thorac Dis, 10*(Suppl 8), S910-s923. doi: 10.21037/jtd.2018.02.85

Velez, D. E., Mestre-Cordero, V. E., Hermann, R., Perego, J., Harriet, S., Fernandez-Pazos, M. L. M., . . . Marina-Prendes, M. G. (2020). Rosuvastatin protects isolated hearts against ischemia-reperfusion injury: role of Akt-GSK-3beta, metabolic environment, and mitochondrial permeability transition pore. *J Physiol Biochem, 76*(1), 85-98. doi: 10.1007/s13105-019-00718-z

Vercaemst, L. (2008). Hemolysis in cardiac surgery patients undergoing cardiopulmonary bypass: a review in search of a treatment algorithm. *J Extra Corpor Technol, 40*(4), 257-267.

Vermeulen Windsant, I. C., Hanssen, S. J., Buurman, W. A., & Jacobs, M. J. (2011). Cardiovascular surgery and organ damage: time to reconsider the role of hemolysis. *J Thorac Cardiovasc Surg, 142*(1), 1-11. doi: 10.1016/j.jtcvs.2011.02.012

Verrier, E. D., Shernan, S. K., Taylor, K. M., Van de Werf, F., Newman, M. F., Chen, J. C., . . . Levy, J. H. (2004). Terminal complement blockade with pexelizumab during coronary artery bypass graft surgery requiring cardiopulmonary bypass: a randomized trial. *Jama, 291*(19), 2319-2327. doi: 10.1001/jama.291.19.2319

Videm, V., & Albrigtsen, M. (2008). Soluble ICAM-1 and VCAM-1 as markers of endothelial activation. *Scand J Immunol, 67*(5), 523-531. doi: 10.1111/j.1365-3083.2008.02029.x

Vimercati, C., Qanud, K., Mitacchione, G., Sosnowska, D., Ungvari, Z., Sarnari, R., . . . Recchia, F. A. (2014). Beneficial effects of acute inhibition of the oxidative pentose phosphate pathway in the failing heart. *Am J Physiol Heart Circ Physiol, 306*(5), H709-717. doi: 10.1152/ajpheart.00783.2013

Virani, S. A., Bains, M., Code, J., Ducharme, A., Harkness, K., Howlett, J. G., . . . Zieroth, S. (2017). The Need for Heart Failure Advocacy in Canada. *Can J Cardiol, 33*(11), 1450-1454. doi: 10.1016/j.cjca.2017.08.024

Vitkova, V., Panek, M., Janec, P., Sibikova, M., Vobruba, V., Haluzik, M., . . . Janota, J. (2018). Endothelial Microvesicles and Soluble Markers of Endothelial Injury in Critically Ill Newborns. *2018*, 1975056. doi: 10.1155/2018/1975056

Vohra, H. A., Whistance, R., Modi, A., & Ohri, S. K. (2009). The inflammatory response to miniaturised extracorporeal circulation: a review of the literature. *Mediators Inflamm, 2009*, 707042. doi: 10.1155/2009/707042

Wan, S., DeSmet, J.-M., Barvais, L., Goldstein, M., Vincent, J.-L., & LeClerc, J.-L. (1996). Myocardium is a major source of proinflammatory cytokines in patients undergoing cardiopulmonary bypass. *J Thorac Cardiovasc Surg, 112*(3), 806-811. doi: https://doi.org/10.1016/S0022-5223(96)70068-5

Wang, S., Krawiec, C., Patel, S., Kunselman, A. R., Song, J., Lei, F., . . . Undar, A. (2015). Laboratory evaluation of hemolysis and systemic inflammatory response in neonatal nonpulsatile and pulsatile extracorporeal life support systems. *Artif Organs, 39*(9), 774-781. doi: 10.1111/aor.12466

Warren, O., Alexiou, C., Massey, R., Leff, D., Purkayastha, S., Kinross, J., . . . Athanasiou, T. (2007). The effects of various leukocyte filtration strategies in cardiac surgery. *Eur J Cardiothorac Surg, 31*(4), 665-676. doi: 10.1016/j.ejcts.2006.12.034

Warren, O. J., Smith, A. J., Alexiou, C., Rogers, P. L., Jawad, N., Vincent, C., . . . Athanasiou, T. (2009). The inflammatory response to cardiopulmonary bypass: part 1--mechanisms of pathogenesis. *J Cardiothorac Vasc Anesth, 23*(2), 223-231. doi: 10.1053/j.jvca.2008.08.007

Warren, O. J., Watret, A. L., de Wit, K. L., Alexiou, C., Vincent, C., Darzi, A. W., & Athanasiou, T. (2009). The inflammatory response to cardiopulmonary bypass: part 2--anti-inflammatory therapeutic strategies. *J Cardiothorac Vasc Anesth, 23*(3), 384-393. doi: 10.1053/j.jvca.2008.09.007

Weerasinghe, A., & Taylor, K. M. (1998). The platelet in cardiopulmonary bypass. *Ann Thorac Surg, 66*(6), 2145-2152. doi: 10.1016/s0003-4975(98)00749-8

