Molecular injury and repair assessment of *ex vivo* perfused heart transplants

By

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Abstract

Cardiac transplantation is a life-saving treatment for patients with end-stage heart disease, but it is limited by a shortage of suitable donor hearts. Strategies to expand the donor organ pool include the use of extended donor criteria and hearts donated after circulatory determination of death. The current clinical standard for organ preservation, cold static preservation (CSP), is limited to only 4-6 h, and prolonged periods of preservation have been associated with poor post-transplant outcomes. Additionally, the inability to measure cardiac function in CSP has contributed to excessive organ discard and the overall shortage of hearts for transplantation. *Ex vivo* heart perfusion (EVHP) represents a promising alternative for organ preservation, repair, and functional assessment. However, previous studies have reported progressive functional decline during EVHP, the underlying mechanisms of which remain poorly understood.

The overall objective of this thesis was to assess the feasibility of using gene expression to measure cardiac tissue injury and repair during EVHP. A novel literature-based cardiac specific injury and repair gene set was developed. Biopsies were obtained from porcine hearts either in vivo (IV, n=7) or after 12 h of EVHP (n=32) or combined heart and liver perfusion (H+L, n=7). Functional parameters were recorded during perfusion. Histology was assessed for features of cardiac injury. A gene expression profiling platform (NanoString nCounter) was used to measure the changes in cardiac injury and repair gene transcripts. The differential expression of these mRNA transcripts was correlated with cardiac function and histology.

Exploratory analysis demonstrated distinct clustering of the biopsy sample groups based on gene expression patterns. A total of 44 genes were significantly upregulated and 12 genes were significantly downregulated in EVHP versus IV group. Aggregate upregulated and downregulated gene sets showed higher and lower expression, respectively, in EVHP versus IV, EVHP versus

H+L, and H+L versus IV. Except for mild interstitial edema in EVHP biopsies, no significant histologic alterations were identified. Gene set expression correlated with various functional parameters and histologic interstitial edema. Most of the upregulated genes included inflammatory and tissue remodeling genes, whereas downregulated genes predominantly represented structural genes, suggesting that pro-inflammatory and pro-fibrotic genes are the main drivers of cardiac injury during EVHP.

The work presented in this thesis demonstrates that EVHP induces a molecular injury response that correlates with functional and histologic features of cardiac injury in a porcine model. Molecular assessment appears more sensitive and specific for measuring tissue injury compared with function and histology. This thesis thus presents a novel approach for assessing organ viability and the mechanisms of tissue injury and repair during EVHP. Additionally, this thesis highlights molecular pathways that may be further exploited to develop therapeutics which could improve cardiac function during EVHP, ultimately optimizing this promising technology and expanding the donor organ pool.

Preface

This thesis is an original work by Silas Kiptoo Rotich. The research project, in which this thesis is a part, was performed following ethics approval granted by the Animal Care and Use Committee at the University of Alberta (Protocol numbers AUP00001491 and AUP00001492) under the project title, "MOLECULAR INJURY AND REPAIR ASSESSMENT IN EX VIVO PERFUSED HEART TRANSPLANTS", APRIL 25, 2017.

The research conducted for this thesis was performed through a research collaboration between Dr. Benjamin Adam from the Department of Laboratory Medicine and Pathology, University of Alberta, and Dr. Darren Freed from the Department of Surgery, University of Alberta.

In Chapters 3 and 4, animal care, surgical procedures, *ex vivo* perfusion procedures, functional data collection and biopsy collection described were performed by Dr. Sanaz Hatami, PhD student, under the supervision of Dr. Freed and Dr. Jayan Nagendran. In Chapters 3 and 4, tissue sectioning, processing, and histology slide preparation were performed by Shalawny Miller, medical laboratory technologist in the LMP Pathology Core Lab at the University of Alberta.

I performed the RNA isolation, probe hybridization and NanoString gene expression assays and analyses described in Chapter 3 with assistance from Kim Formenti, medical laboratory technologist, Department of Laboratory Medicine and Pathology Core Lab, and Nicole Herbers, undergraduate student at the University of Alberta. I performed all other work described in Chapters 1-5, including the literature review, experimental design, laboratory work, histology scoring, data analysis and thesis preparation under the supervision of Dr. Adam. This thesis was also co-supervised by Drs. Michael Mengel and Jelena Holovati.

A portion of the results presented in Chapter 3 (Figures 3.5 and 3.6) have been published in abstract form as Rotich S, Herbers N, Hatami S, Nagendran J, Mengel M, Freed D, Adam B.

Correlation between Gene Expression and Cardiac Function in *ex vivo* Perfusion Hearts [abstract]. *Am J Transplant.* 2019;19 (suppl 3) and have been reproduced here with the permission of the American Society of Transplantation and Elsevier Inc.

A portion of the results presented in Chapter 4 (Table 4.3) have been published in abstract form as Rotich S, Herbers N, Hatami S, Nagendran J, Mengel M, Freed DH, Adam BA. Gene expression in ex vivo perfused porcine hearts: identification of a molecular injury-repair response [abstract]. J Heart Lung Transplant. 2019 Apr;38(4): S183-S184) and have been reproduced here with the permission of the American Society of Transplant Surgeons and Elsevier Inc.

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Dedication

This thesis is dedicated to all the people who have supported me throughout my education.

Thanks for making me see this adventure through to the end.

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I would like to express my heartfelt gratitude to all the people that contributed to the completion of this work, directly or indirectly.

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List of abbreviations

ACTA1	Actin, alpha 1, skeletal muscle
ACTA2	Actin, alpha 2, smooth muscle, aorta
ADAM8	ADAM metallopeptidase domain 8
AGP2	Alpha-1 glycoprotein 2
ANKRD1	Ankyrin repeat domain 1
ATF3	Activating transcription factor 3
ATP	Adenosine triphosphate
BMPER	Bone morphogenetic protein-binding endothelial regulator
BTG2	BTG anti-proliferation factor 2
Ca	Calcium
CASP8	Caspase 8
CCL2	C-C motif chemokine ligand 2
CCR2	C-C motif chemokine receptor 2
CD68	CD68 molecule
CD8A	CD8a molecule
cDNA	Complementary deoxyribonucleic acid
COL1A1	Collagen type I alpha 1 chain
COL4A1	Collagen type IV alpha 1 chain
CORUM	Comprehensive resource of mammalian protein complexes
CSP	Cold static preservation
CTGF	Connective tissue growth factor
CTSS	Cathepsin S

CXCL10	C-X-C motif chemokine ligand 10
CXCL11	C-X-C motif chemokine ligand 11
CXCL2	C-X-C motif chemokine ligand 2
CXCL9	C-X-C motif chemokine ligand 9
CYR61	Cysteine rich angiogenic inducer 61
DAMPS	Danger associated molecular patterns
DCD	Donation after circulatory death
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGLN3	Egl-9 family hypoxia inducible factor 3
EGR1	Early growth response 1
ERK	Extracellular-signal-regulated kinase
EVHP	*Ex vivo* heart perfusion
FCER1G	Fc fragment of IgE receptor Ig
FCGR2B	Fc fragment of IgG receptor IIb
FDR	False discovery rate
FFPE	Formalin fixed paraffin embedded
FGF21	Fibroblast growth factor
FN1	Fibronectin 1
FOS	Fos proto-oncogene, AP-1 transcription factor subunit
FWIT	Functional warm ischemia
GADD45B	Growth arrest and DNA damage inducible beta
GDF15	Growth differentiation factor 15

GO	Gene ontology
h	hour, hours
H	Hydrogen
H&E	hematoxylin and eosin
H+L	Heart and liver
HGP	Human genome project
HMGB	High mobility group box
HMOX1	Heme oxygenase 1
HSP90AA1	Heat shock protein 90 alpha family class A member 1
ICAM1	Intercellular adhesion molecule 1
ICU	Intensive care unit
IGF1	Insulin like growth factor 1
IL6	Interleukin 6
IRI	Ischemic reperfusion injury
IRS1	Insulin receptor substrate 1
ITGB2	Integrin subunit beta 2
IV	In vivo
JNK	Jun N-terminal kinase
JUN	Jun proto-oncogene, AP-1 transcription factor subunit
K	Potassium
LOX	Lysyl oxidase
MAPK	Mitogen activated protein kinase
MCM6	Minichromosome maintenance complex component 6

MCODE	Molecular complex detection
MEF2C	Myocyte enhancer factor 2C
min	minute, minutes
MMP12	Matrix metallopeptidase 12
MMP14	Matrix metallopeptidase 14
mRNA	Messenger ribonucleic acid
MSigDB	Molecular signature database
MYC	MYC proto-oncogene, bHLH transcription factor
MYH7	Myosin heavy chain 7
MYL9	Myosin light chain 9
MYOM2	Myomesin 2
Na	Sodium
NDD	Neurological determination of death
NFATC4	Nuclear factor of activated T cells 4
NFKBIA	NFKB inhibitor alpha
NPPB	Natriuretic peptide B
NR4A1	Nuclear receptor subfamily 4 group A member 1
NRG4	Neuregulin 4
NRP	Normothermic regional perfusion
OCS	Organ care system
OCT	Optimal cutting temperature
PAMPS	Pathogen associated molecular patterns
PC	Principal component

PECAM1	Platelet and endothelial cell adhesion molecule 1
PGD	Primary graft dysfunction
PGF	Placental growth factor
PTGDS	Prostaglandin D2 synthase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RTN4	Reticulon 4
RT-PCR	Real-time polymerase chain reaction
S100A8	S100 calcium binding protein A8
S100A9	S100 calcium binding protein A9
SELE	Selectin E
SERPINE1	Serpin family E member 1
SLC2A4	Solute carrier family 2 member 4
SOCS3	Suppressor of cytokine signaling 3
SPEED	Signaling pathway enrichment using experimental data
STRING	Search tool for the retrieval of interacting genes
TFF3	Trefoil factor 3
TGFB1	Transforming growth factor beta 1
THBS1	Thrombospondin 1
TIMP1	TIMP metallopeptidase inhibitor 1
TLR	Toll like receptor
TNC	Tenascin C
TNF	Tumor necrosis factor

TNFAIP3	TNF alpha induced protein 3
TRDN	Triadin
TUNEL	Terminal deoxynucleotidyl transferase (TdT dUTP Nick-End Labeling)
UW	University of Wisconsin
VCAM1	Vascular cell adhesion molecule 1
VEGFC	Vascular endothelial growth factor C
WLST	Withdrawal of life support therapy

Chapter 1

Introduction

1.1 Cardiac transplantation

Cardiac transplantation is a life-saving surgical intervention that is often the only remaining therapeutic option in patients with end-stage heart failure (1). It is a surgical procedure that involves explanting a functional heart from a recently deceased organ donor and implanting it into a patient with advanced heart failure where other medical, surgical or device-based therapeutic attempts have already been optimized but remain inadequate (2). The procedure takes approximately 4-8 h, with the heart implantation itself taking about 70 to 90 min. The ultimate goal of cardiac transplantation is to provide advanced heart failure patients with a near-normal quality of life.

Heart failure is a complex, heterogenous clinical syndrome that is caused by a functional or structural impairment of the heart (3). Clinically, heart failure manifests as fatigue, shortness of breath, exercise intolerance, edema, chronic cough, lack of appetite, confusion, delirium, and/or high heart rate (3, 4). Patients with end-stage heart failure demonstrate progressive morbidities and very poor prognosis, with heart transplant and mechanical circulatory support as the only available advanced therapeutic options in selected patients (5). Heart failure is a major global health concern with an increasing prevalence, estimated to affect more than 37.7 million people globally. Currently, about 600,000 Canadians are diagnosed with heart failure and the trend is increasing at a rate of about 90,000 new cases per year (6). It is the second most common cause of death after cancer and the leading cause of hospitalization in Canada. Readmission rates are greater than 20% at 30 days and mortality rates are in excess of 30% at one. The overall burden to the Canadian healthcare system is estimated to be more than 2.8 billion dollars per year (7).

Despite representing the current standard of care for the management of end-stage heart failure, cardiac transplantation is faced with critical challenges. This includes a growing number of patients in need of heart transplantation without a corresponding increase in the number of

suitable donor organs. The complexity of heart transplant candidates has also increased, with the patient population becoming older, more immunologically sensitized, and increasingly in need of complex mechanical circulatory support (8). The number of heart transplants performed worldwide is currently estimated to be 5500 procedures annually, constituting less than 10% of the global need (9, 10). Increasing demand for cardiac transplantation and the current limited supply of suitable donor hearts is estimated to result in an overall global one-year mortality rate of 17% for patients on the waiting list (11). In Canada, approximately 180 heart transplant procedures are performed per year, with a wait time of up to two years. About 15% of the wait listed patients die before they can receive a transplant and a similar percentage get removed from the waiting list because they become too sick to proceed with the surgery. Other unresolved challenges with cardiac transplantation include the association between ischemic injury sustained during donor organ preservation and inferior clinical outcomes post-transplantation (9). However, ongoing research efforts are expected to address these limitations through the development of novel approaches and technological advances, including the use of *ex vivo* heart perfusion devices, expansion of donor organ acceptance criteria, and introduction of the concept of donation after circulatory death (8).

1.2 History of cardiac transplantation

In 1964, Dr. James D. Hardy of the University of Mississippi Medical Centre transplanted a chimpanzee heart into a human patient by using a defibrillator to resuscitate the organ prior to implantation. Although the patient survived for only 90 min, this groundbreaking procedure paved the way for human cardiac transplantation. Three years later, in 1967, the world's first inter-human heart transplant was performed at the Groote Schuur Hospital, South Africa, with Dr. Christiaan Barnard as the lead surgeon (12). The recipient was a 54-year old man, Louis Washkansky with

end stage ischemic cardiomyopathy, who received a donation from Denise Darvall, the first human heart donor, after she was hit by a drunk driver (13). The second inter-human heart transplant was a pediatric case that took place three days later at Maimonides Hospital, Brooklyn, New York. This procedure involved an 18-day-old baby with Epstein anomaly who received a donor heart from an anencephalic baby and survived for six and half hours (14). One year later, in 1968, the first heart transplant in Canada was performed at the Montreal Heart Institute (Montreal, Quebec) with Dr. Pierre Grondin as the lead surgeon (15).

Although hundreds of heart transplants were performed over the subsequent decade, more than 70% of the transplant recipients did not survive beyond three months. However, the introduction of cyclosporine as a novel immunosuppressive medication in 1980 was a major breakthrough for the field of solid organ transplantation, with subsequent reductions of post-transplant complications such as allograft dysfunction, acute rejection and systemic infections. This resulted in the median post-heart transplant survival increasing from only three months to more than 8 years (9, 16, 17). Since then, more than 100,000 patients have been transplanted worldwide, with a global estimate of approximately 5500 transplant procedures per year (9, 18). Currently, it is estimated that approximately 86% of patients survive beyond one year post-transplantation, with 75%, 62%, and 36% still alive at 5, 10 and 20 years, respectively, for an overall median survival in excess of 12 years (9). In comparison, the majority of these advanced heart failure patients would not have survived one year without heart transplantation.

However, not all patients diagnosed with advanced heart failure are eligible to receive a transplant. Patients with a predicted life-expectancy less than two years are generally considered ineligible (19). Such patients often suffer from systemic diseases such as advanced pulmonary insufficiency, recurrent infections, malignancy, amyloidosis, hypertension, autoimmune disease,

and/or irreversible liver and kidney disease (20, 21). Mechanical circulatory support can be utilized as a destination therapy (i.e. a treatment for the remainder of their life) in such patients who are ineligible for cardiac transplantation (20). However, mechanical circulatory support therapies confer a number of potential complications, including bleeding and clotting, infections, or even failure of the device, thus resulting in these patients having a poor quality of life and prolonged hospitalizations (22). Such patients could have benefited from cardiac transplantation, but the shortage of suitable donor organs unfortunately limits recipient eligibility criteria, resulting in a significant number of patients dying or being removed from the waiting list because of deteriorating health status (18).

1.3 Cardiac donation

Organ donation refers to the process when a person permits removal and transplantation of their own organs to another person, either by providing consent while still alive or with the approval of their next of kin following death. The primary goal of organ donation is to reduce morbidity and mortality in patients with end-stage organ failure. However, exercising one's choice to donate their organs may help a living individual fulfil their own personal wish to donate, or support a bereaved family and help them fulfil the previously expressed wishes of their loved one to become an organ donor (23, 24). Cardiac donation involves the retrieval of hearts from deceased donors either through donation after neurologic determination of death (NDD) or donation after circulatory determination of death (DCD) (10). Coordinated efforts by critical care practitioners, transplant teams and organ donation organizations are required to effectively manage potential donors and successfully retrieve suitable organs for transplantation. Collaboration between these groups is imperative in facilitating successful organ recovery under optimal conditions.

Ideally, optimizing the conversion from potential deceased donor to actual donor involves addressing organ donation at multiple levels along the path to successful transplantation, including proper donor identification, death declaration, organ procurement, and preservation. Despite concerted efforts to improve public awareness about the benefits of transplantation, deceased organ donation rates have remained relatively low around the world because of a failure to transform potential donors into actual donors (25). Currently, it is estimated that only about 17% of potential donors end up becoming actual donors. Failed donor referrals, lack of consent, or missed donor identification are some of the purported reasons for this very low rate of donor conversion (26). The recent re-adoption of DCD organs is intended to increase donor conversion for potential donors who fail to meet the standard criteria for organ donation following brain death; however, the complexities surrounding this criterion continue to limit its application (26, 27).

While there are growing technological advances to support DCD organ donation, the associated ethical issues remain a major concern. In the anticipatory period of death following the decision to withdraw life sustaining therapy, some potential donors may take longer for the heart to stop beating, thus disqualifying them from donating because of the extended warm ischemic period (28, 29). Public trust is very critical in this scenario to avoid the misconception that withdrawal of life sustaining therapy (WLST) was performed to accelerate the occurrence of death in favor of organ retrieval. Determinations on the care of potential organ donors and the management of prospective recipients are thus vital and should be transparently separated to avoid possible conflicts of interests and ultimately increase organ donation rates (26, 30).

Currently, there is no universally accepted criteria for clinical triggers or definitions for identifying potential deceased organ donors, thus leading to confusion among critical care teams accountable for instigating the process of organ donation, and consequently contributing to missed

opportunities for organ donation (31). Successful cardiac transplantation requires prudent selection of the potential donor, thorough evaluation of the explanted organ, and allocation to the appropriate transplant recipient (18). The two main types of organ donation, NDD and DCD, are determined based on whether or not the heart is beating at the time of death (28, 32).

1.3.1 Donation after neurologic determination of death

Death is the greatest inevitability in life (33). Neurological death, commonly referred to as "brain death" -- and perhaps better understood as "brain arrest" -- is defined as the eventual clinical expression of complete and irreversible cessation of cerebral and brain stem functions (34). The concept of brain death originated in the 1950s following developments and discovery in the critical care field related to seemingly "alive" patients on life sustaining therapy long after clinical cessation of brain function (35).

Notwithstanding general consensus on the concept of neurologic determination of death, there are substantial international variations in the diagnosis of death using these criteria (36). To ensure that there is uniform practice in the recognition, diagnosis and documentation of NDD in Canada, the Canadian Council for Donation and Transplantation hosted a national conference of experts in Vancouver, British Columbia in April 2003 (32). This forum recommended use of the following minimum clinical criteria as Canadian Medical Standard criteria for NDD (32):

- An established etiology that is likely to cause neurologic death in the absence of reversible conditions that can mimic neurologic death.
- Deep unresponsive coma with respective absence of motor responses, excluding spinal reflexes.
- Absence of brain stem reflexes (which include corneal reflex, cough reflex, facial motor response to painful stimuli, gag reflex, and pupillary response to light) and apnea testing.

- Ancillary tests: cerebral perfusion scintigraphy, cerebral angiography.

NDD represents the traditional gold standard of organ donation (1). It is the most commonly used source of organs from deceased donors across the world, primarily because the presence of intact cardiorespiratory function allows for organ evaluation before organ procurement (8, 37). NDD donors are generally preferred for cardiac transplantation and are thus perpetually recruited by transplant programs (8, 38). Most often, it is critically brain-injured patients that progress to neurological death that ultimately become eligible for organ donation (32). Patients with traumatic brain injury, subarachnoid and intracerebral hemorrhage, and anoxic brain injuries may progress to neurologic death if they experience progressive cerebral edema and herniation (39, 40). The changing incidence and effectiveness of treatments for these different causes of brain injury, however, has been reported to influence the number of patients who may progress to neurologic death, thus affecting the number of potential deceased organ donors. Hospital mortality for patients with traumatic brain injury and stroke (subarachnoid hemorrhage and intracerebral hemorrhage) has been reported to have decreased over time, whereas mortality for those with anoxic brain injury appears to have increased (40); this provides a possible explanation for why the donation of organs using these criteria has remained steady.

Brain dead donors have paralyzed hypothalamic thermoregulatory function, which, as a result, leads to gradual hypothermia to equilibrate with the temperature of the surrounding environment (41). This gradual hypothermia consequently causes undesirable effects of hemodynamic instability, coagulopathy, and acidosis. Combined hypothermia, hypokalemia and acidosis are highly detrimental to cardiac physiology and function, which is particularly problematic for cardiac transplantation. Collaborative efforts between critical care physicians and

transplant surgeons are therefore necessary to optimize the management of such patients for optimal organ retrieval.

1.3.2 Donation after circulatory death

Donation after circulatory death (DCD) is the process of procuring organs from donors that have been declared dead based on cardiocirculatory criteria, rather than NDD (42). Cardiocirculatory arrest refers to the abrupt cessation of blood flow to the body as a result of sudden failure of the heart's pumping potential (43). This condition may progress to death if there are no rapid attempts to resuscitate cardiac function. Declaration of death in DCD includes cardiopulmonary assessment with the presence of apnea and circulatory arrest after a defined standoff period (8).

Non-heart beating donor, a term previously used to describe organ donors following DCD criteria, was first adopted in 1995 at the first international conference on non-heart beating donation that led to the development of the Maastricht classification (44). Since modified in 2013, the Maastricht classification is a system used for classifying DCD donors into four categories (I-IV) (Table 1.1) (28).

Table 1.1. Modified Maastricht DCD classification

Category	Type	Circumstances	Type of location
I	Uncontrolled	Found dead No attempt at resuscitation	IA. Out-of-hospital IIB. In-hospital
II	Uncontrolled	Witnessed cardiac arrest Unsuccessful attempt to resuscitate	IIA. Out-of-hospital IIB. In-hospital
III	Controlled	Planned withdrawal of life sustaining therapy	ICU
IV	Uncontrolled controlled	Sudden cardiac arrest in a brain-dead patient	ICU

According to the modified Maastricht classification, category I describes donors declared dead on arrival, with no attempt at resuscitation by a medical team; category II involves unsuccessful resuscitation; category III refers to anticipated cardiac arrest following withdrawal of life support treatment in an ICU patient; and category IV refers to an unanticipated cardiac arrest in a brain dead patient during management for organ procurement using NDD criteria (44).

Donation through uncontrolled DCD presents many challenges, including established warm ischemic injury at the time that the potential for organ donation is identified, the requirement for rapid mobilization of organ procurement services, and the time needed to approach the bereaved family for consent processing (42). During functional warm ischemic time (FWIT), the DCD heart functions in an escalating hypoxemic environment while aiming to keep oxygen delivery to the system (45). FWIT, commonly used in the Maastricht controlled category III, refers to the period between when the donor's systolic blood pressure drops to 50 mmHg following WLST to when the heart stops beating (1).

Continuous low oxygen and high carbon dioxide in the blood results in pulmonary vasoconstriction and right ventricular distention, causing a catecholamine surge and terminal cardiac hyperactivity (8, 45). Therefore, at the time of organ retrieval, DCD hearts will have tolerated significant ischemic injury and consequent anaerobic metabolism, which causes depletion of ATP energy stores and intracellular acidosis (8). Continuous degradation of ATP results in the progressive accumulation of reactive oxygen species (46). Additionally, depletion of energy stores results in failure of ATP-dependent ionic pumps, failure of Na^+/K^+ and Ca^{2+} pumps, and loss of transmembrane ionic gradients resulting in myocyte sodium influx (47). Reperfusion during organ retrieval causes further sodium influx, and this increasing intracellular sodium causes reversal of the Na^+/Ca^{2+}pump and consequent calcium influx (1, 8, 48). Increasing intracellular

Ca^{2+} accumulation promotes myocyte death through production of additional reactive oxygen species and activation of apoptotic and necrotic pathways (49). Resuscitating the DCD heart prior to procurement is therefore essential for limiting the severity of ischemia-reperfusion injury (IRI) and optimizing functional recovery. Given that DCD donors suffer from cardiac arrest following the withdrawal of life-support therapy, donor hearts can only be evaluated after resuscitation to confirm the retrieval of viable organs (8).

Current reports document over 70 successful DCD heart transplants around the world since 2014, with an estimated potential to eventually increase this source of donor hearts to at least 17-30% (50, 51). Four approaches for DCD organ procurement have been reported, including: i) direct procurement and cold static preservation (CSP), ii) direct procurement and *ex vivo* heart perfusion (EVHP) preservation, iii) normothermic regional perfusion (NRP) and CSP, and iv) NRP and EVHP preservation (50, 52-54). Direct procurement is the most commonly used technique for DCD hearts and involves the delivery of cold cardioplegic solution into the organ at the time of retrieval to optimize ischemic post-conditioning and subsequent preservation in either cold static storage or an EVHP device (8). Application of cold cardioplegic solution prevents contraction of the myocardium following reperfusion, thus promoting recovery of myocardial ATP and restoration of ionic pumps, ultimately avoiding hyper-contracture (55, 56). Another technique, NRP, involves reperfusion of the DCD donor, excluding brain circulation, following the declaration of circulatory death (57). During this technique, transplant surgeons perform a median sternotomy on the donor followed by clamping of the arch vessels to prevent circulation into the brain, and subsequently place the donor on extracorporeal life support for one hour before weaning. NRP is aimed at re-establishing myocardial energy stores, decreasing ATP degradation and increasing antioxidant concentrations (58). In NRP, heart function is assessed *in situ*, then arrested

using cold cardioplegic solution and subsequent preservation in CSP or perfusion in an *ex vivo* device.

The first human-to-human heart transplantation performed by Dr. Christiaan Barnard in 1967 utilized a DCD donor (12, 59). However, upon the introduction of the practice of using organs from NDD donors in 1968, the use of DCD hearts was largely abandoned (60). DCD criteria were later readopted and, currently, transplant centers are increasingly utilizing DCD donors in an attempt to address the increasing crisis related to donor organ shortages (42). Canada introduced the use of DCD donor criteria in 2005. Since then, DCD donation has increased by 429%, accounting for 21% of all deceased donors over the last 10 years, according to a report by the Canadian Institute for Health Information (61). However, only kidneys, livers and lungs from these DCD donors have been used for transplantation, not hearts. This limitation in the use of DCD hearts for transplantation is in part due to the warm ischemic time which induces myocardial injury, resulting in suboptimal organ quality and increased risk of graft dysfunction. The standard protocol for DCD organ donation includes an evaluation of the patient status as fully dependent on life-support therapy with a consensus agreement to WLST, a consented option to donate organs, ultimate WLST, declaration of death, and finally organ retrieval (8).

1.4 Myocardial protection and preservation

Protection of the myocardium of donor hearts from ischemic injury prior to transplantation is a critical determinant of a transplant recipient's quality of life. The most important factors in myocardial protection include the procurement period duration and the manner of preservation prior to transplantation. Ischemia induces a cascade of biochemical reactions including a shift to anaerobic metabolism (62). The repercussions of these changes include depletion of ATP and accumulation of lactate, which lead to further detrimental effects such as myocardial edema,

acidosis and irreversible structural damage to the myocardium. Myocardial protection strategies are geared towards obviating reperfusion injury and suppressing anaerobic metabolism (63).

The use of cardioplegia prior to organ procurement and preservation represents one of the key strategies in protecting the myocardium. Cold cardioplegia has been reported to lower oxygen consumption and confer protection to the myocardium by stimulating electromechanical arrest, thus making ischemia less harmful (64, 65). The success of donor heart preservation is critical for the ultimate success of cardiac transplantation, including graft function and long-term survival (66). Donor heart function is directly proportional to the duration of ischemia, with ischemic times exceeding 4 h reported to have a higher risk of primary graft dysfunction with associated prolonged lengths of hospital stay and increased mortality post-transplantation (9, 67). Currently, there are two methods of organ preservation that aim to minimize this ischemic injury: cold static preservation and *ex vivo* heart perfusion (68).

1.4.1 Cold static preservation

Cold static preservation (CSP) is the current clinical standard for myocardial protection during the interval between donor organ retrieval and transplantation. It involves storing the organ in an inactive state under hypothermic conditions (4^{o}C) to minimize cardiac metabolic demands and injury (69). CSP is a universally employed strategy for organ preservation used by almost all heart transplant programs around the world due to its simplicity, reliability and affordability (70). This type of organ preservation combines the use of hypothermic conditions, which decreases myocardial metabolism, and various ionic solutions, which facilitate the rapid cessation of electromechanical cardiac activity (71). These ionic solutions are classified as either intracellular or extracellular based on concentrations of <70 mmol/L of Na^+ and 30-125 mmol/L of K^+, or ≥70 mmol/L of Na^+ and 5-30mmol/L of K^+, respectively (71).

Prolonged CSP beyond 4-6 h has been reported to cause ATP depletion, elevated reactive oxygen species (ROS), Na^+/K^+ ATPase dysfunction, alteration of Ca^{2+} homeostasis, and increased levels of xanthine oxidase: the key elements that alter myocardial contractility and viability (72, 73). Furthermore, hypothermia has been reported to cause myocyte swelling and increased H^+ levels (74). Severely elevated H^+ levels result in activation of proteases and phospholipases, which in turn causes lysosomal degeneration and eventual myocyte death (75). Accordingly, most cardioplegic solutions have been modified with metabolic substrates, colloids, impermeants, buffering systems, pharmacological agents and ROS scavengers, which allow for deleterious hypothermic and ischemic effects to be partially reduced (76).The University of Wisconsin (UW) cold storage solution and histidine-tryptophan ketoglutarate (HTK), both intracellular solutions, and Celsior, an extracellular solution, are the most common CSP solutions used by clinical transplant programs (66). These solutions induce a rapid cessation of cardiac electromechanical activity by depolarizing myocyte membranes through reduction of the transmembrane K^+ gradient (71, 77). UW solution contains allopurinol and glutathione as scavengers for ROS, adenosine as an energy precursor, and hydroxyethyl starch as a colloid to maintain oncotic pressure (74). However, despite the application of hypothermic conditions and the utility of these specialized cardioplegic solutions, there remains low levels of anaerobic metabolism during CSP. As a result, prolonged CSP has been associated with accumulating cardiac injury and increased risk of primary graft failure (69).

The limited safe preservation time associated with CSP means that it can only be utilized for donor hearts that do not need to be transported long distances. Close coordination between organ retrieval and transplant institutions is required to minimize cold ischemic time (70). Additionally, CSP does not allow for pre-transplant functional assessment or resuscitation of

suboptimal organs and, as such, is not ideal for extended donation criteria organs, including those from DCD donors. Some transplant centers have attempted to mitigate these limitations by applying NRP for graft reconditioning, followed by preservation with CSP. Ayyaz *et al.* reported a case of full cardiac recovery following application of NRP in a DCD donor with a warm ischemia time of 23 min (78). Roberto *et al.* applied the same principle in a porcine animal model, with NRP followed by CSP storage prior to transplantation. Their results demonstrated non-inferior myocardial function with DCD hearts treated in this manner, compared with NDD donors from the same pre-clinical model (57). Subsequently, Messer *et al.* reported successful human DCD cardiac transplantation utilizing the NRP-CSP technique with a short ischemic time of 15 min (50). Other advantages of this NRP approach include simultaneous perfusion and functional assessment of other organs such as the liver, kidneys, lungs and pancreas, making it relatively affordable compared to EVHP devices limited to one organ at a time (79). In the United Kingdom, use of NRP has been reported to have increased heart transplant activity by 45% (80).

Despite these promising reports, the use of NRP is surrounded by ethical concerns, including the potential for cerebral reperfusion through collateral circulation, thus limiting its widespread adoption in organ transplantation (78). Furthermore, the application of NRP and short periods of CSP in DCD donors is limited to scenarios with donor-patient colocation for optimal post-transplant outcomes. Therefore, its application is likely not feasible in countries like Canada, where minimum standoff periods are mandatory and long distances between potential donors and recipients are common. Such limitations ultimately limit the size of the donor pool for transplant programs utilizing CSP as their only method of myocardial protection.

1.4.2 *Ex vivo* heart perfusion

Ex vivo heart perfusion (EVHP) is a novel method for preserving donor hearts for potentially longer periods than CSP by providing organs with a continuous supply of oxygen and nutrient-rich perfusate in a normothermic beating and near-physiological state. The use of EVHP for 12 h has been reported to be associated with myocardial functional recovery and maintenance of endothelial cell function. This strategy has the potential to limit the duration of pre-transplant cold ischemia through the continuous supply of oxygenated perfusate, and thus reduce cardiac tissue injury and consequent primary graft dysfunction (18, 81, 82). In recent years, there has been significant global scientific interest in EVHP, with the hope of reducing cold ischemia times, facilitating recovery of myocardial function, allowing the use of marginal donor organs, and ultimately increasing the donor organ pool and improving pre and post-transplant survival (83).

Langendorff perfusion, a technique developed by Oscar Langendorff in 1895, is one of the most commonly used methods for *ex vivo* perfusion and examination of explanted hearts (84-86). The Langendorff method involves perfusing the heart in the reverse direction through the ascending aorta, so that the backward-flow blocking action of the aortic valves causes a pressure build up in the aorta, which forces perfusate into the coronary arteries (70). In this technique, an isolated heart in an empty and beating state (i.e. non-working mode) is thus perfused with donor blood flowing through the aorta, into the coronary artery, and out the coronary sinus (84, 85).

The only currently clinically accepted and available *ex vivo* perfusion device for human cardiac transplantation is the Transmedics Organ Care System (OCS). This is a transportable platform designed to perfuse an explanted heart in a Langendorff, normothermic, unloaded beating state with oxygenated and nutrient-rich donor blood in near-physiologic conditions (87). OCS was developed and tested in the PROCEED II clinical trial and has been shown to be non-inferior to

standard CSP for preserving NDD donor hearts (86). The advantage of this EVHP technique over CSP is attributed to a reduction in ischemia-reperfusion injury because of the re-establishment of aerobic metabolism and the warm environment that helps mitigate the deleterious effects of hypothermia, such as myocardial cellular edema, as well as the provision of nutrient rich, high energy products and elimination of metabolic waste products (69, 88).

However, although EVHP technologies may serve as an alternative for CSP, the most promising benefit is its potential ability to facilitate expanded use of extended criteria organs, such as those from DCD donors (69). As previously discussed, DCD donor hearts undergo an inevitable warm ischemia period, and additional cold ischemia resulting from CSP is undesirable. In addition, DCD hearts are associated with unknown functional status, representing a further risk of occult pathology (53). Novel strategies, such as EVHP, are thus needed to minimize ischemic injury, identify occult pathology, and allow for optimal recovery of the myocardium. Furthermore, EVHP can potentially facilitate organ transportation between different geographical regions that would traditionally not be possible as a result of unacceptably long cold ischemia times (18). Also, the longer safe preservation times that are potentially feasible with EVHP could allow for optimal preparation by the transplant team and more time for appropriate patient matching (18).

The perfusate solution utilized in the OCS system contains insulin, adenosine, antibiotics, bicarbonate salts, methylprednisolone, vitamins, and freshly collected and leucocyte-free blood from the corresponding donor (89). Usually, 1200 to 1500 mL of fresh blood is collected from the donor just before aortic cross-clamping but after systemic heparinization (70). The donor heart is retrieved using the standard surgical explantation procedure by flushing with cold cardioplegic solution, such as UW or Celsior, through the aortic root. This achieves cold cardioplegic arrest and represents the only cold ischemic period, which is usually limited to half an hour (89). With closed

superior and inferior vena cava, the donor heart is then connected to the OCS circuit, which works by pumping perfusate solution into the aorta and then the coronary arteries (87). Deoxygenated blood from the coronary veins flow back into the right ventricle via the tricuspid valves and out into a reservoir, via an oxygenator, through a pulmonary artery cannula (70). A pulsatile pump ejects perfusate solution through a heat exchanger for warming and back into the aorta for a continuous cycle of perfusion (Figure 1.1) (90). Lactate profiles, aortic pressure and coronary flow can be continuously assessed throughout the perfusion period (91).

Although OCS-EVHP is now clinically accepted for normothermic cardiac preservation, left ventricular functional assessment remains elusive with the non-loaded beating state that this system currently employs. Furthermore, there are emerging reports that the use of lactate levels in OCS as a marker for cardiac function might be limited. This has led to the development of alternative systems that allow hearts to be perfused in a loaded beating state, which provide the opportunity for more sophisticated assessment of cardiac functional parameters (86). However, despite the theoretical benefits and preliminary success of these EVHP systems, the mechanisms of EVHP-related repair remain poorly understood and there is a need for more precise tools for measuring and studying tissue injury and repair during EVHP. Fundamental to the advancement of such technologies is the utility of preclinical models, such as large animals that model human physiological processes, to facilitate the translation from basic research into clinical practice (92).

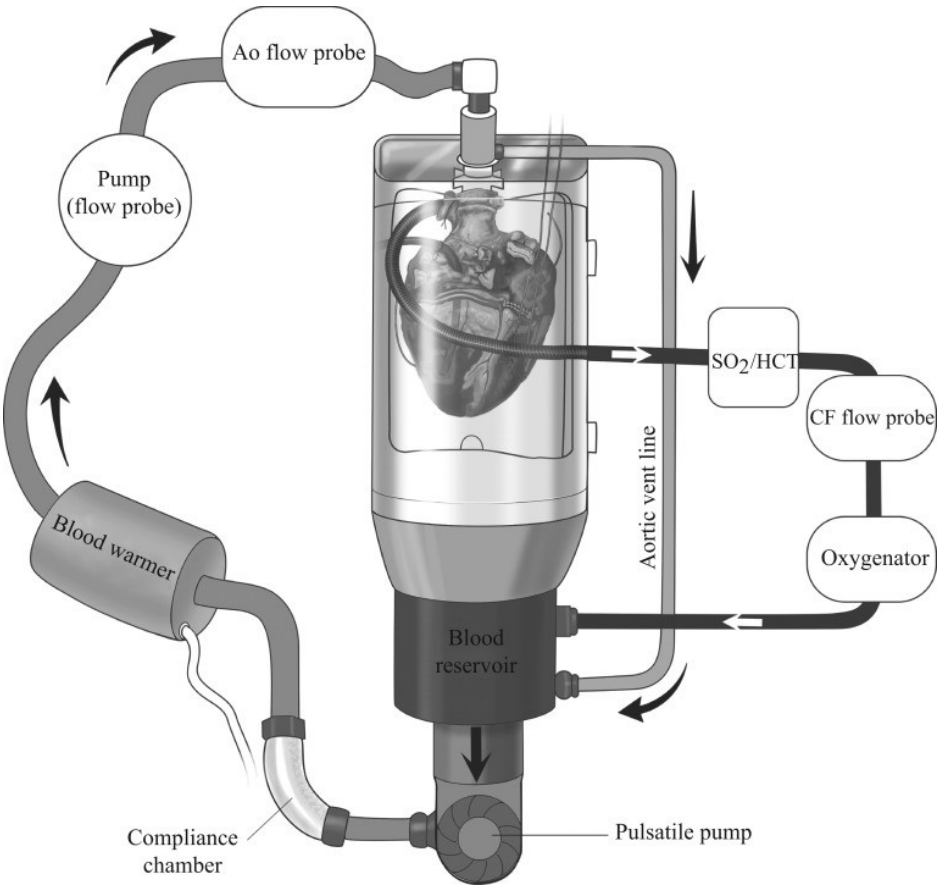

Figure 1.1. Organ Care System. Schematic of circuit flow in the normothermic non-loaded heart beating state. Reproduced with permission from Elsevier Inc. (90)

1.5 Animal model

Given the complexity of the human cardiovascular system, it is necessary to use a pre-clinical model with similar biological characteristics to advance our knowledge of potential therapeutic interventions to preserve and repair damaged hearts (93). An ideal pre-clinical model for cardiovascular studies should be comparable to human subjects in both pathophysiologic and metabolic functions (93). It is critical that, for investigations aimed at eventual clinical translation, initial results from small animal model studies be confirmed with large animal pre-clinical models that are more analogous to humans with a high percentage of genetic similarity (94, 95).

A model system should ideally be low-cost, reproducible, ethically appropriate, easily manipulated, and biologically illustrative of human disease (93). Pigs are the most commonly used

large animal models for cardiovascular studies because they mimic humans in coronary anatomy, organ size, immunology and physiology (96, 97). Porcine models offer simplicity in the implementation of methods and devices from human healthcare facilities (98). Human and porcine myocardium share a high degree of molecular similarity, including in the ventricular expression of β-MHC and excitation-contraction coupling (93, 99).

1.6 Assessment of donor heart viability

Although EVHP represents a promising strategy for increasing the size of the donor organ pool through resuscitation of hearts that would have otherwise been discarded, reliable methods of assessing organ viability prior to cardiac transplantation are necessary (52). The development of EVHP has provided an opportunity to more precisely evaluate and potentially resuscitate such hearts (90). The currently clinically available OCS, as discussed previously in this chapter, allows preservation of hearts in a non-loaded mode. This limits organ assessment to only metabolic parameters, such as lactate concentration, oxygen saturation, aortic pressure and coronary flow rate, but not functional parameters (52). However, alternative EVHP devices have recently been developed which allow preservation of hearts in a working mode, hence facilitating the monitoring of both metabolic and functional parameters, including left ventricular assessment (86). With the availability of these newer devices, reports have emerged demonstrating an invariable decline in myocardial function during EVHP, which presents a significant challenge to the broader clinical implementation of this technology. Specifically, Hatami *et al* found that EVHP induces a time-dependent decline in cardiac function (100). Understanding the mechanism of ongoing cardiac injury during EVHP is thus critical. One potential strategy to address this limitation could be to use modern molecular pathology tools to measure gene expression and correlate it with functional, metabolic, and histological parameters. This approach has previously been used to monitor injury

and repair in other organs, including porcine lungs preserved with *ex vivo* lung perfusion, and could thus similarly be applied to EVHP (101).

1.7 NanoString nCounter

NanoString nCounter is a high throughput gene expression platform capable of detecting up to 800 genes in one reaction with both high sensitivity and reproducibility. The principle of the nCounter assay is the utilization of target-specific, color-coded probe pairs (i.e. a biotin-labeled target-specific capture probe and a unique color-coded reporter probe) for direct digital detection of mRNA molecules, without the need for cDNA generation or pre-amplification. These fluorescent barcodes, which are designed to be specific to particular nucleic acid sequences of interest, allow for simultaneous, automated, non-amplified measurement of multiple gene targets from a single sample (102-104). Biotin labeling of capture probes enables the formed probe-target complex to be immobilized for the collection of data, whereas the color-coding of the reporter probes provides unique signals for detection (105, 106).

NanoString nCounter has been reported to work reliably with formalin-fixed, paraffin-embedded (FFPE) tissue, is similar in sensitivity to real-time polymerase chain reaction (RT-PCR) and is more sensitive than microarrays, thus bridging the gap between genome-wide arrays and quantitative RT-PCR (103). Its reliability for analyzing low quality RNA biospecimens, such as those derived from FFPE tissue, provides a novel opportunity for both retrospective and prospective studies aimed at understanding the molecular mechanisms of disease development, prognostication, therapeutic response, and diagnostic assay validation for use in clinical pathology applications (102). The nCounter platform is able to analyze very short RNA fragments with great specificity due to the fact that it does not require pre-amplification steps and thus avoids

amplification-associated biases and error (103, 107). Additionally, the sensitivity of target detection with the nCounter platform is maintained at very low input RNA amounts (107).

There are four main steps in nCounter-based assays, including hybridization, purification and immobilization, digital counting and analysis (Figure 1.2) (108). During hybridization, the sample RNA is mixed with the probe code-set (i.e. a solution containing the unique pairs of reporter and capture probes) (108). The target-specific probes hybridize directly to the gene of interest without the need for enzymatic reaction. The mixture is then transferred to the nCounter Prep Station where excess probes are removed after hybridization and the probe-target complexes are bound, immobilized and aligned on the nCounter cartridge, which is then transferred to the nCounter Digital Analyzer for digital counting of the color-coded probes. The data are then normalized using NanoString nSolver software for further analysis.

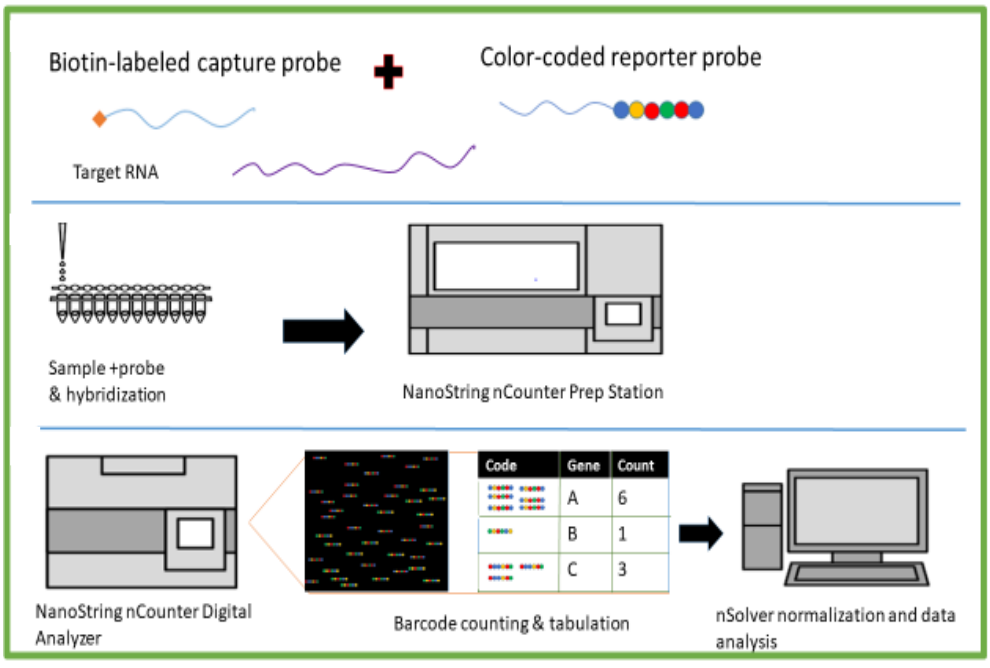

Figure 1.2. NanoString nCounter Gene Expression System.

The greatest benefit of the nCounter methodology is its feasibility for gene profiling of FFPE tissues, which allows for direct correlation between gene expression and histological morphologies on the same tissue sample (109). Furthermore, this platform provides the opportunity to reliably perform retrospective gene expression profiling on archival FFPE biospecimens, thus facilitating immediate correlation with long term clinical follow-up (110). Through the use of molecular signatures/sets of genes as markers for particular tissue phenotypes, this technology has the potential to allow for more precise, objective and mechanistic evaluation of heart tissue during organ retrieval, preservation and transplantation (111).

1.8 Thesis approach

Cardiac transplantation remains the gold standard treatment for end-stage heart failure. The demand for heart transplantation has been increasing while the supply of donor hearts has remained relatively constant. Researchers around the world are thus exploring novel strategies to expand the donor organ pool. The development of *ex vivo* heart perfusion (EVHP) devices, which will potentially allow for extended preservation, functional evaluation and resuscitation of donor organs (18), along with the growing acceptance and utilization of DCD hearts for transplantation, represent major areas of focus for increasing global heart transplant activity. Despite these promising efforts, however, little is known about the molecular mechanisms of cardiac injury and repair during EVHP. Gene expression analysis offers a potential novel approach for addressing this limitation, but no gene signatures have been derived to monitor the quality of hearts in *ex vivo* perfusion. Development of such a tool may allow us to further optimize and implement EVHP technologies.

The second Chapter of this thesis focuses on the development of a literature-based gene set, which will be used in chapter 3 to assess the molecular injury-repair response in porcine hearts

after 12 h of normothermic EVHP. The fourth chapter will focus on the correlation between gene expression and cardiac functional and histological parameters. NanoString nCounter will be used for the measurement of this gene set as a potential novel diagnostic tool for the quantitative assessment of cardiac injury and repair in *ex vivo* perfusion.

1.9 Hypothesis and Thesis Objectives

This thesis will test the hypothesis that gene expression can be applied as a molecular assay for the quantitative assessment of cardiac injury and repair during *ex vivo* heart perfusion. The thesis consists of literature-based and experimental studies with three specific research objectives:

Objective 1: To develop a literature-based, cardiac-specific gene set for measuring tissue injury and repair before, during and after EVHP (Chapter 2)

Objective 2: To assess the expression of this gene set in a porcine model for EVHP (Chapter 3)

Objective 3: To correlate gene set expression with cardiac function and histology (Chapter 4)

1.10 References

1. Page A, Messer S, Large SR. Heart transplantation from donation after circulatory determined death. Ann Cardiothorac Surg. 2018;7(1):75-81.
2. Harris C, Cao C, Croce B, Munkholm-Larsen S. Heart transplantation. Ann Cardiothorac Surg. 2018;7(1):172.
3. Yancy CW, Jessup M, Bozkurt B, Butler J, Casey DE, Jr., Drazner MH, 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. J Am Coll Cardiol. 2013;62(16):e147-239.
4. Hunt SA, Abraham WT, Chin MH, Feldman AM, Francis GS, Ganiats TG, et al. ACC/AHA 2005 Guideline Update for the Diagnosis and Management of Chronic Heart Failure in the Adult: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Update the 2001 Guidelines for the Evaluation and Management of Heart Failure): developed in collaboration with the American College of Chest Physicians and the International Society for Heart and Lung Transplantation: endorsed by the Heart Rhythm Society. Circulation. 2005;112(12):e154-235.
5. Fang JC, Ewald GA, Allen LA, Butler J, Westlake Canary CA, Colvin-Adams M, et al. Advanced (stage D) heart failure: a statement from the Heart Failure Society of America Guidelines Committee. J Card Fail. 2015;21(6):519-34.
6. Government of Canada. Heart Disease in Canada. https://www.canada.ca/en/public-health/services/publications/diseases-conditions/heart-disease-canada.html [Accessed 9 January 2020]

7. Virani SA, Bains M, Code J, Ducharme A, Harkness K, Howlett JG, et al. The Need for Heart Failure Advocacy in Canada. Can J Cardiol. 2017;33(11):1450-4.