Weigt, S. S., Palchevskiy, V., & Belperio, J. A. (2017). Inflammasomes and IL-1 biology in the pathogenesis of allograft dysfunction. *J Clin Invest, 127*(6), 2022-2029. doi: 10.1172/jci93537

Weiss, R. G., Gerstenblith, G., & Bottomley, P. A. (2005). ATP flux through creatine kinase in the normal, stressed, and failing human heart. *Proc Natl Acad Sci U S A, 102*(3), 808-813. doi: 10.1073/pnas.0408962102

Werns, S. W., Fantone, J. C., Ventura, A., & Lucchesi, B. R. (1992). Myocardial glutathione depletion impairs recovery of isolated blood-perfused hearts after global ischaemia. *J Mol Cell Cardiol, 24*(11), 1215-1220.

Wettersten, N., & Maisel, A. (2015). Role of Cardiac Troponin Levels in Acute Heart Failure. *Card Fail Rev, 1*(2), 102-106. doi: 10.15420/cfr.2015.1.2.102

White, C., Ambrose, E., Muller, A., Hatami, S., Li, Y., Le, H., . . . Freed, D. (2017). Impact of reperfusion calcium and pH on the resuscitation of hearts donated after circulatory death. *Ann Thorac Surg, 103*(1), 122-130. doi: 10.1016/j.athoracsur.2016.05.084

White, C. W., Ali, A., Hasanally, D., Xiang, B., Li, Y., Mundt, P., . . . Freed, D. H. (2013). A cardioprotective preservation strategy employing ex vivo heart perfusion facilitates successful transplant of donor hearts after cardiocirculatory death. *J Heart Lung Transplant, 32*(7), 734-743. doi: 10.1016/j.healun.2013.04.016

White, C. W., Ambrose, E., Muller, A., Li, Y., Le, H., Hiebert, B., . . . Freed, D. (2015a). Assessment of donor heart viability during ex vivo heart perfusion. *Can J Physiol Pharmacol, 93*(10), 893-901. doi: 10.1139/cjpp-2014-0474

White, C. W., Ambrose, E., Muller, A., Li, Y., Le, H., Hiebert, B., . . . Freed, D. (2015b). Assessment of donor heart viability during ex vivo heart perfusion. *Canadian Journal of Physiology and Pharmacology, 93*(10), 893-901. doi: 10.1139/cjpp-2014-0474

White, C. W., Ambrose, E., Muller, A., Li, Y., Le, H., Thliveris, J., . . . Freed, D. H. (2016). Avoidance of Profound Hypothermia During Initial Reperfusion Improves the Functional Recovery of Hearts Donated After Circulatory Death. *Am J Transplant, 16*(3), 773-782. doi: 10.1111/ajt.13574

White, C. W., Hasanally, D., Mundt, P., Li, Y., Klein, J., Xiang, B., . . . Freed, D. H. (2014). A wholeblood–based perfusate provides superior preservation of myocardial function during ex vivo heart perfusion. *J Heart Lung Transplant, S1053-2498*(14), 01356-01354.

White, C. W., Messer, S. J., Large, S. R., Conway, J., Kim, D. H., Kutsogiannis, D. J., . . . Freed, D. H. (2018). Transplantation of hearts donated after circulatory death. *Front Cardiovasc Med, 5*, 8. doi: 10.3389/fcvm.2018.00008

White, C. W., Shan, S., Hatami, S., Gurtu, V., Kinnard, A., Matsumura, N., . . . Nagendran, J. (2015). Ex vivo perfusion in a loaded state improves the preservation of donor heart function. *Canadian Journal of cardiology, 31*(10), s202.

Whitlock, R. P., Chan, S., Devereaux, P. J., Sun, J., Rubens, F. D., Thorlund, K., & Teoh, K. H. (2008). Clinical benefit of steroid use in patients undergoing cardiopulmonary bypass: a meta-analysis of randomized trials. *Eur Heart J, 29*(21), 2592-2600. doi: 10.1093/eurheartj/ehn333

Wicomb, W. N., Cooper, D. K., Novitzky, D., & Barnard, C. N. (1984). Cardiac transplantation following storage of the donor heart by a portable hypothermic perfusion system. *Ann Thorac Surg, 37*(3), 243-248.

Wilhelm, M. J., Pratschke, J., Laskowski, I. A., Paz, D. M., & Tilney, N. L. (2000). Brain death and its impact on the donor heart-lessons from animal models. *J Heart Lung Transplant, 19*(5), 414-418. doi: 10.1016/s1053-2498(00)00073-5

Williams, D. C., Turi, J. L., Hornik, C. P., Bonadonna, D. K., Williford, W. L., Walczak, R. J., . . . Cheifetz, I. M. (2015). Circuit oxygenator contributes to extracorporeal membrane oxygenation-induced hemolysis. *Asaio j, 61*(2), 190-195. doi: 10.1097/mat.0000000000000173

Wittwer, T., & Wahlers, T. (2008). Marginal donor grafts in heart transplantation: lessons learned from 25 years of experience. *Transpl Int, 21*(2), 113-125.