8. White CW, Messer SJ, Large SR, Conway J, Kim DH, Kutsogiannis DJ, et al. Transplantation of Hearts Donated after Circulatory Death. Front Cardiovasc Med. 2018;5:8.

9. Chambers DC, Yusen RD, Cherikh WS, Goldfarb SB, Kucheryavaya AY, Khusch K, et al. The Registry of the International Society for Heart and Lung Transplantation: Thirty-fourth Adult Lung And Heart-Lung Transplantation Report-2017; Focus Theme: Allograft ischemic time. J Heart Lung Transplant. 2017;36(10):1047-59.

10. Citerio G, Cypel M, Dobb GJ, Dominguez-Gil B, Frontera JA, Greer DM, et al. Organ donation in adults: a critical care perspective. Intensive Care Med. 2016;42(3):305-15.

11. Goldstein BA, Thomas L, Zaroff JG, Nguyen J, Menza R, Khush KK. Assessment of Heart Transplant Waitlist Time and Pre- and Post-transplant Failure: A Mixed Methods Approach. Epidemiology. 2016;27(4):469-76.

12. Brink JG, Hassoulas J. The first human heart transplant and further advances in cardiac transplantation at Groote Schuur Hospital and the University of Cape Town - with reference to : the operation. A human cardiac transplant: an interim report of a successful operation performed at Groote Schuur Hospital, Cape Town. Cardiovasc J Afr. 2009;20(1):31-5.

13. Stolf NAG. History of Heart Transplantation: a Hard and Glorious Journey. Braz J Cardiovasc Surg. 2017;32(5):423-7.

14. Kantrowitz A, Haller JD, Joos H, Cerruti MM, Carstensen HE. Transplantation of the heart in an infant and an adult. Am J Cardiol. 1968;22(6):782-90.

15. Jacques F, Carrier M, Pelletier GB, White M, Racine N, Pellerin M, et al. Two decades of cardiac transplantation at the Montreal Heart Institute. Can J Cardiol. 2008;24(3):217-21.

16. Kim IC, Youn JC, Kobashigawa JA. The Past, Present and Future of Heart Transplantation. Korean Circ J. 2018;48(7):565-90.

17. Sivathasan C. Experience with cyclosporine in heart transplantation. Transplant Proc. 2004;36(2 Suppl):346s-8s.

18. Beuth J, Falter F, Pinto Ribeiro RV, Badiwala M, Meineri M. New Strategies to Expand and Optimize Heart Donor Pool: Ex Vivo Heart Perfusion and Donation After Circulatory Death: A Review of Current Research and Future Trends. Anesth Analg. 2019;128(3):406-413

19. Kirklin JK. Terminal heart failure: who should be transplanted and who should have mechanical circulatory support? Curr Opin Organ Transplant. 2014;19(5):486-93.

20. Freeman R, Koerner E, Clark C, Halabicky K. The Path From Heart Failure to Cardiac Transplant. Crit Care Nurs Q. 2016;39(3):207-13.

21. Prieto D, Correia P, Antunes P, Batista M, Antunes MJ. Results of heart transplantation in the urgent recipient--who should be transplanted? Braz J Cardiovasc Surg. 2014;29(3):379-87.

22. Lui C, Suarez-Pierre A, Zhou X, Crawford TC, Fraser CD, Giuliano K, et al. Effects of Systemic and Device-Related Complications in Patients Bridged to Transplantation With Left Ventricular Assist Devices. J Surg Res. 2020;246:207-12.

23. Ralph A, Chapman JR, Gillis J, Craig JC, Butow P, Howard K, et al. Family perspectives on deceased organ donation: thematic synthesis of qualitative studies. Am J Transplant. 2014;14(4):923-35.

24. Merchant SJ, Yoshida EM, Lee TK, Richardson P, Karlsbjerg KM, Cheung E. Exploring the psychological effects of deceased organ donation on the families of the organ donors. Clin Transplant. 2008;22(3):341-7.

25. Rudge C, Matesanz R, Delmonico FL, Chapman J. International practices of organ donation. Brit J Anaesth. 2012;108 Suppl 1:i48-55.

26. Girlanda R. Deceased organ donation for transplantation: Challenges and opportunities. World J Transplant. 2016;6(3):451-9.

27. Saidi RF. Changing pattern of organ donation and utilization in the USA. Int J Organ Transplant Med. 2012;3(4):149-56.

28. Thuong M, Ruiz A, Evrard P, Kuiper M, Boffa C, Akhtar MZ, et al. New classification of donation after circulatory death donors definitions and terminology. Transpl Int . 2016;29(7):749-59.

29. Reznik ON, Skvortsov AE, Reznik AO, Ananyev AN, Tutin AP, Kuzmin DO, et al. Uncontrolled donors with controlled reperfusion after sixty minutes of asystole: a novel reliable resource for kidney transplantation. PloS One. 2013;8(5):e64209.

30. Nakagawa TA, Shemie SD, Dryden-Palmer K, Parshuram CS, Brierley J. Organ Donation Following Neurologic and Circulatory Determination of Death. Pediatr Crit Care Med. 2018;19(8S Suppl 2):S26-s32.

31. Da Silva IR, Frontera JA. Worldwide barriers to organ donation. JAMA Neurol. 2015;72(1):112-8.

32. Shemie SD, Doig C, Dickens B, Byrne P, Wheelock B, Rocker G, et al. Severe brain injury to neurological determination of death: Canadian forum recommendations. Can Med Assoc J. 2006;174(6):S1-13.

33. Shemie SD. Trends in deceased organ donation in Canada. Can Med Assoc J. 2017;189(38):E1204-e5.

34. Wijdicks EF. Brain death. Handb Clin Neurol. 2013;118:191-203.

35. Smith M. Brain death: time for an international consensus. Brit J Anaesth. 2012;108 Suppl 1:i6-9.

36. Gardiner D, Shemie S, Manara A, Opdam H. International perspective on the diagnosis of death. Brit J Anaesth. 2012;108 Suppl 1:i14-28.

37. Kramer AH, Baht R, Doig CJ. Time trends in organ donation after neurologic determination of death: a cohort study. CMAJ. 2017;5(1):E19-e27.

38. Nguan C. Trying to meet the demands in organ transplantation. Can Urol Assoc J. 2012;6(6):453-4.

39. Cloutier R, Baran D, Morin JE, Dandavino R, Marleau D, Naud A, et al. Brain death diagnoses and evaluation of the number of potential organ donors in Quebec hospitals. Can J Anaesth. 2006;53(7):716-21.

40. Kramer AH, Zygun DA, Doig CJ, Zuege DJ. Incidence of neurologic death among patients with brain injury: a cohort study in a Canadian health region. Can Med Assoc J. 2013;185(18):E838-45.

41. Westphal GA, Caldeira Filho M, Fiorelli A, Vieira KD, Zaclikevis V, Bartz M, et al. Guidelines for maintenance of adult patients with brain death and potential for multiple organ donations: the Task Force of the Brazilian Association of Intensive Medicine the Brazilian Association of Organs Transplantation, and the Transplantation Center of Santa Catarina. Transplant Proc. 2012;44(8):2260-7.

42. Manara AR, Murphy PG, O'Callaghan G. Donation after circulatory death. Br J Anaesth. 2012;108 Suppl 1:i108-21.

43. Patel K, Hipskind JE. Cardiac Arrest. StatPearls. : StatPearls Publishing LLC.; 2019.

44. Kootstra G, Daemen JH, Oomen AP. Categories of non-heart-beating donors. Transplant Proc. 1995;27(5):2893-4.

45. White CW, Lillico R, Sandha J, Hasanally D, Wang F, Ambrose E, et al. Physiologic Changes in the Heart Following Cessation of Mechanical Ventilation in a Porcine Model of Donation After Circulatory Death: Implications for Cardiac Transplantation. Am J Transplant. 2016;16(3):783-93.

46. Harvey PR, Iu S, McKeown CM, Petrunka CN, Ilson RG, Strasberg SM. Adenine nucleotide tissue concentrations and liver allograft viability after cold preservation and warm ischemia. Transplantation. 1988;45(6):1016-20.

47. Gomez L, Li B, Mewton N, Sanchez I, Piot C, Elbaz M, et al. Inhibition of mitochondrial permeability transition pore opening: translation to patients. Cardiovascr Res. 2009;83(2):226-33.

48. Heusch G. Molecular basis of cardioprotection: signal transduction in ischemic pre-, post-, and remote conditioning. Circ Res. 2015;116(4):674-99.

49. Garcia-Dorado D, Ruiz-Meana M, Inserte J, Rodriguez-Sinovas A, Piper HM. Calcium-mediated cell death during myocardial reperfusion. Cardiovasc Res. 2012;94(2):168-80.

50. Messer S, Page A, Colah S, Axell R, Parizkova B, Tsui S, et al. Human heart transplantation from donation after circulatory-determined death donors using normothermic regional perfusion and cold storage. J Heart Lung Transplant. 2018;37(7):865-9.

51. Noterdaeme T, Detry O, Hans MF, Nellessen E, Ledoux D, Joris J, et al. What is the potential increase in the heart graft pool by cardiac donation after circulatory death? Transpl Int. 2013;26(1):61-6.

52. Messer SJ, Axell RG, Colah S, White PA, Ryan M, Page AA, et al. Functional assessment and transplantation of the donor heart after circulatory death. J Heart Lung Transplant. 2016;35(12):1443-52.

53. Dhital KK, Iyer A, Connellan M, Chew HC, Gao L, Doyle A, et al. Adult heart transplantation with distant procurement and ex-vivo preservation of donor hearts after circulatory death: a case series. Lancet. 2015;385(9987):2585-91.

54. Boucek MM, Mashburn C, Dunn SM, Frizell R, Edwards L, Pietra B, et al. Pediatric heart transplantation after declaration of cardiocirculatory death. N Engl J Med. 2008;359(7):709-14.

55. Siegmund B, Klietz T, Schwartz P, Piper HM. Temporary contractile blockade prevents hypercontracture in anoxic-reoxygenated cardiomyocytes. Am J Physiol. 1991;260(2 Pt 2):H426-35.

56. Osaki S, Ishino K, Kotani Y, Honjo O, Suezawa T, Kanki K, et al. Resuscitation of non-beating donor hearts using continuous myocardial perfusion: the importance of controlled initial reperfusion. Ann Thorac Surg. 2006;81(6):2167-71.

57. Ribeiro RVP, Alvarez JS, Yu F, Paradiso E, Adamson MB, Maria Ruggeri G, et al. Hearts Donated After Circulatory Death and Reconditioned Using Normothermic Regional Perfusion Can Be Successfully Transplanted Following an Extended Period of Static Storage. Circ Heart Fail. 2019;12(4):e005364.

58. Aguilar A, Alvarez-Vijande R, Capdevila S, Alcoberro J, Alcaraz A. Antioxidant patterns (superoxide dismutase, glutathione reductase, and glutathione peroxidase) in kidneys from non-heart-beating-donors: experimental study. Transplant Proc. 2007;39(1):249-52.

59. Barnard CN. The operation. A human cardiac transplant: an interim report of a successful operation performed at Groote Schuur Hospital, Cape Town. S Afr Med J. 1967;41(48):1271-4.

60. Diagnosis of brain death. Lancet. 1976;2(7994):1069-70.

61. Canadian Institute of Health Information. Organ replacement in Canada: CORR annual statistics, 2019. 2019.

62. Angeli E, Lueck S, Gargiulo GD. Different strategies of myocardial protection: the age of perfectionism. J Thorac Dis. 2018;10(3):1211-3.

63. Vinten-Johansen J, Thourani VH. Myocardial protection: an overview. J Extra Corpor Technol. 2000;32(1):38-48.

64. Nardi P, Pisano C, Bertoldo F, Vacirca SR, Saitto G, Costantino A, et al. Warm blood cardioplegia versus cold crystalloid cardioplegia for myocardial protection during coronary artery bypass grafting surgery. Cell Death Discov. 2018;4:23.

65. Baikoussis NG, Papakonstantinou NA, Verra C, Kakouris G, Chounti M, Hountis P, et al. Mechanisms of oxidative stress and myocardial protection during open-heart surgery. Ann Card Anaesth. 2015;18(4):555-64.

66. Demmy TL, Biddle JS, Bennett LE, Walls JT, Schmaltz RA, Curtis JJ. Organ preservation solutions in heart transplantation--patterns of usage and related survival. Transplantation. 1997;63(2):262-9.

67. Stehlik J, Edwards LB, Kucheryavaya AY, Benden C, Christie JD, Dipchand AI, et al. The Registry of the International Society for Heart and Lung Transplantation: 29th official adult heart transplant report--2012. J Heart Lung Transplant. 2012;31(10):1052-64.

68. Belzer FO, Southard JH. Principles of solid-organ preservation by cold storage. Transplantation. 1988;45(4):673-6.

69. Iyer A, Gao L, Doyle A, Rao P, Cropper JR, Soto C, et al. Normothermic ex vivo perfusion provides superior organ preservation and enables viability assessment of hearts from DCD donors. Am J Transplant. 2015;15(2):371-80.

70. Messer S, Ardehali A, Tsui S. Normothermic donor heart perfusion: current clinical experience and the future. Transpl Int. 2015;28(6):634-42.

71. Jahania MS, Sanchez JA, Narayan P, Lasley RD, Mentzer RM, Jr. Heart preservation for transplantation: principles and strategies. Ann Thorac Surg. 1999;68(5):1983-7.

72. Guibert EE, Petrenko AY, Balaban CL, Somov AY, Rodriguez JV, Fuller BJ. Organ Preservation: Current Concepts and New Strategies for the Next Decade. Transfus Med Hemother. 2011;38(2):125-42.

73. Schipper DA, Marsh KM, Ferng AS, Duncker DJ, Laman JD, Khalpey Z. The Critical Role of Bioenergetics in Donor Cardiac Allograft Preservation. J Cardiovasc Tansl Res. 2016;9(3):176-83.

74. Salehi S, Tran K, Grayson WL. Advances in Perfusion Systems for Solid Organ Preservation. Yale J Biol Med. 2018;91(3):301-12.

75. Bonventre JV, Cheung JY. Effects of metabolic acidosis on viability of cells exposed to anoxia. Am J Physiol. 1985;249(1 Pt 1):C149-59.

76. Michel P, Vial R, Rodriguez C, Ferrera R. A comparative study of the most widely used solutions for cardiac graft preservation during hypothermia. J Heart Lung Transplant. 2002;21(9):1030-9.

77. Teh ES, Zal F, Polard V, Menasche P, Chambers DJ. HEMO2life as a protective additive to Celsior solution for static storage of donor hearts prior to transplantation. Artif Cells Nanomed Biotechnol. 2017;45(4):717-22.

78. Ali A, White P, Dhital K, Ryan M, Tsui S, Large S. Cardiac recovery in a human non-heart-beating donor after extracorporeal perfusion: source for human heart donation? J Heart Lung Transplant. 2009;28(3):290-3.

79. Minambres E, Suberviola B, Dominguez-Gil B, Rodrigo E, Ruiz-San Millan JC, Rodriguez-San Juan JC, et al. Improving the Outcomes of Organs Obtained From Controlled Donation After Circulatory Death Donors Using Abdominal Normothermic Regional Perfusion. Am J Transplant. 2017;17(8):2165-72.

80. Messer S, Page A, Axell R, Berman M, Hernandez-Sanchez J, Colah S, et al. Outcome after heart transplantation from donation after circulatory-determined death donors. J Heart Lung Transplant. 2017;36(12):1311-8.

81. Freed DH, White CW. Donor heart preservation: straight up, or on the rocks? Lancet. 2015;385(9987):2552-4.

82. Trahanas JM, Witer LJ, Alghanem F, Bryner BS, Iyengar A, Hirschl JR, et al. Achieving 12 Hour Normothermic Ex Situ Heart Perfusion: An Experience of 40 Porcine Hearts. ASAIO J. 2016;62(4):470-6.

83. Ardehali A, Esmailian F, Deng M, Soltesz E, Hsich E, Naka Y, et al. Ex-vivo perfusion of donor hearts for human heart transplantation (PROCEED II): a prospective, open-label, multicentre, randomised non-inferiority trial. Lancet. 2015;385(9987):2577-84.

84. Liao R, Podesser BK, Lim CC. The continuing evolution of the Langendorff and ejecting murine heart: new advances in cardiac phenotyping. Am J Physiol Heart C. 2012;303(2):H156-67.

85. Rivard AL, Gallegos R, Ogden IM, Bianco RW. Perfusion preservation of the donor heart: basic science to pre-clinical. J Extra Corpor Technol. 2009;41(3):140-8.

86. Hatami S, White CW, Ondrus M, Qi X, Buchko M, Himmat S, et al. Normothermic Ex Situ Heart Perfusion in Working Mode: Assessment of Cardiac Function and Metabolism. JoVE-J Vis Exp. 2019(143).

87. Leprince P, Popov AF, Simon AR, Benk C, Siepe M. Ex vivo perfusion of the heart with the use of the Organ Care System. Eur J Cardio-thorac Surg. 2016;49(5):1318-20.

88. Smulowitz PB, Serna DL, Beckham GE, Milliken JC. Ex vivo cardiac allograft preservation by continuous perfusion techniques. ASAIO J. 2000;46(4):389-96.

89. Monteagudo Vela M, Garcia Saez D, Simon AR. Current approaches in retrieval and heart preservation. Ann Cardiothorac Surg. 2018;7(1):67-74.

90. Sunjaya AF, Sunjaya AP. Combating Donor Organ Shortage: Organ Care System Prolonging Organ Storage Time and Improving the Outcome of Heart Transplantations. Cardiovasc Therapeut. 2019;2019:9482797.

91. Garcia Saez D, Zych B, Sabashnikov A, Bowles CT, De Robertis F, Mohite PN, et al. Evaluation of the organ care system in heart transplantation with an adverse donor/recipient profile. Ann Thorac Surg. 2014;98(6):2099-105; discussion 105-6.

92. Suzuki Y, Yeung AC, Ikeno F. The representative porcine model for human cardiovascular disease. J Biomed Biotechnol. 2011;2011:195483.

93. Camacho P, Fan H, Liu Z, He JQ. Large Mammalian Animal Models of Heart Disease. J Cardiovasc Dev Dis. 2016;3(4).

94. Chong JJ, Murry CE. Cardiac regeneration using pluripotent stem cells--progression to large animal models. Stem Cell Res. 2014;13(3 Pt B):654-65.

95. Graves JA. Background and Overview of Comparative Genomics. ILAR J. 1998;39(2-3):48-65.

96. Cui J, Li J, Mathison M, Tondato F, Mulkey SP, Micko C, et al. A clinically relevant large-animal model for evaluation of tissue-engineered cardiac surgical patch materials. Cardiovasc Revasc Med. 2005;6(3):113-20.

97. Hearse DJ, Sutherland FJ. Experimental models for the study of cardiovascular function and disease. Pharmacol Res. 2000;41(6):597-603.

98. Lelovas PP, Kostomitsopoulos NG, Xanthos TT. A comparative anatomic and physiologic overview of the porcine heart. J Am Assoc Lab Anim Sci. 2014;53(5):432-8.

99. Milani-Nejad N, Janssen PM. Small and large animal models in cardiac contraction research: advantages and disadvantages. Pharmacol Therapeut. 2014;141(3):235-49.

100. Hatami S, White CW, Shan S, Haromy A, Qi X, Ondrus M, et al. Myocardial Functional Decline During Prolonged Ex Situ Heart Perfusion. Ann Thorac Surg. 2019;108(2):499-507.

101. Dromparis P, Aboelnazar NS, Wagner S, Himmat S, White CW, Hatami S, et al. Ex vivo perfusion induces a time- and perfusate-dependent molecular repair response in explanted porcine lungs. Am J Transplant. 2019;19(4):1024-36.

102. Adam B, Afzali B, Dominy KM, Chapman E, Gill R, Hidalgo LG, et al. Multiplexed color-coded probe-based gene expression assessment for clinical molecular diagnostics in formalin-fixed paraffin-embedded human renal allograft tissue. Clin Transplant. 2016;30(3):295-305.

103. Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N, Dunaway DL, et al. Direct multiplexed measurement of gene expression with color-coded probe pairs. Nat Biotechnol. 2008;26(3):317-25.

104. Reis PP, Waldron L, Goswami RS, Xu W, Xuan Y, Perez-Ordonez B, et al. mRNA transcript quantification in archival samples using multiplexed, color-coded probes. BMC Biotechnol. 2011;11:46.

105. Cesano A. nCounter((R)) PanCancer Immune Profiling Panel (NanoString Technologies, Inc., Seattle, WA). J Immunother Cancer. 2015;3:42.

106. Kulkarni MM. Digital multiplexed gene expression analysis using the NanoString nCounter system. Curr Prot Mol Biol. 2011;Chapter 25:Unit25B.10.

107. Veldman-Jones MH, Brant R, Rooney C, Geh C, Emery H, Harbron CG, et al. Evaluating Robustness and Sensitivity of the NanoString Technologies nCounter Platform to Enable Multiplexed Gene Expression Analysis of Clinical Samples. Cancer Res. 2015;75(13):2587-93.

108. Tsang HF, Xue VW, Koh SP, Chiu YM, Ng LP, Wong SC. NanoString, a novel digital color-coded barcode technology: current and future applications in molecular diagnostics. Expert Rev Mol Diagn. 2017;17(1):95-103.

109. Adam BA, Smith RN, Rosales IA, Matsunami M, Afzali B, Oura T, et al. Chronic Antibody-Mediated Rejection in Nonhuman Primate Renal Allografts: Validation of Human Histological and Molecular Phenotypes. Am J Transplant. 2017;17(11):2841-50.

110. Adam B, Mengel M. Molecular nephropathology: ready for prime time? Am J Physiol-Renal. 2015;309(3):F185-8.

111. Ruppert V, Maisch B. Molecular signatures and the study of gene expression profiles in inflammatory heart diseases. Herz. 2012;37(6):619-26.

Chapter 2

Gene set development for the assessment of cardiac injury and repair during *ex vivo* heart perfusion[1]

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2.1 Introduction

Ex vivo heart perfusion (EVHP) devices represent a promising alternative to cold static storage for the preservation of donor hearts prior to heart transplantation. The greatest potential benefit of this technology, as discussed in chapter one of this thesis, is the opportunity to evaluate, repair and preserve 'extended criteria' donor hearts, including those from donation after circulatory death (DCD) donors. Despite preliminary success with EVHP for short-term organ preservation, emerging reports demonstrating progressive functional decline (of normally working hearts pre-retrieval) after 12 h of perfusion highlight the need for additional optimization before the potential benefits of this technology can be fully realized. In particular, the molecular mechanisms of donor heart injury and repair during EVHP remain poorly characterized. However, previous research focused on analogous processes of cardiac ischemia and reperfusion provide existing knowledge from which gene signatures can be developed and used as novel tools for organ assessment during preservation.

Ideally, any biological process, including cellular response to a stimulus or a pathological condition, can be measured or reflected by changes in the expression levels of genes (1). The central dogma of molecular biology describes the flow of information stored within genetic DNA being transcribed into mRNA, and then translated into proteins (2). Expression of this genetic information following modification by environmental factors phenotypically characterizes an organism (3). Gene expression profiling provides an opportunity to better understand these biological changes. The level of expression of a particular gene can be determined by quantifying the number of corresponding messenger RNA (mRNA) molecules present in a sample. However, expression of a single gene may not necessarily define biological conditions. Therefore, it is often prudent to utilize gene signatures associated with a biological condition to better define and understand the molecular mechanisms of biological changes. Accordingly, gene signatures have

become critical tools for assessing molecular changes representing specific cellular conditions or disease states, in order to address research questions or evaluate responses to clinical therapeutic interventions (4).

The completion of the Human Genome Project (HGP) in April 2003 following its inception in 1990, with the aim of sequencing and mapping all genes of the entire human genome, has catalyzed a tremendous transformation in medicine (5). With an initial quest to better understand human cancer, the HGP brought together expertise from multiple areas of science including engineers, computer specialists, statisticians and biologists, to ensure the project was successful (6, 7). The HGP led to the development of new technologies and analytical tools that facilitated open data sharing through user-friendly software such as GenBank, ultimately making HGP data available to all (8). Additionally, the HGP led to the development of high-throughput technologies facilitating the sequencing of non-human pre-clinical models, thus benefiting translation from basic research to clinical practice (6). The availability of genome-wide sequences provides the opportunity to transform our increasingly sophisticated knowledge of human diseases into the provision of predictive, preventive, and personalized medicine.

The ability to identify gene signatures reflecting specific biological processes provides the opportunity to perform informative gene expression profiling with high-throughput technologies such as microarrays, RNA sequencing and NanoString nCounter. Quantitative information representing changes in expressed genes can be used to link the relationship between biological processes with distinct perturbations. Although all three of the previously mentioned transcriptomics platforms can be used to measure the expressions of large numbers of genes simultaneously, this thesis focuses on the use of NanoString nCounter technology. As discussed in chapter one, this is a robust high-throughput gene expression profiling platform capable of

measuring mRNA levels of specific gene signatures in a flexible, simple and cost-effective manner, including from formalin-fixed paraffin-embedded (FFPE) tissue samples.

Developing informative gene signatures requires identifying sets of genes that reliably represent the biological processes of interest. Ideally, this involves the use of genome-wide microarray or RNA sequencing data to define gene signatures that can be utilized as postulates of a given study characteristic or biological state of interest (1). Gene signatures can either be developed from experimental databases such as PubMed or *a priori* knowledge such as gene ontologies and signaling pathways. However, this process of gene set development is hindered by functional redundancy of genes, which can potentially result in the exclusion of relevant genes from the signature list. The Molecular Signature Database Hallmarks (MSigDB H), an enrichment analysis database, represents one of the currently available platforms that can be used to identify robust and non-redundant gene expression signatures (9). The MSigDB H collection was derived and refined by merging compositionally redundant gene sets based on their ability to distinguish related biological process (10).

As discussed in the first chapter of this thesis, limited understanding of and insufficient tools for studying the mechanisms of EVHP-related cardiac injury and repair remain a barrier to the successful clinical implementation of this promising technology. This chapter is aimed at developing a literature-based, cardiac-specific, injury and repair-related gene set from the PubMed database that can be used to measure molecular responses before, during and after EVHP. Specifically, a PubMed search was performed to retrieve articles describing significantly differentially expressed genes associated with various clinical and experimental models of acute cardiac injury and repair. Several additional databases, including MSigDB Hallmarks, SPEED, STRING, Metascape, Cytoscape and Gene Card, were utilized to further characterize the

differentially expressed genes identified from the PubMed database. Furthermore, signaling pathways associated with the selected genes were studied through enrichment analysis. Also, Metascape was used to identify possible protein complexes via the automated MCODE algorithm. Finally, possible functions of the identified MCODE protein complexes were explored, including their mechanisms and the signaling pathways involved.

2.2 Materials and Methods

2.2.1 Data sets

This study focuses on previously published articles reporting microarray data related to acute injury/stress, ischemia/reperfusion, or repair in human or animal cardiac tissue or cells. Specifically, PubMed was searched using the following terms: 1) *"acute AND (heart OR cardiac OR myocardial) AND (injury OR failure) AND microarray",* 2) *"(heart OR cardiac OR myocardial) AND repair AND microarray",* and *3) "(heart OR cardiac OR myocardial) AND ischemia AND reperfusion AND microarray"* as assessed on June 1, 2018. Studies focusing on chronic heart disease were excluded. For all studies meeting these inclusion criteria, all genes reported to be statistically significantly differentially expressed were recorded. These genes were then categorized based on the number of studies in which they were independently reported. To balance comprehensiveness and functional redundancy, the final gene set used for downstream analysis was derived from the reporting frequency category containing 50 to 100 genes.

2.2.2 Gene ontology enrichment analysis

The Metascape database (http://metascape.org) was utilized to evaluate the overriding Gene Ontology (GO) biological network categories represented by the final gene set (11). The GO sources utilized by the Metascape database for this analysis included the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://genome.jp/kegg), Gene Ontology (GO) Biological Processes

(http://geneontology.org/), Canonical Pathways, Reactome Gene sets (http://reactome.org) and the comprehensive resource of mammalian protein complexes (CORUM; http://mips.gsf.de/genre/proj/corum/index.html) (11, 12). This database uses the whole genome as the enrichment background. P-values were calculated based on accumulative hypergeometric distribution (13). A minimum of three terms with P-value <0.01 and enrichment factor greater than 1.5 were applied to group the associations into clusters based on terms with similar characteristics. Kappa scores were used to perform hierarchical clustering of the terms, with a score greater than 0.3 representing a significant cluster (14). For visualization of gene co-expression networks, and for better understanding of the enriched biological states, Cytoscope version 3.7.2 (http://cytoscape.org) was used (15, 16). Functional annotations were further manually refined using MSigDB H, Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) (http://string-db.org), and Gene Card database (GeneAlaCart file (date: January 2020). GO structures were obtained from xlsx files (9, 17).

2.2.3 Signaling pathway enrichment analysis

Signaling Pathway Enrichment using Experimental Data sets (SPEED) (http://speed.sys-bio.net/), which supports humans genes and signaling pathways only, was used to perform pathway enrichment analysis. “Maximum absolute Z-score percentile” of 1% (more stringent) and “Minimum percent overlap across experiments” of 33% (more lenient) was used. A stringent Z-score percentile limits the number of regulated genes, thus accepting highly differentially expressed genes as gene signatures, whereas a lenient minimum percent overlap across experiments allows a greater number of less consistent genes to be considered, overall balancing the number of genes accepted with a reasonable level of confidence (18). To assess the balance relative to experimental background noise, “Maximum expression level percentile” was set at 40%

with all available pathways selected for use. The complete list of genes in the SPEED database was included to determine statistical significance.

2.2.4 Construction of protein-protein interaction network

To better understand the potential functions of the target gene products in the final gene set, STRING was used to construct a protein-protein interaction (PPI) network. The STRING database PPI network is based on seven parameters, including: i) curated database which is enriched with known metabolic and signal transduction pathways and protein complexes, ii) experimentally derived data including yeast two-hybrid, co-crystallization, co-purification and genetic interactions, iii) text-mining which incorporates an unsupervised automated data search of proteins that are commonly mentioned together, iv) co-expression, which identifies genes that are co-regulated over a large number of experiments, v) co-occurrence, which identifies groups of genes with similar patterns of existence across genomes, vi) neighborhood, which identifies groups of genes that are regularly observed together in their neighborhood, and vii) fusion, a parameter that identifies sets of genes that are occasionally present in one open reading frame (17). A median confidence of combined score greater than 0.4 was set to explore the regulatory mechanisms of the final gene set. Additionally, a search for genes that form protein complexes (i.e. a network that contains between 3 to 500 gene products) was performed using the Metascape database (11).

2.3 Results

2.3.1 Identification of acute cardiac injury and repair associated genes

The search terms identified a total of 56 studies reporting significant differential gene expression patterns in relevant clinical or experimental models. Specifically, *"acute AND (heart OR cardiac OR myocardial) AND (injury OR failure) AND microarray"* identified 18 studies (19-36), *"(heart OR cardiac OR myocardial) AND repair AND microarray"* identified 20 studies (37-

56), and *"(heart OR cardiac OR myocardial) AND ischemia AND reperfusion AND microarray"* identified 18 studies (57-73). These 56 studies reported a total of 1735 unique genes with significant differential expression.

These genes were grouped according to the number of articles describing significant differential expression for each of them. *TIMP1* was the most frequently reported gene, being described in nine out of 56 studies. Two genes (*CXCL10* and *HMOX1*) were reported in seven studies, two genes (*CCL2* and *NR4A1*) were reported in six studies, five genes (*ATF3, BTG2, CXCL2, THBS1, VCAM1*) were reported in five studies, and 15 genes (*CCR2, CTGF, EGR1, FOS, GADD45B, ICAM1, IL6, LOX, MMP12, MYC, RTN4, S100A9, SELE, SERPINE1, SOCS3*) were reported in four studies. An additional 43 genes were reported in three studies, 201 genes in two studies, and 1466 genes in only one study (Table 2.1). Given the goal gene set size of 50-100 genes, those described in at least three articles (n=68 genes) were selected for further analysis (Table 2.2).

Table 2.1. Summary of the reporting frequency of 1735 significantly differentially expressed cardiac injury and repair genes in 56 studies as of June 1, 2018.

Number of articles describing significant differential expression for a specific gene	Total number of unique genes reported
≥1	1735
≥2	269
≥3	68
≥4	25
≥5	10
≥6	5
≥7	3
≥8	1
≥9	1

Table 2.2. Summary of the transcripts included in the literature-derived 68-gene set for cardiac injury and repair.

Gene Symbol	Gene Name	Functional Annotation	Reference
ACTA1	Actin, alpha 1, skeletal muscle	Cell structure	(29, 38, 46)
ACTA2	Actin, alpha 2, smooth muscle, aorta	Cell structure	(29, 39, 63)
ADAM8	ADAM metallopeptidase domain 8	Tissue remodeling	(24, 37, 57)
ANKRD1	Ankyrin repeat domain 1	Signal transduction	(29, 33, 39)
ATF3	Activating transcription factor 3	Signal transduction	(25, 30, 62, 68)
BTG2	BTG anti-proliferation factor 2	Apoptosis	(25, 29, 33, 36, 64)
CASP8	Caspase 8	Apoptosis	(29, 65, 68)
CCL2	C-C motif chemokine ligand 2	Immune response	(19, 20, 24, 25, 61, 73)
CCR2	C-C motif chemokine receptor 2	Immune response	(24, 29, 45, 57)
CD68	CD68 molecule	Immune response	(19, 29, 57)
CD8A	CD8a molecule	Immune response	(19, 29, 40)
COL1A1	Collagen type I alpha 1 chain	Tissue remodeling	(24, 31, 41)
COL4A1	Collagen type IV alpha 1 chain	Tissue remodeling	(24, 29, 41)
CTGF	Connective tissue growth factor	Tissue remodeling	(25, 29, 47, 50)
CTSS	Cathepsin S	Immune response	(19, 37, 50)
CXCL10	C-X-C motif chemokine ligand 10	Immune response	(19, 24, 29, 45, 47, 51, 57)
CXCL11	C-X-C motif chemokine ligand 11	Immune response	(19, 51, 59)
CXCL2	C-X-C motif chemokine ligand 2	Immune response	(25, 29, 33, 59, 62)
CXCL9	C-X-C motif chemokine ligand 9	Immune response	(19, 29, 51)
CYR61	Cysteine rich angiogenic inducer 61	Cell proliferation	(25, 29, 60)
EGLN3	Egl-9 family hypoxia inducible factor 3	Oxidative stress	(29, 33, 63)
EGR1	Early growth response 1	Signal transduction	(29, 33, 48, 62)
FCER1G	Fc fragment of IgE receptor Ig	Immune response	(19, 24, 29)
FCGR2B	Fc fragment of IgG receptor IIb	Immune response	(24, 29, 45)
FN1	Fibronectin 1	Tissue remodeling	(24, 31, 50)
FOS	Fos proto-oncogene, AP-1 transcription factor subunit	Signal transduction	(33, 62, 73)
GADD45B	Growth arrest and DNA damage inducible beta	Apoptosis	(29, 30, 32, 56)
GDF15	Growth differentiation factor 15	Signal transduction	(47, 60, 63)
HMOX1	Heme oxygenase 1	Oxidative stress	(24, 33, 41, 57, 59)
HSP90AA1	Heat shock protein 90 alpha family class A member 1	Signal transduction	(29, 64, 73)
ICAM1	Intercellular adhesion molecule 1	Immune response	(28, 47, 62, 73)
IGF1	Insulin like growth factor 1	Cell proliferation	(48, 53, 55)
IL6	Interleukin 6	Immune response	(29, 44, 68, 73)
IRS1	Insulin receptor substrate 1	Signal transduction	(29, 47, 63)
ITGB2	Integrin subunit beta 2	Immune response	(19, 24, 57)
JUN	Jun proto-oncogene, AP-1 transcription factor subunit	Signal transduction	(29, 73)
LOX	Lysyl oxidase	Tissue remodeling	(24, 33, 57, 71)
MCM6	Minichromosome maintenance complex component 6	Signal transduction	(24, 29, 34)
MEF2C	Myocyte enhancer factor 2C	Signal transduction	(116-118)

Table 2.2. Summary of the transcripts included in the literature-derived 68-gene set for cardiac injury and repair (continued)

Gene Symbol	Gene Name	Functional Annotation	Reference
MMP12	Matrix metallopeptidase 12	Tissue remodeling	(37, 40, 47, 57)
MMP14	Matrix metallopeptidase 14	Tissue remodeling	(29, 37, 47)
MYC	MYC proto-oncogene, bHLH transcription factor	Cell proliferation	(29, 32, 64, 73)
MYH7	Myosin heavy chain 7	Cell structure	(29, 50, 61)
MYL9	Myosin light chain 9	Cell structure	(29, 50, 59)
MYOM2	Myomesin 2	Cell structure	(29, 34, 61)
NFATC4	Nuclear factor of activated T cells 4	Signal transduction	(119-121)
NFKBIA	NFKB inhibitor alpha	Immune response	(27, 33, 35)
NPPB	Natriuretic peptide B	Signal transduction	(20, 25, 61)
NR4A1	Nuclear receptor subfamily 4 group A member 1	Signal transduction	(29, 33, 62, 64)
PECAM1	Platelet and endothelial cell adhesion molecule 1	Immune response	(29, 46, 62)
PGF	Placental growth factor	Cell proliferation	(33, 57, 60)
PTGDS	Prostaglandin D2 synthase	Signal transduction	(29, 34, 61)
RTN4	Reticulon 4	Signal transduction	(29, 59, 62, 64)
S100A8	S100 calcium binding protein A8	Immune response	(23, 73)
S100A9	S100 calcium binding protein A9	Immune response	(24, 25, 29, 73)
SELE	Selectin E	Immune response	(25, 29, 59, 73)
SERPINE1	Serpin family E member 1	Tissue remodeling	(25, 29, 37, 47)
SLC2A4	Solute carrier family 2-member 4	Signal transduction	(29, 33, 60)
SOCS3	Suppressor of cytokine signaling 3	Signal transduction	(21, 29, 44, 50)
TGFB1	Transforming growth factor beta 1	Tissue remodeling	(33, 47, 50)
THBS1	Thrombospondin 1	Tissue remodeling	(19, 24, 25, 29, 50)
TIMP1	TIMP metallopeptidase inhibitor 1	Tissue remodeling	(21, 25, 31, 37, 47, 50, 57, 65, 71)
TNC	Tenascin C	Tissue remodeling	(29, 50, 54)
TNF	Tumor necrosis factor	Immune response	(29, 44, 73)
TNFAIP3	TNF alpha induced protein 3	Immune response	(19, 27, 35)
TRDN	Triadin	Cell structure	(29, 34, 63)
VCAM1	Vascular cell adhesion molecule 1	Immune response	(29, 40, 47, 57, 69)
VEGFC	Vascular endothelial growth factor C	Tissue remodeling	(29, 51, 67)

2.3.2 Gene Ontology (GO) analysis of cardiac injury-repair gene set

To understand the biological functions of the 68 genes identified from the literature, GO analysis was performed using the Metascape database. From the enrichment analysis, many biological processes associated with acute cardiac tissue injury and repair were confirmed,

including leukocyte migration, response to low oxygen levels, extracellular matrix organization, tissue morphogenesis, cell death regulation, epithelial cell proliferation, muscle structure development, and response to growth factor (Figure 2.1A). The interconnection of the GO associated terms was assessed using Cytoscape, with nodes representing GO terms that are interconnected based on similar biological processes (Figure 2.1B). Further refinement of these terms using the MSgDB H, GeneCards and STRING databases resulted in each gene being associated with one of six major functional categories, including immune response (n=22 genes), tissue remodelling (n=14), signal transduction (n=17), apoptosis (n=3), cell structure (n=6), cell proliferation (n=4), and oxidative stress (n=2) (Table 2.2).

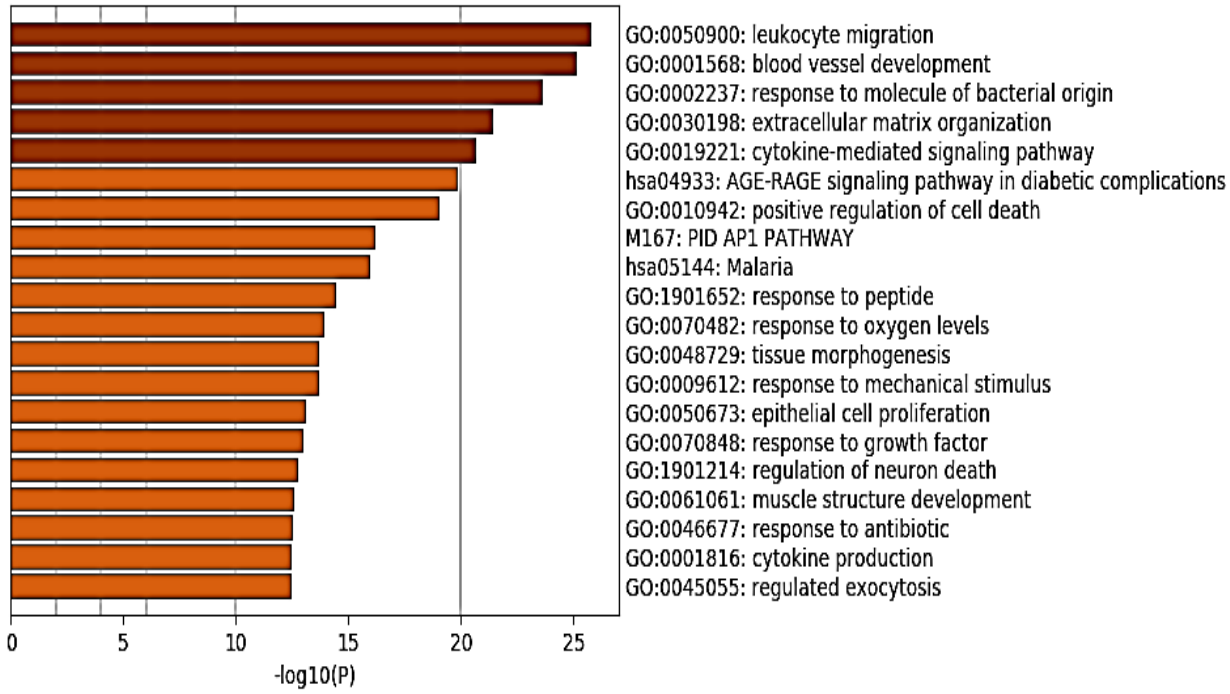

Figure 2.1 A. Gene Ontology analysis of 68 genes associated with acute cardiac injury and repair. Top 20 most significant GO functional processes associated with the 68 genes.

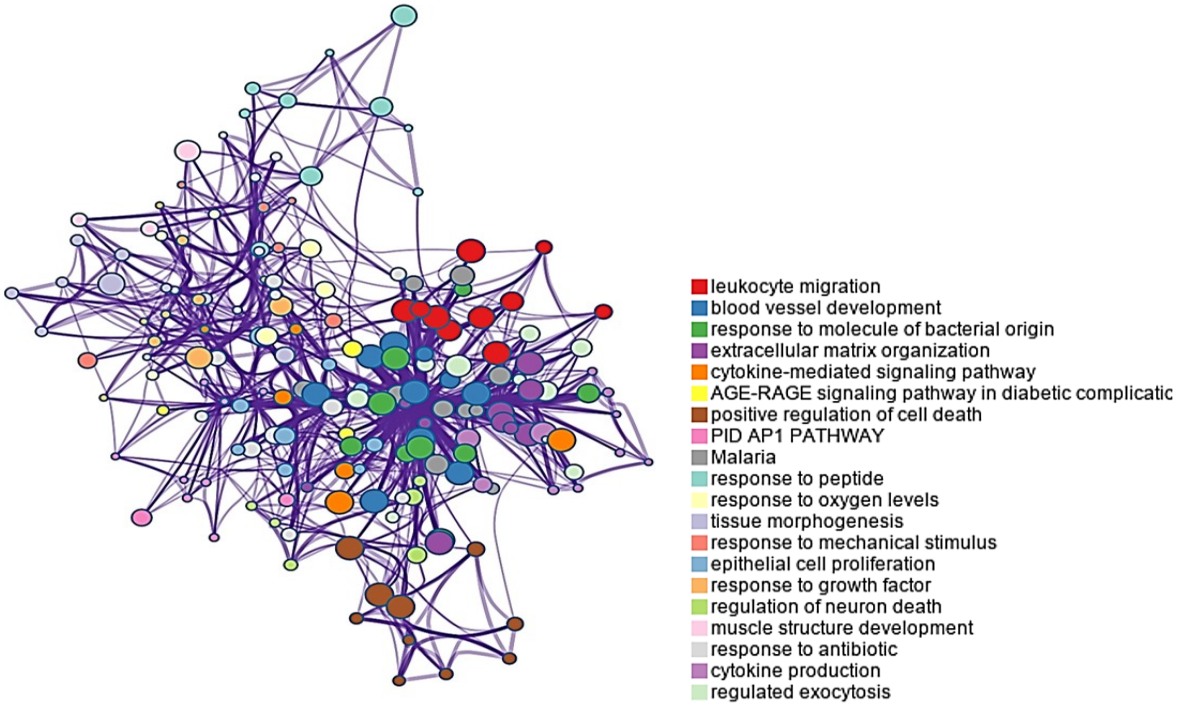

Figure 2.1 B. Gene Ontology analysis of 68 genes associated with acute cardiac injury and repair. Network organization of all the clusters produced with the complete list of genes. Created by Metascape. Each enriched functional category is represented by one node. Node size is directly proportional to the number of genes, whereas color indicates cluster identity.

2.3.3 Signaling Pathways

The SPEED database was used to deduce common signaling pathways causing the regulation of the 68-gene set. This database uses an algorithm that allows for the identification of signaling pathways which cause a distinctive pattern of gene regulation in humans (18). It utilizes microarray data to identify signature genes that are consistently regulated by specific signaling pathways, established from pathway perturbation experiments involving TLR, TGFB, H_20_2, MAPK, IL-1, PI3K, Wnt, VEGF, JAK-STAT and TNF alpha (74-80). SPEED analysis of the 68-gene set identified 67 human genes associated with several signaling pathways involved in acute tissue injury and repair, including the IL-1, TNFa, MAPK-PI3K, TLR and JAK-STAT signaling pathways (Table 2.3, Figure 2.2).

Table 2.3. Signaling pathway enrichment analysis using SPEED database.

Pathway	Genes in List	Genes in Background	P-Value	FDR
IL-1	17	125	8.58e-24	1.59e-23
TNFa	13	109	1.42e-17	1.16e-17
MAPK_PI3K	9	41	5.16e-15	7.15e-15
TLR	9	62	2.84e-15	2e-13
JAK-STAT	7	27	1.76e-12	9.93e-13
H202	6	41	3.02e-09	1.82e-09
Wnt	6	80	1.83e-07	1.38e-07
TGFB	3	32	1.35e-04	6.4e-05
VEGF	1	2	6.23e-03	2.86e-03
PI3K_only	1	16	4.88e-02	1.73e-02
MAPK_only	1	20	6.06e-02	2.58e-02

Genes in list, n=67; genes in background, n=21485. FDR, false discovery rate.

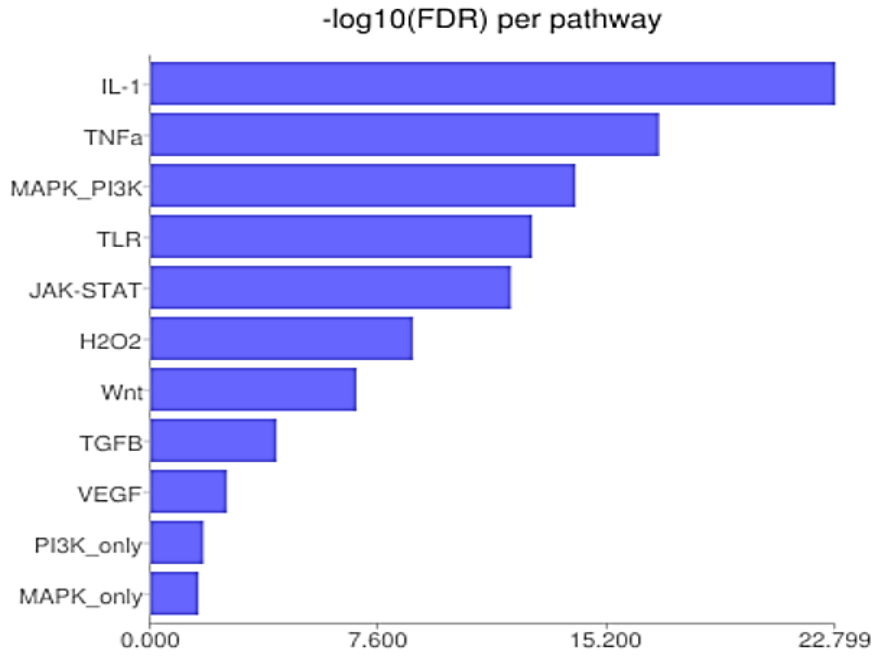

Figure 2.2. Bar Chart representing -log10 false discovery rate (FDR) in each pathway of the SPEED signaling enrichment analysis for the 67 genes.

To determine non-random associations between the genes, SPEED applies Fisher's exact test and correction with -log10 false discovery rate (FDR) (18). The IL-1 signalling pathway was found to be the most significantly represented by the 68-gene set, including the following 17 transcripts: *MYC, CCL2, VCAM1, EGR1, SELE, GADD45B, CXCL10, JUN, GDF15, 1L6, NFKBIA, AFT3, TNF, ICAM1, BTG2, TNFAIP3*, and *CXL2*. The other pathways that were most significantly associated with the 68-gene set included TLR signaling (9 genes: *IL6, GADD45B, CXCL10, TNFAIP3, TNF, ICAM1, NFKBIA, CXCL2, SOCS3*), TNF alpha-signalling (12 genes: *CYR61, EGR1, IL6, CXCL2, CCL2, JUN, FOS, AFT3, TNFAIP3, ICAM1, NFKBIA, SERPINE1*) and MAPK-PI3K (*CYR61, EGR1, FOS, JUN, GDF15, THBS1, CTGF, AFT3, NR4A1*). (Table 2.4).