Wollborn, J., Siemering, S., Steiger, C., Buerkle, H., Goebel, U., & Schick, M. A. (2019). Phosphodiesterase-4 inhibition reduces ECLS-induced vascular permeability and improves microcirculation in a rodent model of extracorporeal resuscitation. *Am J Physiol Heart Circ Physiol, 316*(3), H751-h761. doi: 10.1152/ajpheart.00673.2018

Wu, Z. J., Gartner, M., Litwak, K. N., & Griffith, B. P. (2005). Progress toward an ambulatory pump-lung. *J Thorac Cardiovasc Surg, 130*(4), 973-978. doi: 10.1016/j.jtcvs.2005.04.032

Xia, Z., Huang, Z., & Ansley, D. M. (2006). Large-dose propofol during cardiopulmonary bypass decreases biochemical markers of myocardial injury in coronary surgery patients: a comparison with isoflurane. *Anesth Analg, 103*(3), 527-532. doi: 10.1213/01.ane.0000230612.29452.a6

Xin, L., Gellner, B., Ribeiro, R. V. P., Ruggeri, G. M., Banner, D., Meineri, M., . . . Badiwala, M. V. (2017). A new multi-mode perfusion system for ex vivo heart perfusion study. *Journal of Medical Systems, 42*(2), 25. doi: 10.1007/s10916-017-0882-5

Yamaura, K., Akiyoshi, K., Irita, K., Taniyama, T., & Takahashi, S. (2001). Effects of olprinone, a new phosphodiesterase inhibitor, on gastric intramucosal acidosis and systemic inflammatory responses following hypothermic cardiopulmonary bypass. *Acta Anaesthesiol Scand, 45*(4), 427-434. doi: 10.1034/j.1399-6576.2001.045004427.x

Yancy, C. W., Jessup, M., Bozkurt, B., Butler, J., Casey, D. E., Jr., Drazner, M. H., . . . Wilkoff, B. L. (2013). 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, 128*(16), e240-327.

Yu, J., Wang, L., Akinyi, M., Li, Y., Duan, Z., Zhu, Y., & Fan, G. (2015). Danshensu protects isolated heart against ischemia reperfusion injury through activation of Akt/ERK1/2/Nrf2 signaling. *Int J Clin Exp Med, 8*(9), 14793-14804.

Zakkar, M., Guida, G., Suleiman, M. S., & Angelini, G. D. (2015). Cardiopulmonary bypass and oxidative stress. *Oxid Med Cell Longev, 2015*, 189863. doi: 10.1155/2015/189863

Zanotti-Cavazzoni, S. L., & Hollenberg, S. M. (2009). Cardiac dysfunction in severe sepsis and septic shock. *Curr Opin Crit Care, 15*(5), 392-397. doi: 10.1097/MCC.0b013e3283307a4e

Zhang, C. (2008). The role of inflammatory cytokines in endothelial dysfunction. *Basic Res Cardiol, 103*(5), 398-406. doi: 10.1007/s00395-008-0733-0

Zhang, C., Syed, T. W., Liu, R., & Yu, J. (2017). Role of endoplasmic reticulum stress, autophagy, and inflammation in cardiovascular disease. *Front Cardiovasc Med, 4*, 29. doi: 10.3389/fcvm.2017.00029

Zhang, X. Q., Tang, R., Li, L., Szucsik, A., Javan, H., Saegusa, N., . . . Selzman, C. H. (2013). Cardiomyocyte-specific p65 NF-kappaB deletion protects the injured heart by preservation of calcium handling. *Am J Physiol Heart Circ Physiol, 305*(7), H1089-1097. doi: 10.1152/ajpheart.00067.2013

Zhang, Y., Fang Liu, F., Bi, X., Wang, S., Wu, X., & Jiang, F. (2015). The antioxidant compound tert-butylhydroquinone activates Akt in myocardium, suppresses apoptosis and ameliorates pressure overload-induced cardiac dysfunction. *Sci Rep, 5*, 13005. doi: 10.1038/srep13005

Zhao, X., Lu, S., Nie, J., Hu, X., Luo, W., Wu, X., . . . Yang, Z. (2014). Phosphoinositide-dependent kinase 1 and mTORC2 synergistically maintain postnatal heart growth and heart function in mice. *Mol Cell Biol, 34*(11), 1966-1975. doi: 10.1128/mcb.00144-14

Zhou, H., Zhou, D., Lu, J., Wu, C., & Zhu, Z. (2019). Effects of pre–cardiopulmonary bypass administration of dexmedetomidine on cardiac injuries and the inflammatory response in valve replacement surgery with a sevoflurane postconditioning protocol: a pilot study. *Journal of Cardiovascular Pharmacology, 74*(2), 91-97. doi: 10.1097/fjc.0000000000000698