Table 2.4. Genes associated with each of the SPEED signaling pathways.

Signaling Pathway	Genes (transcription factors)
H202	*EGR1, JUN, GDF15, IL6, AFT3, HMOX1,*
IL-1	*MYC, CCL2, VCAM1, EGR1, SELE, GADD45B, CXCL10, JUN, GDF15, 1L6, NFKBIA, AFT3, TNF, ICAM1, BTG2, TNFAIP3, CXL2*
JAK-STAT	*CCL2, GADD45B, CXL10, FOS, ICAM1, CXCL9, SOCS3*
MAPK-PI3K	*CYR61, EGR1, FOS, JUN, GDF15, THBS1, CTGF, AFT3, NR4A1*
MAPK-only	*THBS1*
PIK3-only	*CYR61*
TGFB	*GADD45B, SERPINE, EGR1,*
TLR	*IL6, GADD45B, CXCL10, TNFAIP3, TNF, ICAM1, NFKBIA, CXCL2, SOCS3*
TNFa	*CYR61, EGR1, IL6, CXCL2, CCL2, JUN, FOS, AFT3, TNFAIP3, ICAM1, NFKBIA, SERPINE1*
VEGF	*EGR1*
Wnt	*JUN, AFT3, FOS, IRS1, TNFAIBP3, HSP90AA1*

2.3.4 Protein-Protein Interaction

To understand the protein interaction network of the 68 gene set, these transcripts were further analyzed using the STRING network. Protein-protein interaction analysis demonstrated moderate overall correlation (Pearson's correlation coefficient = 0.601, $p<0.0001$) based on all of the association evidence available in the STRING database, including: (i) three prediction channels (neighborhood, gene fusion, and co-occurrence), (ii) text-mining, iii) co-expression, (iv) experiments (biochemical/genetic data), and (v) databases (previous reports of curated pathways and protein-protein complexes) (Figure 2.3). The clustering coefficients (Pearson's correlation coefficients) for channels (ii) to (v) were 0.630, 0.366, 0.142, and 0.484 ($p<0.0267$), respectively. Although the STRING interaction scores do not reflect strength or specificity of the underlying evidence, they do represent approximate confidence of the associations in a scale of zero to 1 based on the available evidence (17).

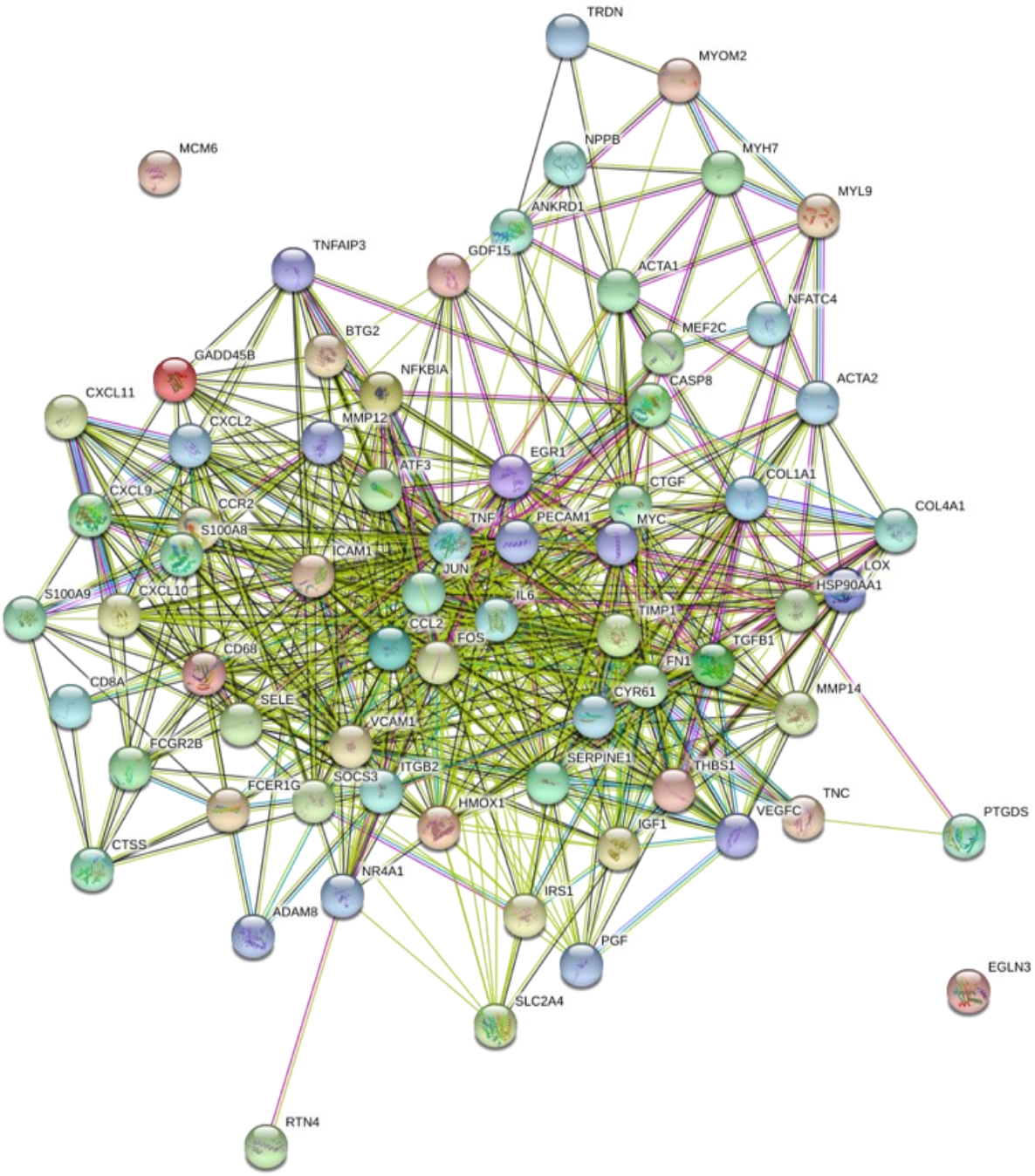

Figure 2.3. STRING interaction network of the 68 genes. Functional association evidence based on: (i) text mining, (ii) experiments, (iii) co-expression, (iv) co-occurrence, (v) gene fusion, (vi) neighborhood, vii) databases. Confidence cut-off for showing interaction set at median confidence (0.400). Each node represents a gene, and the edges represent the type of association. Number of nodes: 68, Number of edges: 560, Average node degree: 17.4, Average local clustering coefficient: 0.601.

A further analysis of the protein-protein interaction using the Metascape database, which applies Molecular Complex Detection (MCODE) algorithm, resulted in the identification of three densely connected MCODE components, including: 1) *CXCL2, CCR2, CXCL9, CXCL11* and *CXCL10*, 2) *TNF, ACTA1, SLC2A4, ACTA2* and *MYH7*, and 3) *JUN, FOS* and *ATF3* (Figure 2.4 and Table 2.5) (81).

A

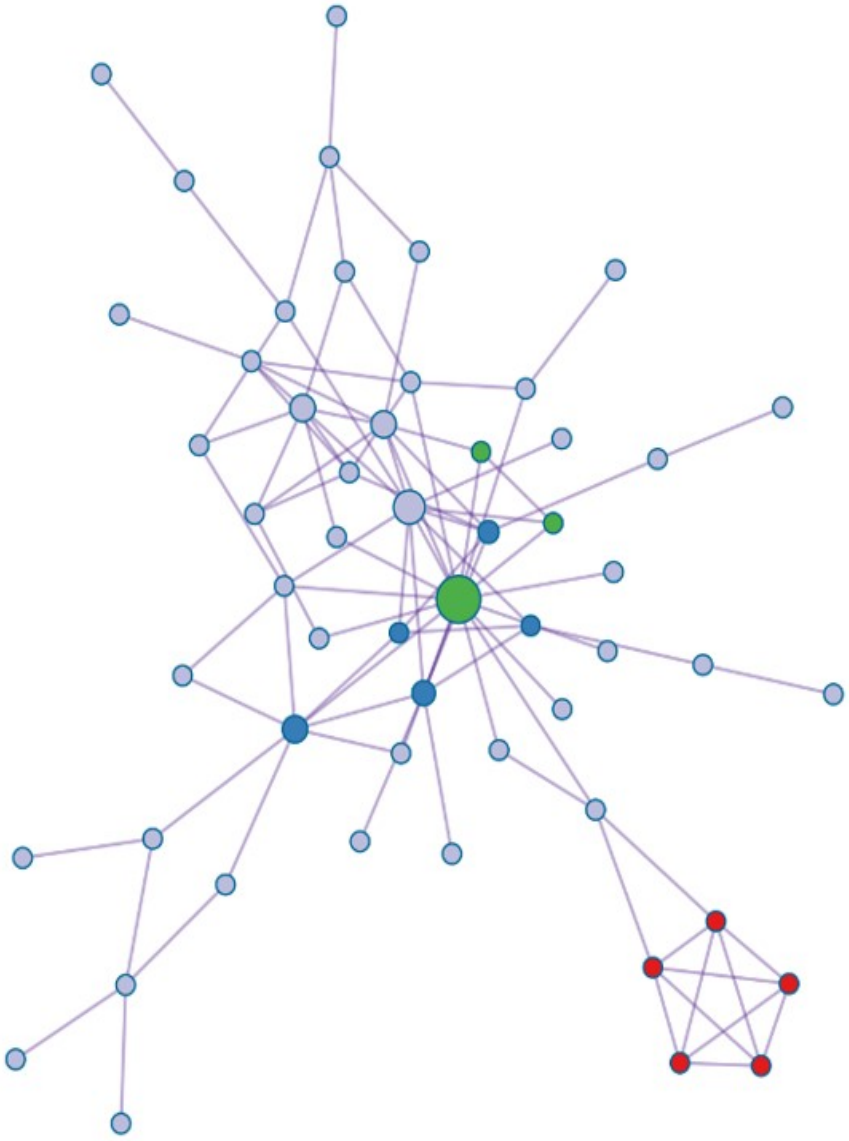

Figure 2.4. A) Protein-protein interaction network

B

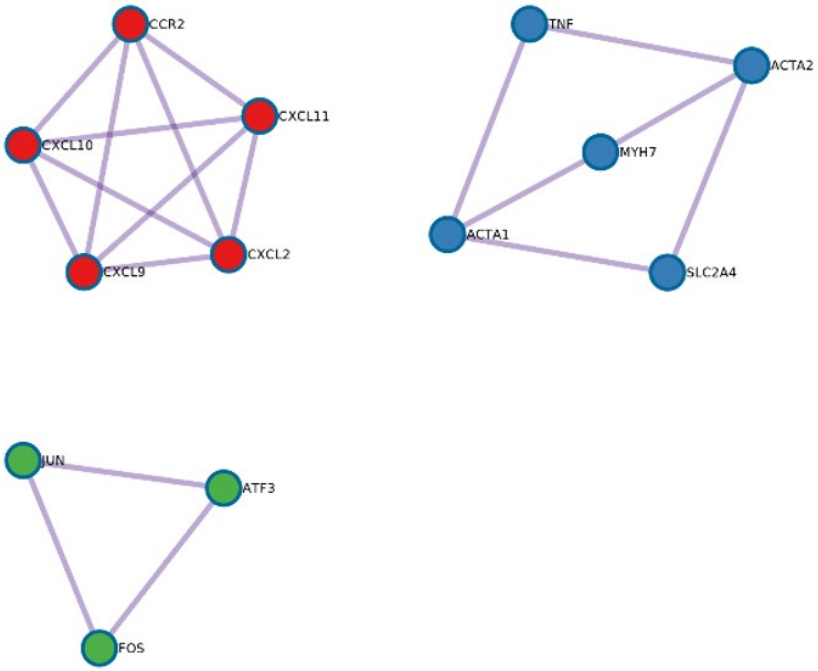

Figure 2.4. B) MCODE components identified in the 68-gene set.

Table 2.5. Functional descriptions represented by the three MCODE components in the 68-gene set

Color	MCODE	GO	Description	Log10(P)
	MCODE_1	R-HAS-380108	Chemokine receptors bind chemokines	-13.6
	MCODE_1	GO:0070098	chemokine-mediated signalling pathway	-12.2
	MCODE_1	GO:1990868	Response to chemokine	-12.0
	MCODE_2	GO:0048729	Tissue morphogenesis	-5.5
	MCODE_2	GO:0006936	Muscle contraction	-4.5
	MCODE_2	GO:0003012	Muscle synthesis process	-4.2
	MCODE_3	M166	PID AFT2 pathway	-7.9
	MCODE_3	M167	PID AP1 pathway	-7.6
	MCODE_3	M2	PID SMA2 3 nuclear pathway	-7.4

2.4 Discussion

EVHP represents a promising tool for the evaluation, preservation, and repair of donor hearts prior to transplantation. However, the benefits of this emerging technology have not yet been fully realized, primarily due to functional decline during prolonged organ perfusion, unlike in the preservation of other organs such as lungs. Understanding the mechanisms of myocardial functional deterioration during EVHP has the potential to facilitate its further development and clinical implementation, and thus provide the opportunity for improved assessment of organ quality and development of therapeutic interventions to optimize donor heart quality. Gene expression signatures can be used as a robust tool for understanding and discovery of functional correlations between genes, disease states and therapeutic interventions (1). The objective of this study was to design a literature-based, cardiac-specific gene set that could be used to further understand the mechanisms of injury and repair, and to assess the viability of donor hearts during EVHP.

The identification of 'immune response', 'tissue remodeling' and 'signal transduction as the main subset of gene lists with most transcripts in the identified 68-gene set reflect common phases including acute inflammatory and reparative processes, that occur following cardiac tissue injury (82). A further analysis of signaling pathway enrichment demonstrated IL-1 to be the top-ranked pathway represented by the 68-gene set, with a large number of genes (*MYC, CCL2, VCAM1, EGR1, SELE, GADD45B, CXCL10, JUN, GDF15, 1L6, NFKBIA, AFT3, TNF, ICAM1, BTG2, TNFAIP3, CXL2*) identified to be the main causal influence. IL-1 signaling has been strongly implicated in facilitating the pro-inflammatory response following myocardial infarction (83). Notably, it has been previously reported that the myocardial environment may act as a local immune system that regulates immune response and inflammation (84). Following ischemia, resident myocardial immune cells generate danger signals called damage-associated molecular

patterns (DAMPs), which are recognized by pattern recognition receptors (PRRs) (82, 84). One major class of PRRs is the Toll-like Receptors (TLRs), ten of which have been identified in humans (TLR 1-10). TLR1, TLR2, TLR4, TLR6 and TLR10 are located on cell surfaces, whereas TLR3, TLR7, TLR8 and TLR9 are expressed intracellularly and recognized by nucleic acid structures (85, 86). TLR2 and TLR4 are mostly expressed in cardiac cells and trigger signalling cascades that activate NF-kB and interferon regulatory transcription factors, which in turn regulate target genes encoding pro-inflammatory cytokines and interferons in the myocardium (87). A range of potential endogenous ligands have been identified, including: 1) products of necrosis and cellular constituents resulting from ischemia, such as heat shock proteins (HSP90AA1, HSP60, HSP70) and high mobility group box (HMGB); 2) products of extracellular matrix (ECM) alterations due to ischemia-reperfusion, such as S100 proteins, fibronectin, fibrinogen, heparin; and 3) reactive oxygen species (ROS) (84).

Furthermore, the production of ROS by myocardial mitochondria during periods of ischemia and organ reperfusion causes the activation of inflammatory molecular cascades, including pro-inflammatory signaling pathways such as IL-1, TNFα and MAPK pathways (88, 89). These signaling pathways are consistent with the findings of this study, including the identification of IL-1, TNFα and MAPK-PIK3 as three of the top-ranking upstream signaling pathways in SPEED enrichment analysis. MAPK, an intracellular signal transduction pathway, is a key player in the regulation of cell function and survival during ischemia-reperfusion injury (IRI) (90, 91). The MAPK pathway consists of a cascade of kinase signaling including c-Jun N-terminal, extracellular-signal-regulated Kinase (ERK) and p38 (92). Whereas MAPK-ERK has been reported to confer cardioprotective properties to the myocardium following ischemia and IRI, MAPK-JNK activation has been shown to reduce IRI and infarct size as well as induction of

apoptosis (93). Previous studies on small animal models (rat) reported the existence of two JNK isoforms (JNK-1 and JNK-2), with knockdown of JNK-1 and retention of JNK-2 preventing cardiomyocyte IRI-induced apoptosis (94). On the other hand, MAPK-p38 activation has been reported to contribute to myocardial tissue injury through activation of the TNFα pathway in response to progressive accumulation of ROS produced during ischemia-reperfusion (89, 93, 95). The MAPK-p38 signaling pathway has been thoroughly studied resulting in the development of a MAPK-p38 inhibitor, which is administered during reperfusion for the recovery of myocardial function and attenuation of the generation of IL-6, a proinflammatory cytokine that is a potent inducer of the acute inflammatory response (96, 97). This MAPK-p38 inhibitor has been incorporated into some of the commercially-available cardioplegic solutions, such as Celsior, University of Wisconsin and Euro-Collins, to protect the myocardium against IRI (88, 98, 99). In general, previous studies have reported the MAPK pathway to induce myocardial injury through activation of immune cells, regulation of cytokines, and the induction of apoptosis (88). Therefore, it is not surprising that this pathway is so strongly represented by the 68-gene set derived in this study, including the following transcripts: *EGR1, FOS, JUN, GDF15, AFT3* and *NR4A1*. In particular, activation of the MAPK-ERK signaling pathway has been shown to be involved in the downstream activation of EGR1, a gene reported to be an upstream master regulator of the inflammatory response during myocardial IRI (100). Extensive involvement of this pathway in IRI supports the inclusion of these genes in a molecular signature for the assessment of cardiac injury and repair.

The search for densely connected regions in the 68- gene set protein-protein interaction network using the MCODE algorithm resulted to the identification of three molecular complexes which could potentially represent the main effectors of cellular processes during myocardial injury.

Particularly, *FOS, JUN* and *AFT3* genes identified to form one of the molecular complexes, were found to share common signaling pathways, including the MAPK-PIK3 and AP-1, ATF2 and SMA 2/3. These signaling pathways are critical upstream cascades that influence myocardial tissue remodeling. The MAPK signaling pathway has been shown to play a vital role in the conversion of extracellular stimuli caused by specific stress into consequent cellular responses (101). MAPK signaling has been implicated to cause the downstream regulation of AP-1 signaling, which together with activated NF-kB influences the expression of MMPS and pro-inflammatory cytokines including IL-1, TNFα and IL-6 (102). Notably, activating protein-1(AP-1) is a group of dimeric transcription factors comprising JUN, ATF3 or FOS subunits which bind to common DNA site known as AP-1-binding site (103). Previous experiment on loss and gain of function highlighted the critical roles of *FOS* and *JUN* genes in cardiac injury. *JUN* is noted to play a vital role in promoting sarcomeric protein (actin and myosin) expression while suppressing ECM proteins (104). Thus, an alteration to the regulation of sarcomeric proteins could potentially be deleterious to cardiac contractility. JUN is reported to confer cardio-protection against pathologic remodeling, hence very critical in preventing cardiac dilation and heart failure(103, 104). Furthermore, studies exploring PI3K/Akt pathway have suggested that this signaling pathway plays a critical role in preservation of cardiac function during ischemia and IRI (105).

The second MCODE component constitutes three structural genes (*ACT1, ACT2,* and *MYH7*), an immune response gene (*TNF*) and a signal transduction gene (*SLC2A4*). The described roles of this MCODE complex include tissue morphogenesis and muscle contraction. These structural genes encode myocardial contractile proteins that play a critical role in cell motility and contraction of the myocardium (106, 107). Identification of *TNF*, a pro-inflammatory gene, as a member of this MCODE component suggests its critical role in the downstream regulation of these

structural genes. On the other hand, *SLC2A4* encodes a glucose-transporter protein which, upon insulin stimulation, moves to the myocyte cell surface and begins to transport glucose across the cell membrane for energy metabolism essential for muscle contraction (108, 109). The susceptibility of myocardial contractile proteins to progressive production of ROS and oxidative stress has been reported (110). The consequence of this is increased cardiac relaxation and myocardial contractile dysfunction (111). Therefore, the members of this MCODE protein complex may represent a novel gene set for assessing myocardial contractile function following ischemia and IRI.

The third MCODE complex identified in this study comprised of five genes including *CCR2, CXCL2, CXCL19, CXCL10*, and *CXCL11* all of which are involved in chemokine-mediated signaling and were functionally annotated as immune response genes. Chemokines represent a family of CC and CXC cytokines that are crucial in leukocyte recruitment and the mediation of pro-inflammatory responses (112). CCL2-CCR2 binding facilitates monocyte recruitment thus, the expression of these genes may implicate myocardial pathogenicity that is characterized with monocytic infiltration(113). Analyzing these chemokine and respective receptor may potentially help in detecting the extend of cardiac injury. The ROS and DAMPs produced during ischemia and IRI are recognized upstream effectors of chemokine-mediated signaling cascade (114). Thus, the inclusion of this molecular complex in the 68-gene set is supported as a representative marker of pro-inflammatory effects in EVHP. Previous study suggested that targeting of CCL2/CCR2 system may represent a potential approach for developing effective therapies (115), further suggesting the importance of evaluating these genes to assess cardiac viability and potentially optimize organ management during EVHP.

2.5 Conclusion

The derivation of a novel, evidence-based 68-gene set for cardiac injury and repair, representing multiple pathophysiologically relevant signaling pathways, provides the opportunity to study the mechanisms of acute myocardial injury and repair, as well a possible tool for the assessment of organ quality, during EVHP. It is worth noting that most of the genes identified in this Chapter were previously analyzed based on case-control experiments. Therefore, collective analysis of this large number of genes may provide a comprehensive understanding of cellular processes occurring during machine perfusion. Additionally, the identified MCODE components highlighted chemokine-chemokine receptor interactions, structural alterations and tissue remodeling as the key cellular processes that may potentially help in distinguishing between normal and altered molecular processes in EVHP. These molecular complexes may constitute potential targets for both diagnostic and therapeutic purposes. Further analysis of this set of genes is experimentally exploited in Chapter 3 and Chapter 4.

2.6 References

1. Kulkarni MM. Digital multiplexed gene expression analysis using the NanoString nCounter system. Curr Protoc Mol Biol. 2011;Chapter 25:Unit25B.10.
2. Crick F. Central dogma of molecular biology. Nature. 1970;227(5258):561-3.
3. Kukurba KR, Montgomery SB. RNA Sequencing and Analysis. Cold Spring Harb Protoc. 2015;2015(11):951-69.
4. Mallik S, Zhao Z. Identification of gene signatures from RNA-seq data using Pareto-optimal cluster algorithm. BMC Syst Biol. 2018;12(Suppl 8):126.
5. Green ED, Guyer MS. Charting a course for genomic medicine from base pairs to bedside. Nature. 2011;470(7333):204-13.
6. Hood L, Rowen L. The Human Genome Project: big science transforms biology and medicine. Genome Med. 2013;5(9):79.
7. Dulbecco R. A turning point in cancer research: sequencing the human genome. Science. 1986;231(4742):1055-6.
8. Del Greco MF, Pattaro C, Luchner A, Pichler I, Winkler T, Hicks AA, et al. Genome-wide association analysis and fine mapping of NT-proBNP level provide novel insight into the role of the MTHFR-CLCN6-NPPA-NPPB gene cluster. Hum Mol Genet. 2011;20(8):1660-71.
9. Liberzon A, Birger C, Thorvaldsdottir H, Ghandi M, Mesirov JP, Tamayo P. The Molecular Signatures Database (MSigDB) hallmark gene set collection. Cell Syst. 2015;1(6):417-25.
10. Cantini L, Calzone L, Martignetti L, Rydenfelt M, Bluthgen N, Barillot E, et al. Classification of gene signatures for their information value and functional redundancy. NPJ Syst Biol Appl. 2018;4:2.

11. Zhou Y, Zhou B, Pache L, Chang M, Khodabakhshi AH, Tanaseichuk O, et al. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. Nat Commun. 2019;10(1):1523.

12. Ruepp A, Waegele B, Lechner M, Brauner B, Dunger-Kaltenbach I, Fobo G, et al. CORUM: the comprehensive resource of mammalian protein complexes--2009. Nucleic Acids Res. 2010;38(Database issue):D497-501.

13. Kim B. How to Reveal Magnitude of Gene Signals: Hierarchical Hypergeometric Complementary Cumulative Distribution Function. Evol Bioinform. 2018;14:1176934318797352.

14. Reed JF, 3rd. Homogeneity of kappa statistics in multiple samples. Comput Meth Prog Biomed. 2000;63(1):43-6.

15. Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, et al. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics. 2009;25(8):1091-3.

16. Goenawan IH, Bryan K, Lynn DJ. DyNet: visualization and analysis of dynamic molecular interaction networks. Bioinformatics. 2016;32(17):2713-5.

17. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, et al. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res. 2019;47(D1):D607-d13.

18. Parikh JR, Klinger B, Xia Y, Marto JA, Bluthgen N. Discovering causal signaling pathways through gene-expression patterns. Nucleic Acids Res. 2010;38(Web Server issue):W109-17.

19. Van Aelst LN, Summer G, Li S, Gupta SK, Heggermont W, De Vusser K, et al. RNA Profiling in Human and Murine Transplanted Hearts: Identification and Validation of Therapeutic Targets for Acute Cardiac and Renal Allograft Rejection. Am J Transplant. 2016;16(1):99-110.

20. Siltanen A, Nuutila K, Imanishi Y, Uenaka H, Makela J, Patila T, et al. The Paracrine Effect of Skeletal Myoblasts Is Cardioprotective Against Oxidative Stress and Involves EGFR-ErbB4 Signaling, Cystathionase, and the Unfolded Protein Response. Cell Transplant. 2016;25(1):55-69.

21. Maciejak A, Kiliszek M, Michalak M, Tulacz D, Opolski G, Matlak K, et al. Gene expression profiling reveals potential prognostic biomarkers associated with the progression of heart failure. Genome Med. 2015;7(1):26.

22. Zheng J, Wei CC, Hase N, Shi K, Killingsworth CR, Litovsky SH, et al. Chymase mediates injury and mitochondrial damage in cardiomyocytes during acute ischemia/reperfusion in the dog. PloS One. 2014;9(4):e94732.

23. Wu Y, Li Y, Zhang C, A X, Wang Y, Cui W, et al. S100a8/a9 released by CD11b+Gr1+ neutrophils activates cardiac fibroblasts to initiate angiotensin II-Induced cardiac inflammation and injury. Hypertension. 2014;63(6):1241-50.

24. Cho JH, Lee I, Hammamieh R, Wang K, Baxter D, Scherler K, et al. Molecular evidence of stress-induced acute heart injury in a mouse model simulating posttraumatic stress disorder. Proc Nat Acad Sci USA. 2014;111(8):3188-93.

25. Vikholm P, Schiller P, Hellgren L. A modified Glenn shunt reduces venous congestion during acute right ventricular failure due to pulmonary banding: a randomized experimental study. Interact Cardiovasc Thorac Surg. 2014;18(4):418-25.

26. Ruppert V, Maisch B. Molecular signatures and the study of gene expression profiles in inflammatory heart diseases. Herz. 2012;37(6):619-26.

27. Mathur S, Walley KR, Boyd JH. The Toll-like receptor 9 ligand CPG-C attenuates acute inflammatory cardiac dysfunction. Shock. 2011;36(5):478-83.

28. Wang X, Wang Y, Jiang M, Zhu Y, Hu L, Fan G, et al. Differential cardioprotective effects of salvianolic acid and tanshinone on acute myocardial infarction are mediated by unique signaling pathways. J Ethnopharmacol. 2011;135(3):662-71.

29. Mengel M, Sis B, Kim D, Chang J, Famulski KS, Hidalgo LG, et al. The molecular phenotype of heart transplant biopsies: relationship to histopathological and clinical variables. Am J Transplant. 2010;10(9):2105-15.

30. Kim MY, Seo EJ, Lee DH, Kim EJ, Kim HS, Cho HY, et al. Gadd45beta is a novel mediator of cardiomyocyte apoptosis induced by ischaemia/hypoxia. Cardiovasc Res. 2010;87(1):119-26.

31. Song GY, Wu YJ, Yang YJ, Li JJ, Zhang HL, Pei HJ, et al. The accelerated post-infarction progression of cardiac remodelling is associated with genetic changes in an untreated streptozotocin-induced diabetic rat model. Eur J Heart Fail. 2009;11(10):911-21.

32. Matkovich SJ, Van Booven DJ, Youker KA, Torre-Amione G, Diwan A, Eschenbacher WH, et al. Reciprocal regulation of myocardial microRNAs and messenger RNA in human cardiomyopathy and reversal of the microRNA signature by biomechanical support. Circulation. 2009;119(9):1263-71.

33. Pitts KR, Derry JM, Kerkof K, Lawrence WA, Toombs CF. Differentially regulated functional gene clusters identified during ischemia and reperfusion in isolated cardiac myocytes using coverslip hypoxia. J Pharmacol Toxicol Methods. 2008;57(1):42-51.

34. Schiekofer S, Shiojima I, Sato K, Galasso G, Oshima Y, Walsh K. Microarray analysis of Akt1 activation in transgenic mouse hearts reveals transcript expression profiles associated with compensatory hypertrophy and failure. Physiol Genomics. 2006;27(2):156-70.

35. Lattanzio FA, Jr., Tiangco D, Osgood C, Beebe S, Kerry J, Hargrave BY. Cocaine increases intracellular calcium and reactive oxygen species, depolarizes mitochondria, and activates genes associated with heart failure and remodeling. Cardiovasc Toxicol. 2005;5(4):377-90.

36. Taylor LA, Carthy CM, Yang D, Saad K, Wong D, Schreiner G, et al. Host gene regulation during coxsackievirus B3 infection in mice: assessment by microarrays. Circ Res. 2000;87(4):328-34.

37. Tillmanns J, Hoffmann D, Habbaba Y, Schmitto JD, Sedding D, Fraccarollo D, et al. Fibroblast activation protein alpha expression identifies activated fibroblasts after myocardial infarction. J Mol Cell Cardiol. 2015;87:194-203.

38. Rodius S, Nazarov PV, Nepomuceno-Chamorro IA, Jeanty C, Gonzalez-Rosa JM, Ibberson M, et al. Transcriptional response to cardiac injury in the zebrafish: systematic identification of genes with highly concordant activity across in vivo models. BMC Genomics. 2014;15:852.

39. Ghosh AK, Murphy SB, Kishore R, Vaughan DE. Global gene expression profiling in PAI-1 knockout murine heart and kidney: molecular basis of cardiac-selective fibrosis. PLoS One. 2013;8(5):e63825.

40. Zhao W, Zhao T, Chen Y, Qu Y, Gerling IC, Sun Y. Modification of oxidative stress on gene expression profiling in the rat infarcted heart. Mol Cell Biochem. 2013;379(1-2):243-53.

41. Azuaje F, Zhang L, Jeanty C, Puhl SL, Rodius S, Wagner DR. Analysis of a gene co-expression network establishes robust association between Col5a2 and ischemic heart disease. BMC Med Genomics. 2013;6:13.

42. Ghorbel MT, Mokhtari A, Sheikh M, Angelini GD, Caputo M. Controlled reoxygenation cardiopulmonary bypass is associated with reduced transcriptomic changes in cyanotic tetralogy of Fallot patients undergoing surgery. Physiol Genomics. 2012;44(22):1098-106.

43. Azuaje FJ, Rodius S, Zhang L, Devaux Y, Wagner DR. Information encoded in a network of inflammation proteins predicts clinical outcome after myocardial infarction. BMC Med Genomics. 2011;4:59.

44. Troidl C, Mollmann H, Nef H, Masseli F, Voss S, Szardien S, et al. Classically and alternatively activated macrophages contribute to tissue remodelling after myocardial infarction. J Cell Mol Med. 2009;13(9b):3485-96.

45. Watts JA, Gellar MA, Obraztsova M, Kline JA, Zagorski J. Role of inflammation in right ventricular damage and repair following experimental pulmonary embolism in rats. Int J Exp Pathol. 2008;89(5):389-99.

46. Lara-Pezzi E, Felkin LE, Birks EJ, Sarathchandra P, Panse KD, George R, et al. Expression of follistatin-related genes is altered in heart failure. Endocrinology. 2008;149(11):5822-7.

47. Streblow DN, Kreklywich CN, Andoh T, Moses AV, Dumortier J, Smith PP, et al. The role of angiogenic and wound repair factors during CMV-accelerated transplant vascular sclerosis in rat cardiac transplants. Am J Transplant. 2008;8(2):277-87.

48. Arab S, Konstantinov IE, Boscarino C, Cukerman E, Mori A, Li J, et al. Early gene expression profiles during intraoperative myocardial ischemia-reperfusion in cardiac surgery. J Thorac Cardiovasc Surg. 2007;134(1):74-81, .e1-2.

49. Wang XH, Zhang GP, Jin HM, Chen SF. Dynamic changes in the expression of growth factor receptors in the myocardium microvascular endothelium after murine myocardial infarction. Chin Med J. 2007;120(6):485-90.

50. Naseem RH, Meeson AP, Michael Dimaio J, White MD, Kallhoff J, Humphries C, et al. Reparative myocardial mechanisms in adult C57BL/6 and MRL mice following injury. Physiol Genomics. 2007;30(1):44-52.

51. Ayach BB, Yoshimitsu M, Dawood F, Sun M, Arab S, Chen M, et al. Stem cell factor receptor induces progenitor and natural killer cell-mediated cardiac survival and repair after myocardial infarction. Proc Natl Acad Sci U S A. 2006;103(7):2304-9.

52. Huda R, Chung DH, Mathru M. Ischemic preconditioning at a distance: altered gene expression in mouse heart and other organs following brief occlusion of the mesenteric artery. Heart Lung Circ. 2005;14(1):36-43.

53. Barton PJ, Felkin LE, Birks EJ, Cullen ME, Banner NR, Grindle S, et al. Myocardial insulin-like growth factor-I gene expression during recovery from heart failure after combined left ventricular assist device and clenbuterol therapy. Circulation. 2005;112(9 Suppl):I46-50.

54. Boerma M, van der Wees CG, Vrieling H, Svensson JP, Wondergem J, van der Laarse A, et al. Microarray analysis of gene expression profiles of cardiac myocytes and fibroblasts after mechanical stress, ionising or ultraviolet radiation. BMC Genomics. 2005;6:6.

55. Li T, Chen YH, Liu TJ, Jia J, Hampson S, Shan YX, et al. Using DNA microarray to identify Sp1 as a transcriptional regulatory element of insulin-like growth factor 1 in cardiac muscle cells. Circ Res. 2003;93(12):1202-9.

56. Chen Y, Park S, Li Y, Missov E, Hou M, Han X, et al. Alterations of gene expression in failing myocardium following left ventricular assist device support. Physiol Genomics. 2003;14(3):251-60.

57. Wassenaar JW, Gaetani R, Garcia JJ, Braden RL, Luo CG, Huang D, et al. Evidence for Mechanisms Underlying the Functional Benefits of a Myocardial Matrix Hydrogel for Post-MI Treatment. J Am Coll Cardiol. 2016;67(9):1074-86.

58. Carbe CJ, Cheng L, Addya S, Gold JI, Gao E, Koch WJ, et al. Gi proteins mediate activation of the canonical hedgehog pathway in the myocardium. Am J Physiol-Heart C. 2014;307(1):H66-72.

59. Csonka C, Szucs G, Varga-Orvos Z, Bencsik P, Csont T, Zvara A, et al. Ischemic postconditioning alters the gene expression pattern of the ischemic heart. Exp Biol Med. 2014;239(2):141-50.

60. Zhou L, Zang G, Zhang G, Wang H, Zhang X, Johnston N, et al. MicroRNA and mRNA signatures in ischemia reperfusion injury in heart transplantation. PLoS One. 2013;8(11):e79805.

61. Ashton KJ, Tupicoff A, Williams-Pritchard G, Kiessling CJ, See Hoe LE, Headrick JP, et al. Unique transcriptional profile of sustained ligand-activated preconditioning in pre- and post-ischemic myocardium. PloS one. 2013;8(8):e72278.

62. Wilhide ME, Tranter M, Ren X, Chen J, Sartor MA, Medvedovic M, et al. Identification of a NF-kappaB cardioprotective gene program: NF-kappaB regulation of Hsp70.1

contributes to cardioprotection after permanent coronary occlusion. J Mol Cell Cardiol. 2011;51(1):82-9.

63. Ahmed MS, Gravning J, Martinov VN, von Lueder TG, Edvardsen T, Czibik G, et al. Mechanisms of novel cardioprotective functions of CCN2/CTGF in myocardial ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol. 2011;300(4):H1291-302.

64. Tranter M, Ren X, Forde T, Wilhide ME, Chen J, Sartor MA, et al. NF-kappaB driven cardioprotective gene programs; Hsp70.3 and cardioprotection after late ischemic preconditioning. J Mol Cell Cardiol. 2010;49(4):664-72.

65. Kim YJ, Lim HJ, Choi SU. Effect of propofol on cardiac function and gene expression after ischemic-reperfusion in isolated rat heart. Korean J Anesthesiol. 2010;58(2):153-61.

66. Canatan H. The effect of cardiac ischemic preconditioning on rat left ventricular gene expression profile. Cell Biochem Funct. 2008;26(2):179-84.

67. Wang Y, Gabrielsen A, Lawler PR, Paulsson-Berne G, Steinbruchel DA, Hansson GK, et al. Myocardial gene expression of angiogenic factors in human chronic ischemic myocardium: influence of acute ischemia/cardioplegia and reperfusion. Microcirculation. 2006;13(3):187-97.

68. Roy S, Khanna S, Kuhn DE, Rink C, Williams WT, Zweier JL, et al. Transcriptome analysis of the ischemia-reperfused remodeling myocardium: temporal changes in inflammation and extracellular matrix. Physiol Genomics. 2006;25(3):364-74.

69. Podgoreanu MV, Michelotti GA, Sato Y, Smith MP, Lin S, Morris RW, et al. Differential cardiac gene expression during cardiopulmonary bypass: ischemia-independent upregulation of proinflammatory genes. J Thorac Cardiovasc Surg. 2005;130(2):330-9.

70. Amberger A, Schneeberger S, Hernegger G, Brandacher G, Obrist P, Lackner P, et al. Gene expression profiling of prolonged cold ischemia and reperfusion in murine heart transplants. Transplantation. 2002;74(10):1441-9.

71. Stegall MD, Park WD, Kim DY, Covarrubias M, Khair A, Kremers WK. Changes in intragraft gene expression secondary to ischemia reperfusion after cardiac transplantation. Transplantation. 2002;74(7):924-30.

72. Deng MC, Eisen HJ, Mehra MR, Billingham M, Marboe CC, Berry G, et al. Noninvasive discrimination of rejection in cardiac allograft recipients using gene expression profiling. Am J Transplant. 2006;6(1):150-60.

73. Kearns MJ, Miller SD, Cheung A, Bashir J, Wong S, Seidman MA, et al. A Rodent Model of Cardiac Donation After Circulatory Death and Novel Biomarkers of Cardiac Viability During Ex Vivo Heart Perfusion. Transplantation. 2017;101(8):e231-e9.

74. Classen S, Zander T, Eggle D, Chemnitz JM, Brors B, Buchmann I, et al. Human resting CD4+ T cells are constitutively inhibited by TGF beta under steady-state conditions. J Immunol. 2007;178(11):6931-40.

75. Tullai JW, Schaffer ME, Mullenbrock S, Kasif S, Cooper GM. Identification of transcription factor binding sites upstream of human genes regulated by the phosphatidylinositol 3-kinase and MEK/ERK signaling pathways. J Biol Chem. 2004;279(19):20167-77.

76. Malcolm KC, Arndt PG, Manos EJ, Jones DA, Worthen GS. Microarray analysis of lipopolysaccharide-treated human neutrophils. Am J Physiol-Lung C. 2003;284(4):L663-70.

77. Von Bernuth H, Picard C, Jin Z, Pankla R, Xiao H, Ku CL, et al. Pyogenic bacterial infections in humans with MyD88 deficiency. Science. 2008;321(5889):691-6.

78. Misior AM, Deshpande DA, Loza MJ, Pascual RM, Hipp JD, Penn RB. Glucocorticoid- and protein kinase A-dependent transcriptome regulation in airway smooth muscle. Am J Resp Cell Mol Biol. 2009;41(1):24-39.

79. Indraccolo S, Pfeffer U, Minuzzo S, Esposito G, Roni V, Mandruzzato S, et al. Identification of genes selectively regulated by IFNs in endothelial cells. J Immunol. 2007;178(2):1122-35.

80. Zimmermann M, Beer L, Ullrich R, Lukovic D, Simader E, Traxler D, et al. Analysis of region specific gene expression patterns in the heart and systemic responses after experimental myocardial ischemia. Oncotarget. 2017;8(37):60809-25.

81. Bader GD, Hogue CW. An automated method for finding molecular complexes in large protein interaction networks. BMC bioinformatics. 2003;4:2.

82. Epelman S, Liu PP, Mann DL. Role of innate and adaptive immune mechanisms in cardiac injury and repair. Nat Rev Immunol. 2015;15(2):117-29.

83. Frangogiannis NG. Inflammation in cardiac injury, repair and regeneration. Curr Opin Cardiol. 2015;30(3):240-5.

84. Vilahur G, Badimon L. Ischemia/reperfusion activates myocardial innate immune response: the key role of the toll-like receptor. Front Physiol. 2014;5:496.

85. Mann DL. The emerging role of innate immunity in the heart and vascular system: for whom the cell tolls. Circ Res. 2011;108(9):1133-45.

86. Wang Y, Abarbanell AM, Herrmann JL, Weil BR, Poynter J, Manukyan MC, et al. Toll-like receptor signaling pathways and the evidence linking toll-like receptor signaling to cardiac ischemia/reperfusion injury. Shock. 2010;34(6):548-57.

87. Mann DL, Topkara VK, Evans S, Barger PM. Innate immunity in the adult mammalian heart: for whom the cell tolls. Trans Am Clin Climatol Assoc. 2010; 121:34-50; discussion -1.

88. Vassalli G, Milano G, Moccetti T. Role of Mitogen-Activated Protein Kinases in Myocardial Ischemia-Reperfusion Injury during Heart Transplantation. J Transplant. 2012;2012:928954.

89. Meldrum DR, Dinarello CA, Cleveland JC, Jr., Cain BS, Shames BD, Meng X, et al. Hydrogen peroxide induces tumor necrosis factor alpha-mediated cardiac injury by a P38 mitogen-activated protein kinase-dependent mechanism. Surgery. 1998;124(2):291-6; discussion 7.

90. Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev. 1999;79(1):143-80.

91. Gerits N, Kostenko S, Moens U. In vivo functions of mitogen-activated protein kinases: conclusions from knock-in and knock-out mice. Transgenic Res. 2007;16(3):281-314.

92. Chang L, Karin M. Mammalian MAP kinase signalling cascades. Nature. 2001;410(6824):37-40.

93. Milano G, Morel S, Bonny C, Samaja M, von Segesser LK, Nicod P, et al. A peptide inhibitor of c-Jun NH2-terminal kinase reduces myocardial ischemia-reperfusion injury and infarct size in vivo. Am J Physiol-Heart C. 2007;292(4):H1828-35.

94. Hreniuk D, Garay M, Gaarde W, Monia BP, McKay RA, Cioffi CL. Inhibition of c-Jun N-terminal kinase 1, but not c-Jun N-terminal kinase 2, suppresses apoptosis induced by ischemia/reoxygenation in rat cardiac myocytes. Mol Pharmacol. 2001;59(4):867-74.

95 .Kumar D, Menon V, Ford WR, Clanachan AS, Jugdutt BI. Effect of angiotensin II type 2 receptor blockade on mitogen activated protein kinases during myocardial ischemia-reperfusion. Mol Cell Biochem. 2004;258(1-2):211-8.

96. Clanachan AS, Jaswal JS, Gandhi M, Bottorff DA, Coughlin J, Finegan BA, et al. Effects of inhibition of myocardial extracellular-responsive kinase and P38 mitogen-activated protein kinase on mechanical function of rat hearts after prolonged hypothermic ischemia. Transplantation. 2003;75(2):173-80.

97. Diestel A, Roessler J, Pohl-Schickinger A, Koster A, Drescher C, Berger F, et al. Specific p38 inhibition in stimulated endothelial cells: a possible new anti-inflammatory strategy after hypothermia and rewarming. Vasc Pharmacol. 2009;51(4):246-52.

98. Koike N, Takeyoshi I, Ohki S, Tokumine M, Matsumoto K, Morishita Y. Effects of adding P38 mitogen-activated protein-kinase inhibitor to celsior solution in canine heart transplantation from non-heart-beating donors. Transplantation. 2004;77(2):286-92.

99. Hashimoto N, Takeyoshi I, Yoshinari D, Tsutsumi H, Tokumine M, Totsuka O, et al. Effects of a p38 mitogen-activated protein kinase inhibitor as an additive to Euro-Collins solution on reperfusion injury in canine lung transplantation1. Transplantation. 2002;74(3):320-6.

100. Zhang J, Song J, Xu J, Chen X, Yin P, Lv X, et al. ERK1/2-Egr-1 Signaling Pathway-Mediated Protective Effects of Electroacupuncture in a Mouse Model of Myocardial Ischemia-Reperfusion. Evid-Based Complementary Altern Med. 2014; 2014:253075.

101. Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. Nat Rev Mol Cell Biol. 2006;7(8):589-600.

102. Yu L, Feng Z. The Role of Toll-Like Receptor Signaling in the Progression of Heart Failure. Mediat Inflamm. 2018;2018:9874109.

103. Karin M, Liu Z, Zandi E. AP-1 function and regulation. Curr Opin Cell Biol. 1997;9(2):240-6.

104. Windak R, Müller J, Felley A, Akhmedov A, Wagner EF, Pedrazzini T, et al. The AP-1 transcription factor c-Jun prevents stress-imposed maladaptive remodeling of the heart. PLoS One. 2013;8(9):e73294.

105. Zhao MM, Yang JY, Wang XB, Tang CS, Du JB, Jin HF. The PI3K/Akt pathway mediates the protection of SO(2) preconditioning against myocardial ischemia/reperfusion injury in rats. Acta Pharmacol Sin. 2013;34(4):501-6.

106. Narolska NA, Eiras S, van Loon RB, Boontje NM, Zaremba R, Spiegelen Berg SR, et al. Myosin heavy chain composition and the economy of contraction in healthy and diseased human myocardium. J Muscle Res Cell M. 2005;26(1):39-48.

107. Jung MJ, Murzik U, Wehder L, Hemmerich P, Melle C. Regulation of cellular actin architecture by S100A10. Exp Cell Res. 2010;316(7):1234-40.

108. Malodobra-Mazur M, Bednarska-Chabowska D, Olewinski R, Chmielecki Z, Adamiec R, Dobosz T. Single nucleotide polymorphisms in 5'-UTR of the SLC2A4 gene regulate solute carrier family 2 member 4 gene expression in visceral adipose tissue. Gene. 2016;576(1 Pt 3):499-504.

109. Govers R. Molecular mechanisms of GLUT4 regulation in adipocytes. Diabetes Metab. 2014;40(6):400-10.

110. Steinberg SF. Oxidative stress and sarcomeric proteins. Circ Res. 2013;112(2):393-405.

111. Figueiredo-Freitas C, Dulce RA, Foster MW, Liang J, Yamashita AM, Lima-Rosa FL, et al. S-Nitrosylation of Sarcomeric Proteins Depresses Myofilament Ca2+)Sensitivity in Intact Cardiomyocytes. Antioxid Redox Sign. 2015;23(13):1017-34.

112. Cavalera M, Frangogiannis NG. Targeting the chemokines in cardiac repair. Curr Pharm Des. 2014;20(12):1971-9.

113. O'Connor T, Borsig L, Heikenwalder M. CCL2-CCR2 Signaling in Disease Pathogenesis. Endocr Metab Immune Disord Drug Targets. 2015;15(2):105-18.

114. Lakshminarayanan V, Lewallen M, Frangogiannis NG, Evans AJ, Wedin KE, Michael LH, et al. Reactive oxygen intermediates induce monocyte chemotactic protein-1 in vascular endothelium after brief ischemia. Am J Pathol. 2001;159(4):1301-11.

115. Raghu H, Lepus CM, Wang Q, Wong HH, Lingampalli N, Oliviero F, et al. CCL2/CCR2, but not CCL5/CCR5, mediates monocyte recruitment, inflammation and cartilage destruction in osteoarthritis. Ann Rheum Dis. 2017;76(5):914-22.

116. Pavo N, Lukovic D, Zlabinger K, Lorant D, Goliasch G, Winkler J, et al. Intrinsic remote conditioning of the myocardium as a comprehensive cardiac response to ischemia and reperfusion. Oncotarget. 2017;8(40):67227-40.

117. Xin M, Olson EN, Bassel-Duby R. Mending broken hearts: cardiac development as a basis for adult heart regeneration and repair. Nat Rev Mol Cell Biol. 2013;14(8):529-41.

118. Castaldi A, Dodia RM, Orogo AM, Zambrano CM, Najor RH, Gustafsson AB, et al. Decline in cellular function of aged mouse c-kit (+) cardiac progenitor cells. J Physiol. 2017;595(19):6249-62.

119. Walther S, Awad S, Lonchyna VA, Blatter LA. NFAT transcription factor regulation by urocortin II in cardiac myocytes and heart failure. Am J Physiol-Heart C. 2014;306(6):H856-66.

120. Yu BX, Yuan JN, Zhang FR, Liu YY, Zhang TT, Li K, et al. Inhibition of Orai1-mediated Ca(2+) entry limits endothelial cell inflammation by suppressing calcineurin-NFATc4 signaling pathway. Biochem Biophys Res Commun. 2018;495(2):1864-70.

121. Guan XH, Hong X, Zhao N, Liu XH, Xiao YF, Chen TT, et al. CD38 promotes angiotensin II-induced cardiac hypertrophy. J Cell Mol Med. 2017;21(8):1492-502.

Chapter 3

Gene expression changes

during *ex vivo* heart perfusion[2]

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3.1 Introduction

Heart failure represents a significant global health burden affecting both children and adults, male and female (1, 2). Approximately 1-2% of the global population is estimated to be affected, with a lifetime risk at 55 years of age of 33% in men and 28% in women (3). Cardiac transplantation is the current "gold standard" treatment for patients with end-stage heart disease. The current median survival after cardiac transplantation is 11 years overall and 13 years for patients that survive beyond the first-year post-transplantation (4). It is estimated that more than 50,000 patients around the world would benefit from cardiac transplantation; however, only about 10% successfully receive it, with a resulting global waitlist mortality rate of 17% per year (5). One of the primary reasons for this disappointingly low rate of cardiac transplant activity is a shortage of appropriate donor hearts. Despite the growing wait list for cardiac transplantation, a large number of potential donor hearts are not being used for various reasons, including size mismatch between the donor heart and recipient, advanced donor age, and medical co-morbidities in the donor (6). However, with the growing demand for heart transplantation, transplant programs have commenced significant efforts to address this shortage by expanding acceptance criteria to include donor hearts that would have previously been declined because of either smoking history, donor age, inotropic therapy, intravenous drug abuse or cardiac arrest (7). Reassuringly, despite this expansion of donor organ acceptance criteria, rates of primary graft dysfunction (PGD) have not significantly increased (5). One strategy to further increase the size of the potential donor organ pool is to expand the use of hearts from "donation after circulatory death" (DCD) donors.

Cold static preservation (CSP) represents the current clinical standard for *ex vivo* preservation of donor hearts, during the time between organ retrieval and transplantation into the recipient. This preservation strategy aims to minimize cardiac metabolic activity and thus reduce oxidative stress and ischemic injury prior to transplantation. However, the duration of CSP

correlates with post-transplant mortality, thus limiting this preservation strategy to a maximum of 4-6 h (8). Additionally, increased warm ischemic injury sustained by hearts retrieved from extended criteria donors means that these organs may not be able to sustain additional cold ischemia during CSP. There is thus an urgent need to explore alternative preservation strategies that may be more appropriate for such extended criteria donor hearts.

Ex vivo heart perfusion (EVHP) is an emerging novel technique that provides the opportunity to not only preserve donor hearts but also objectively evaluate the function of, and potentially recover, organs deemed ineligible for transplantation (9). Despite encouraging early results with EVHP, including non-inferior post-transplant outcomes compared with CSP and the ability to facilitate expanded transportation of donor hearts for transplantation (10), there are emerging reports of significant functional decline during EVHP (11) and the mechanisms of cardiac injury and repair in this context remain poorly understood (12). Most preclinical research on EVHP has focused on technical optimization with little attention to developing more precise tools for assessing organ viability prior to heart transplantation (13), which currently include nonspecific metabolic and functional parameters such as lactate, venous and arterial oxygen saturation, coronary flow and arterial pressure (14).

As discussed in Chapter 2 of this thesis, transcriptome-wide microarray studies have previously demonstrated that heart tissue injury and repair stimulate the expression of numerous gene pathways. This study investigated the hypothesis that analysis of the expression level of a prototypical subset of the genes (described in Chapter 2) would allow for more specific, objective and mechanistic assessment of cardiac tissue injury and repair during EVHP. Specifically, this study aimed to assess the feasibility of using gene expression to quantify heart injury and repair in a porcine animal model for EVHP.

3.2 Methods

3.2.1 Animal Model

A total of 46 hearts from female domestic pigs were used for this study. Experiments were performed in accordance with the Animal Care and Use Committee at the University of Alberta. All animals were treated with humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources.

3.2.2 Organ Retrieval and Preparation

Organ retrieval and preparation was performed by Dr. Sanaz Hatami, PhD student, Department of Surgery, University of Alberta. After injection of an intramuscular dose of ketamine (20 mg/kg, Bimeda Inc., Irwindale, CA) and atropine (0.05 mg/kg, Rafter 8, Calgary, AB) as premedication, orotracheal intubation was established, and general anesthesia was maintained with isoflurane (1-2%, Fresenius Kabi Canada Ltd, Toronto, ON). The porcine hearts were procured and prepared for the *ex vivo* perfusion as described in detail before (15). Briefly, an experienced cardiothoracic surgeon performed a standard median sternotomy by incising the skin over, and through the sternum to expose the heart. After intravenous delivery of heparin (1000 U/kg bolus, Fresenius Kabi Canada Ltd, Toronto, ON) and Ringer's lactate solution (1000-1500 mL), a two-stage venous cannula was placed into the right atrium and 1000 mL of whole blood was retrieved and was used to prime the EVHP circuit. *In vivo* biopsy samples were obtained from a random subset of normal hearts that were not utilized for EVHP. The pigs were then exsanguinated (euthanized), and the hearts were excised, weighed, and immediately mounted on a custom EVHP apparatus and perfused for 12 h, either as isolated heart perfusion or combined heart and liver perfusion (Figures 3.1). The porcine livers were procured and prepared as described before (16).

All livers were exposed through a midline laparotomy, and the vascular elements were appropriately isolated. The infra-renal aorta was then cannulated with a 20 French cannula in preparation for cold flush. After exsanguination, the aorta was cross-clamped, and the suprahepatic vena cava was divided in the chest cavity for venous venting. The abdominal viscera were flushed with 2 liters of cold (4ºC) Histidine-Tryptophan-Ketoglutarate (Custodiol HTK, Methapharm Inc., Brantford, ON) and the liver was dissected.

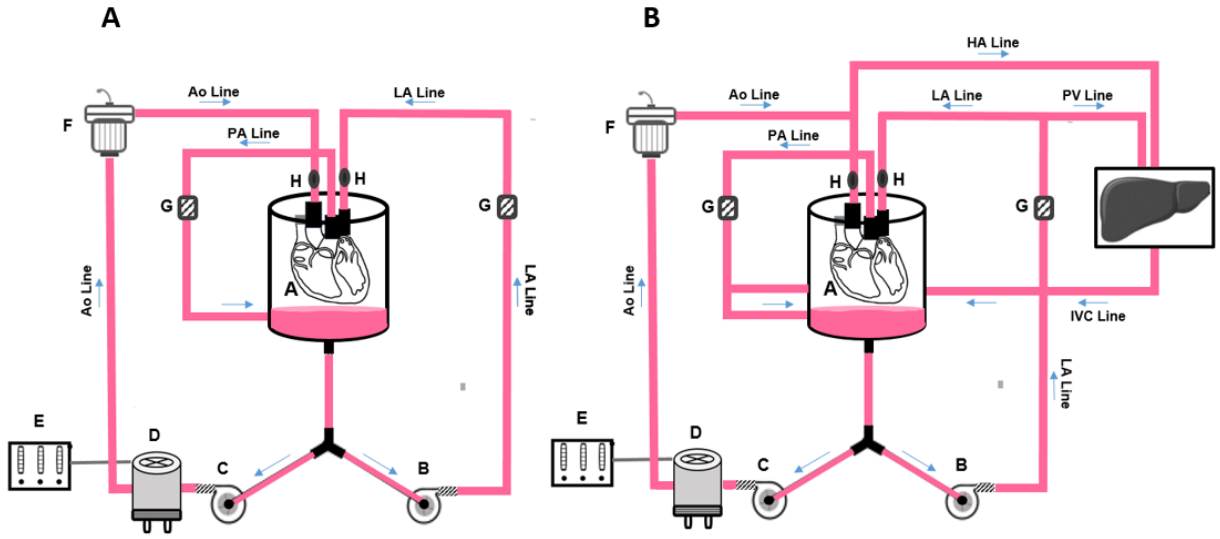

Figure 3.1. Schematic of *ex vivo* perfusion systems. A: *Ex vivo* heart perfusion B: Combined *ex vivo* perfusion of heart and liver. Components: A, venous reservoir; B, left atrial pump; C, Aortic pump; D, oxygen/heat exchanger; E, medical gas mixer; F, leukocyte filter; G, Flow sensor; H, Pressure sensor; Ao, Aorta; HA, hepatic artery; IVC, inferior vena cava; LA, left atrium; PA, pulmonary artery; PV, portal vein. This figure was provided by Dr. Sanaz Hatami, PhD student, Department of Surgery, University of Alberta.

3.2.3 Biopsy collection and processing

Biopsies were collected by Dr. Sanaz Hatami in the Department of Surgery, and later processed by Shalawny Miller, medical laboratory technologist in the Department of Laboratory Medicine and Pathology Core Lab, University of Alberta. A total of 46 porcine heart biopsies were collected, including seven in vivo (IV), 32 after 12 h of heart-only perfusion (EVHP-T12), and seven after 12 h of combined heart-liver perfusion (H+L-T12) (Figure 3.2). The biopsies were

obtained from the left ventricle, briefly rinsed in PBS, placed in optimal cutting temperature (OCT) compound (Sakura Finetek USA, Inc., Torrance, CA) then mounted in a cryostat chuck, and stored in a -80°C freezer.

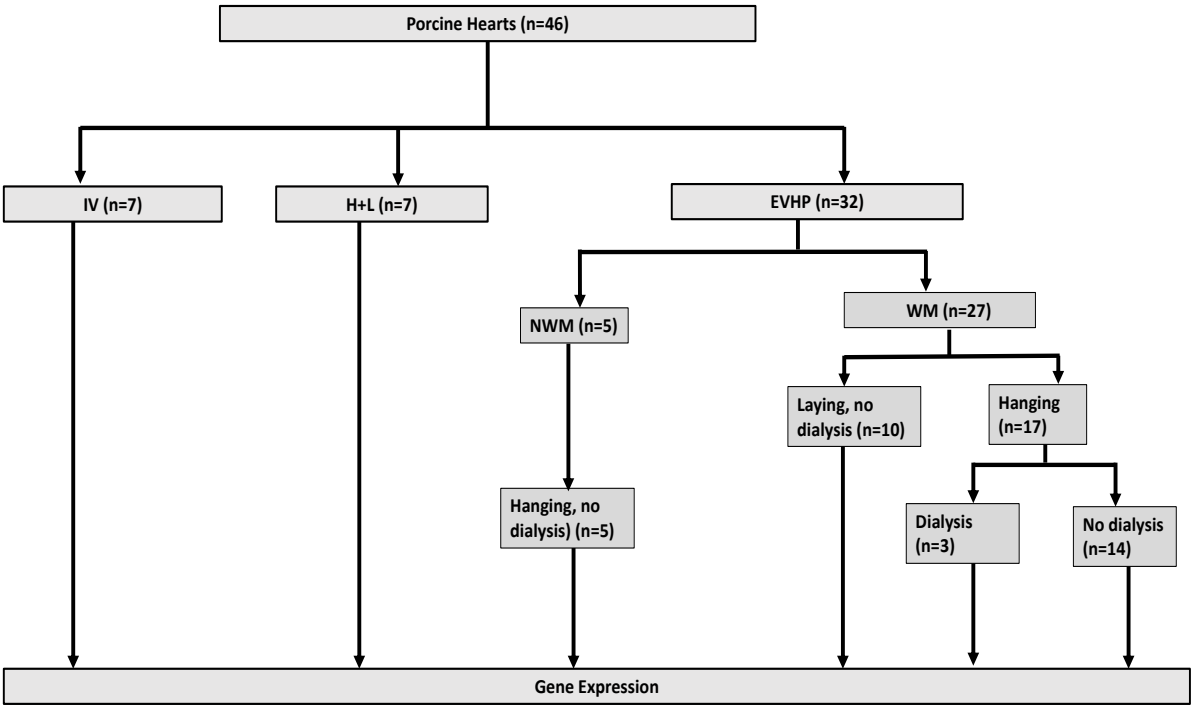

Figure 3.2. Study Design. Experimental groups: IV, *in vivo*; H+L, combined heart and liver perfusion; EVHP, *ex vivo* heart perfusion; NWM, non-working mode; WM, working mode.

The OCT biopsy samples were subsequently thawed by running on warm water, fixed with Surgipath 10% Neutral Buffered Formalin (Leica Biosystems Inc., Ontario, Canada) for 48 h, placed in tissue cassettes and loaded into an automated tissue processor (Leica HistoCore PEARL, Leica Biosystems Inc., Buffalo Groove, IL) for overnight processing (approximately 13 h). The tissue processor was set respectively as follows: formalin fixation for 1 h at 37°C, processing water for 3 min, and dehydration with increasing ethanol concentration (70%, 80%, 95% for 40 min each, and 100% for 3 h) at 45°C. Clearance of alcohol from with xylene was set at 45°C for 3 h followed

by paraffin infiltration for 3.5 h. The tissue cassettes were then removed from the processor and two sets of sections were cut from the formalin-fixed, paraffin-embedded (FFPE) blocks using a rotary microtome (Leica HistoCore AUTOCUT Microtome, Leica Biosystems Inc., Buffalo Groove, IL). The two sets of sections differed in thickness: one set of dedicated sections for gene expression analysis described in this chapter, and another set for histology analysis described in Chapter 4.

3.2.4 RNA extraction and gene expression

An overview of the FFPE sample RNA isolation and gene expression workflow is shown in Figure 3.3.

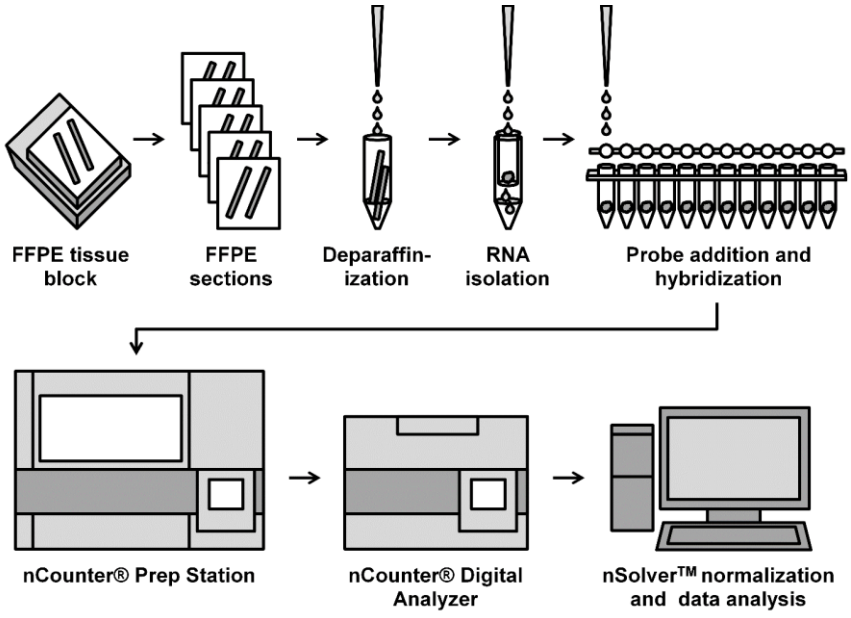

Figure 3.3. Overview of the NanoString nCounter gene expression analysis workflow. Formalin-fixed paraffin-embedded (FFPE) tissue section preparation, RNA isolation, probe hybridization, processing and counting, and data analysis.

Three consecutive 20 μm fresh sections where obtained from each FFPE block and dedicated for RNA isolation. Deparaffinization and RNA extraction were performed using the Qiagen RNeasy FFPE kit (Qiagen, Toronto, ON). Briefly, paraffin was removed from the freshly

cut sections using deparaffinization solution followed by lysis with proteinase K containing buffer at 56°C, and incubation at 80°C to reverse RNA-RNA formalin crosslinking. The mixture was further treated with DNase to remove genomic DNA and/or micro DNA present. The lysate was then mixed with Buffer RBC, followed by the addition of ethanol to provide optimal conditions for RNA binding to the silica membrane in the RNeasy MinElute spin column. RNA was then eluted using RNase-free water. Concentration and purity of the isolated RNA were quantified with a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

Oligonucleotide probes specific to *Sus scrofa* (domestic pigs) were designed (NanoString Technologies, Seattle, WA) and manufactured (Integrated DNA Technologies, Coralville, IA) for the cardiac injury-repair 68-gene set described in the previous chapter. In addition to the 68 experimental genes, *GAPDH, HPRT1, SDHA* and *TBP* were included as housekeeping/reference genes in the final gene set for data normalization purposes (17, 18). Buffer, probe, and RNA sample mixture were added into strip tubes and then hybridized at 65°C for 18 h. A NanoString® nCounter FLEX Analysis System (NanoString Technologies, Seattle, WA) was then utilized to quantify gene expression, as per manufacturer instructions. The hybridized mixture was transferred to the nCounter prep station for processing to eliminate unbound probes and get the purified probe-target complex immobilized and aligned on the nCounter cartridge. The cartridge was then transferred to the nCounter digital analyzer for probe counting (Figure 3.3). NanoString nSolver Analysis Software Version 4.0 (NanoString Technologies, Seattle, WA) was used to perform quality control assessment and data normalization. Briefly, for System QC, flag lanes were considered when percent field of view (FOV) registration was <75 on 'Imaging QC', binding density outside of 0.1-2.25 on 'Binding Density QC', and when positive control R^2 value <0.95 on 'Positive Control Linearity QC'. For mRNA data QC, flag lanes were considered when 0.5 fM positive control is ≤

2 standard deviation the mean of the negative controls on 'Positive Control Limit of Detection QC'.

3.2.5 Statistics

R version 3.4.1 (R Foundation for Statistical Computing, Vienna, Austria) was used for all post-normalization statistical analysis. Log2 normalized transcript counts were used for the analysis of individual genes and mean log2 normalized counts were utilized for the analysis of aggregate gene sets. Exploratory heatmap analysis (heatmap.2 function in gplots package) and principal component analysis (prcomp function in stats package) were performed using unsupervised clustering (18). Volcano plot analysis was used to assess differential gene expression by consolidating fold change and Students *t*-test with a false discovery rate (FDR) threshold of 0.05 (t.test and p.adjust functions in stats package). Comparison of gene set expression between sample groups was performed using Mann-Whitney U-test (Wilcox.test function in stats package). Statistical significance was considered at $P<0.05$.

3.3 Results

3.3.1 RNA and quality control

The mean RNA yield for the 46 FFPE biopsy samples was 61.2 ± 24.9 (mean ± SD) ng/μL with a range of 26.88-114.9 ng/μL. No quality control or normalization flags were encountered. The mean positive control normalization factor was 1.09 ± 0.32 (range: 0.64-1.88; manufacturer-recommended acceptable range: 0.3-3) and mean housekeeping gene normalization factor was 1.07 ± 0.30 (0.56-2.05; acceptable range: 0.1-10).

3.3.2 Differential gene expression during EVHP

Exploratory heatmap analysis demonstrated a distinct pattern of gene expression clustering in 31/32 (96.9%) of the EVHP-T12 samples versus the IV and H+L-T12 sample groups; 5/7 (71.4%) of the H+L-T12 samples versus the IV group; and 37/39 (94.9%) of the combined EVHP-T12 and H+L-T12 samples versus the IV group (Figure 3.4A). The EVHP-T12 cluster demonstrated relatively higher expression of 51 transcripts (Gene Cluster I) and relatively lower expression of 17 transcripts (Gene Cluster II) when compared to the other sample groups (Figure 3.4). Principal component analysis (PCA) confirmed distinct clustering of the sample groups based on the gene expression patterns (Figure 3.5). Principal component 1 (PC1) identified 44.9% of the gene expression variance between samples, primarily representing differences between the IV sample group versus H+L-T12 and EVHP-T12. Principal component 2 (PC2) identified 9.6% of the variance, primarily representing differences within the IV and EVHP-T12 groups. The PCA results showed the largest variation between the IV group and the EVHP-T12 and H+L-T12 sample groups. EVHP-T12 and H+L-T12 demonstrated more similar although still distinct clustering. PCA also identified some of the genes that most strongly influenced the clustering of the sample groups; for example, *EGR1, SLC2A4, THBS1, COL4A1* and *CXCL10* were associated with the EVHP-T12 group, whereas *JUN, IGF1, ACTA2 TRDN*, and *PTGDS* were associated with the H+L-T12 group.

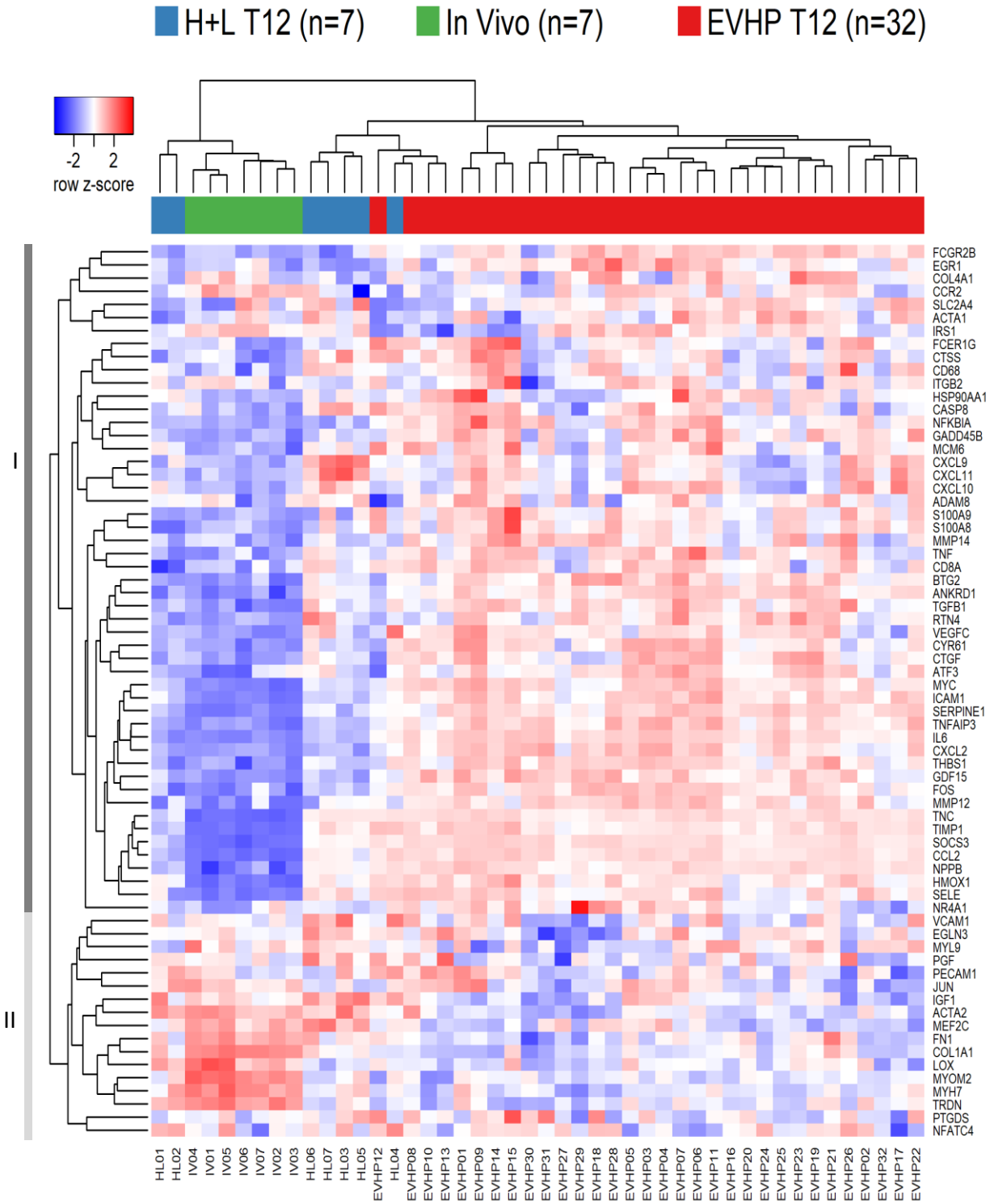

Figure 3.4. Exploratory gene expression analysis. Heatmap showing relative gene expression values (blue represents relative downregulation and red represents relative upregulation in this cohort). Each column represents one sample and each row represents one gene. Blue, green, and red sample labels represent H+L T12, *in vivo* and EVHP T12 sample groups, respectively. I and II represent gene clusters.

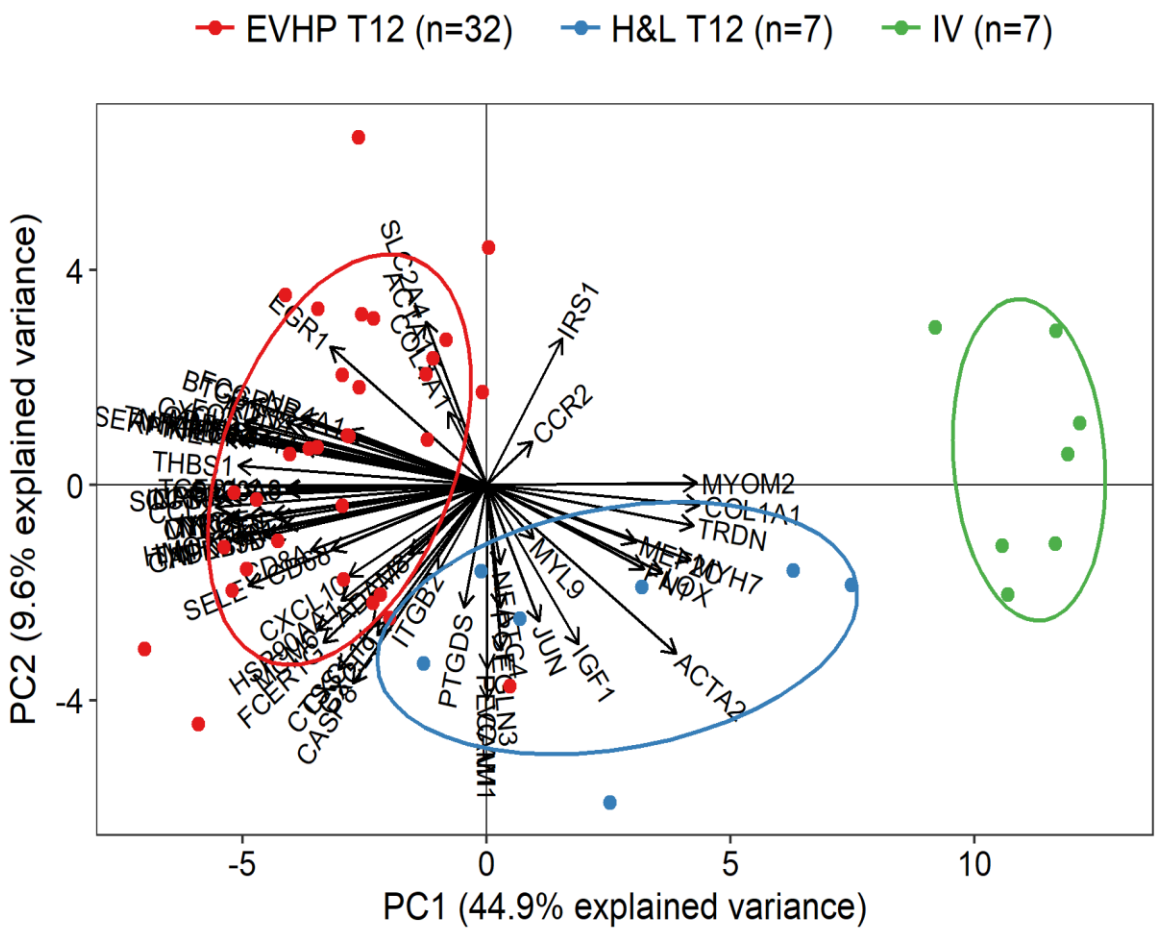

Figure 3.5. Principal component analysis of all 68 genes. Distinct clustering of the sample groups based on the variation in gene expression patterns. Published as per footnote on page 79.

To identify which genes were statistically significantly differentially expressed in response to EVHP, volcano plot analysis was performed to compare the EVHP-T12 versus IV sample groups (Figure 3.6). Forty-four of the previously identified 51 transcripts in "Gene Cluster I" exhibited statistically significantly higher expression in EVHP-T12 versus IV after correcting for multiple comparisons (FDR<0.05). In terms of functional annotation, 18/44 (40.9%) of the upregulated transcripts represented immune response genes, 11/44 (25%) signal transduction, 8/44 (18.2%) tissue remodeling, 3/44 (6.8%) apoptosis, 3/44 (6.8%) cell proliferation and 1/44 (2.3%) oxidative stress. The top 10 most highly upregulated transcripts (log2 fold change greater than 4)

included genes associated with immune response (*IL6, CCL2, CXCL2*, and *SELE*), signal transduction (*NPPB* and *SOCS3*), tissue remodeling (*TNC*, *SERPINE1* and *TIMP1*) and oxidative stress (*HMOX1*). Of the Cluster II genes, 12 were found to be significantly downregulated in EVHP-T12 versus IV (FDR<0.05). These 12 downregulated transcripts included four (33%) cell structure genes (*MYH7, ACTA2, MYOM2*, and *TRDN*), three (25%) signal transduction genes (*IRS1, JUN*, and *MEF2C*), three (25%) tissue remodeling genes (*LOX, FN1* and *COL1A1*), one (8%) cell proliferation gene (*IGF1*), and one (8%) immune response gene (*CCR2*). The remaining 12 transcripts did not show statistically significant differential expression between EVHP-T12 and IV (FDR>0.05).

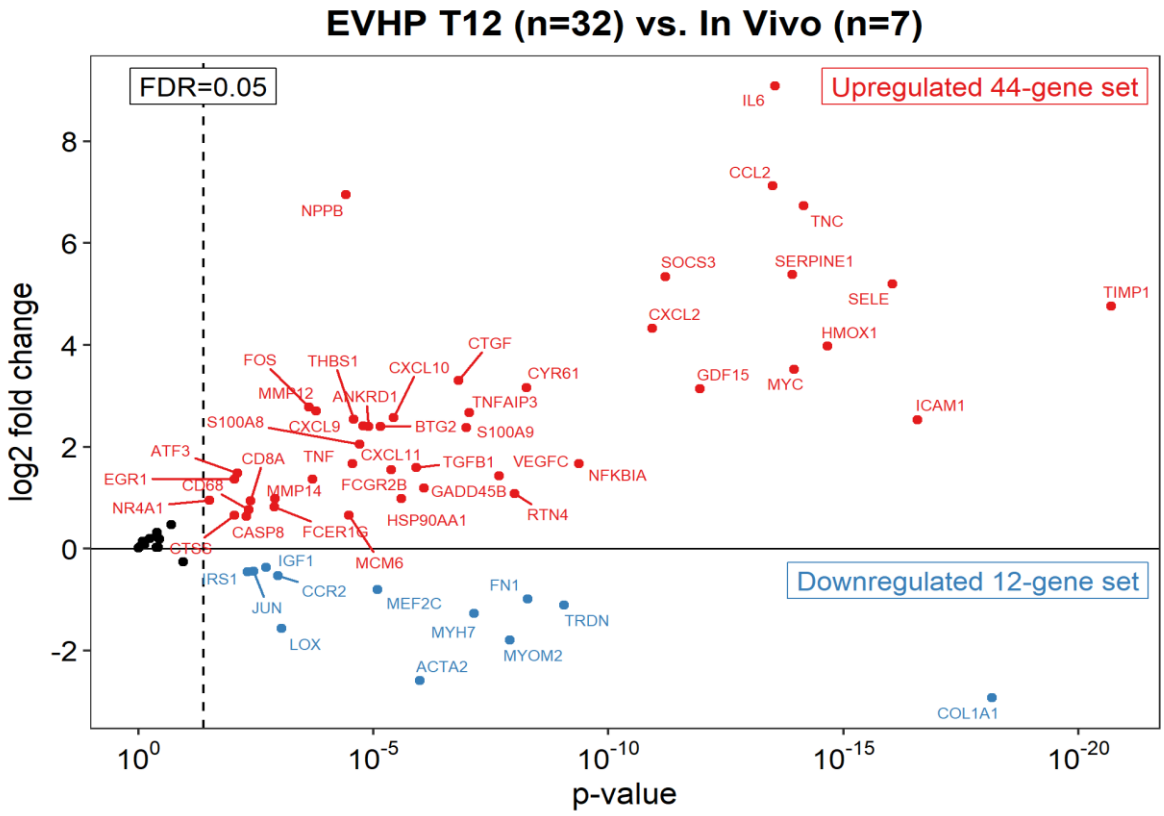

Figure 3.6. Volcano plot demonstrating differential expression of 68 cardiac injury and repair-related genes after 12 h of *ex vivo* heart perfusion. Y-axis represents log2-fold change and X-axis represents Student's t-test P-value between EVHP T12 versus in vivo controls. A false discovery rate (FDR) threshold of 0.05 was used to determine statistically significant differential expression. Published as per footnote on page 79.

3.3.3 Gene set expression versus perfusion strategy

The significantly upregulated and downregulated genes identified with volcano plot analysis were combined into aggregate gene sets by calculating the mean of the log2 normalized counts of the constituent genes. Expression of the aggregate gene sets was then compared between different perfusion strategies: 1) EVHP versus H+L, in comparison with the IV group, 2) hanging versus laying modes, and 3) WM versus NMW. Upregulated 44-gene set expression was significantly higher in both EVHP-T12 and H+L-T12 sample groups versus IV ($P<0.001$), and significantly higher in EVHP-T12 versus H+L-T12 ($P<0.001$) (Figure 3.6A). The opposite pattern was observed with the downregulated 12-gene set, with significantly lower expression in both EVHP-T12 and H+L-T12 groups versus IV ($P<0.001$), and lower expression in EVHP-T12 versus H+L-T12 ($P<0.001$) (Figure 3.6B).

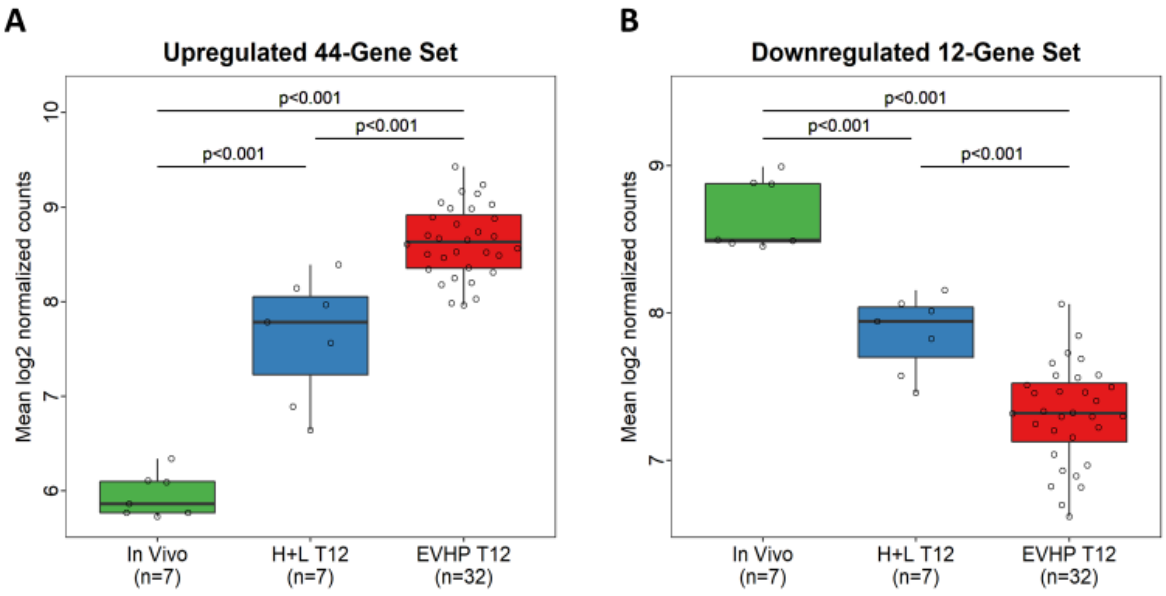

Figure 3.7. Gene set expression changes with *ex vivo* heart perfusion (EVHP) and combined heart-liver perfusion (H+L). Box plot demonstrating aggregate upregulated (A) and downregulated (B) 44-gene set expression with different preservation strategies.

Within the EVHP-T12 sample group, there was no significant difference in upregulated 44-gene set expression between NWM and WM hearts (P=0.096) (Figure 3.8A). However, there was significantly lower downregulated 12-gene set expression in WM versus NWM hearts (P=0.011) (Figure 3.8B). There was significantly higher expression of the upregulated 44-gene set expression in hanging versus laying hearts (P=0.036) (Figure 3.7C), and significantly lower expression of the downregulated 12-gene set in the laying versus hanging modes (P=0.011) (Figure 3.7D). Although the number of hearts exposed to dialysis treatment was small (n=3), no significant differences in gene set expression were identified between the dialysis and no dialysis groups (Figure 3.8 E and F).

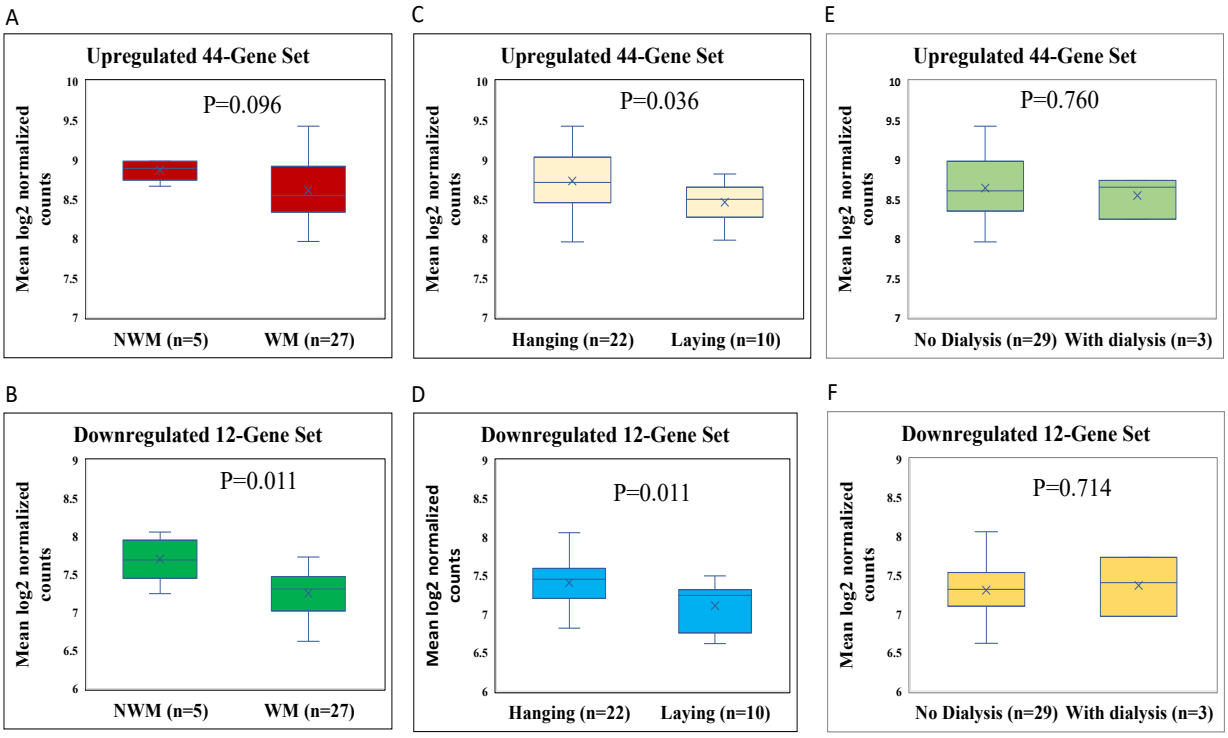

Figure 3.8. Gene set expression changes with different *ex vivo* heart perfusion (EVHP) strategies. Boxes represent interquartile range whereas whiskers show data points within 1.5x interquartile range from upper and lower box limits. NWM= Non-Working Mode, WM=Working Mode.

3.4 Discussion

The shortage of suitable donor hearts for cardiac transplantation is in part due to the relatively short preservation time and limited organ transportation necessitated by the current clinical standard of CSP. EVHP represents a promising alternative that could minimize ischemic injury, allow for organ resuscitation and functional assessment, prolong preservation time, facilitate therapeutic interventions, and ultimately expand the donor organ pool. The current clinically accepted and available *ex vivo* device, Organ Care System (TransMedics Inc., Andover, MA) allows organ preservation in a non-working mode, and myocardial quality assessment is limited to lactate levels. This limitation led to the development of EVHP devices that allow for organ preservation in working mode, thus making it possible to assess several functional parameters. Despite the potential of EVHP demonstrated in animal and early human experiments, details on the molecular mechanisms of cardiac injury and repair during EVHP remain poorly understood. This study investigated gene expression patterns in hearts from a healthy porcine model subjected to 12 h of EVHP.

In this study, 68 transcripts previously reported in several studies to be associated with acute phase of cardiac injury and repair (as discussed in chapter 2 of this thesis) were used to evaluate changes in gene expression during EVHP. Distinct sample group clustering based on gene expression patterns was observed. Further analysis identified 44 significantly upregulated and 12 significantly downregulated genes after 12 h of EVHP. This suggests that EVHP induces a quantifiable molecular response that could potentially be used to more precisely monitor organ status during EVHP and further understand mechanisms of EVHP-related cardiac injury and repair.

Myocardial ischemia induces two inflammatory phases: 1) acute pro-inflammatory phase, which begins following the onset of ischemia to 24 h, and 2) anti-inflammatory reparative phase which begins from the 4^{th} to 7^{th} days following the ischemic insult (19). The pro-inflammatory

phase has been shown to be detrimental to the myocardium whereas the latter phase confers protection (19, 20). The 18/44 significantly upregulated transcripts in this study included pro-inflammatory genes, in keeping with acute molecular injury after 12 h of EVHP. Sang *et al*. reported upregulation of pro-inflammatory genes (*CCL2, IL-6, NFkBIA, S100* family, *CXCL2, CCR2*) following myocardial infarction (19), suggesting that our findings represent progressive pro-inflammatory ischemic injury during EVHP. Notably, previous studies on danger associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs) reported the presence of S100 proteins in DAMPs generated by injured cells (21). The significant upregulation of S100A8 and S100A9 genes after 12 h of EVHP, therefore, suggests accumulation of myocardial injury rather than repair during EVHP. *IL-6*, a pleiotropic cytokine that has been reported to play both pro-inflammatory and anti-inflammatory roles (22) is another upregulated gene in this study. It is a potent inducer of acute phase inflammation and is produced in response to cardiac ischemic injury, thus inducing and directing the trafficking of immune cells. However, it also produces signals conferring a protective and wound healing response to cardiac tissue injury (23). It has been reported that in the acute phase, *IL-6* is cardioprotective, but causes pathogenic inflammation in the chronic phase (24). In acute myocardial ischemic injury, *IL-6* is purported to be overexpressed to protect myocytes against oxidative stress and prevent apoptosis (25). Interestingly, *IL-6* is reported to decrease ventricular contraction through the JAK/STAT signaling pathways, in contrast to the STAT3 and ERK signaling pathway, which confers cardioprotective functions of IL-6 signaling during reperfusion injury (26, 27). In this study, therefore, upregulation of *IL-6* may have represented a reparative response to cardiac injury. However, further study is needed to confirm the downstream pathways induced by upregulation of this gene.

Four genes (*CXCL2, CXCL11, CXCL10* and *CXCL9*) of the MCODE complex previously identified in chapter two of this thesis were significantly upregulated, with significant downregulation of *CCR2*. This MCODE complex plays a critical role in chemokine-mediated signaling pathway. CC and CXC represent a family of chemotactic cytokines that are commonly involved in the chemotactic recruitment of mononuclear leukocytes and neutrophils respectively (28). The production of DAMPs, ROS, and injury of the extracellular matrix following ischemia-reperfusion injury represent upstream effectors of signaling pathways including TLR, IL-1, complement cascade and NF-KB that activate chemokine-mediated pro-inflammatory response (28). Notably, TLR signaling has been implicated in downstream CXC chemokine upregulation (29). The role of CCL2 during inflammation has been reported to be involved in the recruitment of monocytes from the blood stream into the tissues through interaction with its corresponding receptor, CCR2 (30). However, this study identified upregulation of *CCL2* and downregulation of *CCR2* after 12 h of EVHP, which is of uncertain significance and warrants further mechanistic analysis. *CXCL9, CXCL10* and *CXCL11* are implicated in the chemotactic recruitment of lymphocytes, whereas *CXCL2* is involved in the recruitment of neutrophils and monocytes (28, 31-33). The upregulation of these genes therefore indicates the presence of molecular signals promoting lymphocytic and neutrophilic inflammation after 12 h of EVHP.

The AP-1 MCODE complex identified in chapter two of this thesis demonstrated upregulation of *ATF3* and *FOS*, and downregulation of *JUN* with EVHP. Previous AP-1 studies focusing on the deletion of JUN reported fibrosis of the myocardium, alteration of sarcomere organization and cardiomyocyte apoptosis (34). Activation of the MAPK signaling pathway results in the downstream activation of the AP-1 cascade, which then induces the expression of *TGFβ-1* and collagens I and III (35).*TGFβ-1* upregulation in this experiment has been previously reported

to play a critical role in extracellular matrix formation-remodeling (36). Altogether, this suggests that upregulation of both *FOS* and *ATF3* and down regulation of *JUN* might represent the induction of molecular pathways associated with myocardial fibrosis and cardiomyocyte loss. Subsequently, ATF3 homodimerized AP-1 signaling might have induced the upregulation of *TGFβ-1*, thus representing early cardiac remodeling. Evidently, *ATF3* and *GDF3* -- a *TGFβ-1* superfamily member -- have been reported to be upregulated following ischemia-reperfusion, findings that are consistent with this study (37, 38).

Acute myocardial ischemic injury has previously been reported to induce a reduction in oxygen tension within cardiomyocytes, consequently leading to loss of oxidative phosphorylation and decreased ATP production due to structural changes within the cardiomyocytes, including cellular and mitochondrial edema and depletion of energy stores (39). These reports are consistent with our findings of significant downregulation among structural genes (e.g. *COL1A1, MYH7, ACTA2, FN1*), reflecting alterations in extracellular matrix, sarcomeric integrity, myocardial contractility, and mitochondrial structure. Reduced molecular injury in combined heart and liver perfusion may therefore be attributable to hepatic activity, such as liver-derived cardioprotective effectors that enhance cardiomyocyte tolerance to ischemia (40). Specifically, Liu *et al* demonstrated significantly reduced myocardial infarcts due to upregulation of alpha-1 acid glycoprotein 2 (*AGP2*), neuregulin 4 (*NRG4*), bone morphogenetic protein-binding endothelial regulator (*BMPER*), fibroblast growth factor 21 (*FGF21*) and trefoil factor 3 (*TFF3*) by hepatocytes in post-myocardial ischemia (40). Furthermore, significantly lower expression of the downregulated 12-gene set in WM and laying position suggests that these strategies are associated with relatively more myocardial injury. However, further work is needed to understand these

interesting observations and help explain the unresolved mechanisms underlying improved cardiac function observed with EVHP in a loaded physiologic mode (41).

Cardiac fibrosis is one of the defense phenomena that is induced following myocardial injury. The lack of regenerative capacity by adult human cardiac cells shifts the response to loss of cardiomyocytes by a reparative process that causes the formation of fibrous tissue (42). Rapid loss of cardiomyocytes following acute ischemic injury stimulates an intense inflammatory response which ultimately leads to the replacement of the lost cells with fibrous tissue (43). Cytokines such as *TNF-α, IL-6*, chemokines, and growth factors such as *TGF-β* and *GDF-15* have been noted to be the common upstream profibrotic effectors (42). The upregulation of these genes in this study therefore suggest an active stimulation of early fibrotic cardiac tissue remodeling. Notably, coactivation of proinflammatory and profibrotic reactions stimulates a vicious cycle that causes progressive activation of myofibroblasts (42). This results in significant increase of collagen deposition and accumulation in the extracellular matrix (44). Upregulation of matrix metalloproteinases as well as their tissue inhibitors have been reported to be associated with collagen turnover and extracellular matrix (ECM) fibrosis (45). These findings are consistent with the results in this experiment including increased expression levels of *MMP12, MMP14* and *TIMP1*, hence suggesting cardiac remodeling, a process that has been associated with systolic and diastolic dysfunction. Studies on pre-clinical models of end stage heart failure reported similar results on the upregulation of MMPs (46, 47).

ECM turnover is further enhanced with upregulation of *LOX* genes and subsequent increase of lysyl oxidase enzymes that are crucial in collagen cross-linking, an activity that enhances resistance of collagen to matrix metalloproteinase degradation (48, 49). LOX enzymes function by converting soluble collagen into insoluble collagen thus making them resistant to degradation by

matrix proteinases (49). Interestingly, the findings in this study identified a significant downregulation of *LOX*, suggesting an active ECM turnover that is very much susceptible to MMP degradation (50). It is therefore tempting to speculate that EVHP induces an active cardiac repair through the downregulation of *LOX*, however, overwhelmed by other factors causing cardiac fibrosis. Consistent with this hypothesis is a study by El Hajj *et al.* that reported improved cardiac function following application of lysyl oxidase inhibitor (44).

In addition to its inhibitory activity against MMP degradation, Takawale *et al* recently reported that *TIMP1* contributes to cardiac fibrosis through MMP-independent pathways (51). They demonstrated that *TIMP1* is involved in mediating the association between CD63 and integrin-β1 present on cardiac fibroblasts, causing downstream activation and nuclear translocation of Smad2/3 and β-catenins (51). Notably, Smad2/3 are known to be TGF-β signal transducers and, thus, their activation results in increased expression of *TGF-β1* ultimately causing de novo collagen production (51, 52). In particular, *TGF-β1* is reported to be involved in the downstream regulation of the *ATF3* gene, which further activates the upregulation of *TGF-β1* in a positive-feedback loop (53). Together with *TIMP1*, *MMPs* and *TGF-β1, ATF3* was upregulated in this experimental study. This cumulative activation of *TGF-β1* could explain the overwhelming action of downregulated *LOX* gene with less cross-linked ECM turnover during EVHP repair. Studies in tissue inhibitor of metalloproteinases (TIMPs) have demonstrated low expression levels of *TIMP1* in a normal healthy heart and increased expression levels in dysfunctional hearts (54). The overexpression of both *TIMP1* and *MMP14*, however, has been associated with cardiac fibrosis and left ventricular dysfunction in pressure-overloaded human hearts (55) as well as in animal studies (56), further suggesting a pattern of tissue remodelling-mediated myocardial damage during EVHP. Particularly, MMP-14 has been widely studied and reported to degrade

fibronectin and fibrillar collagen type I which are extracellular matrix components that are encoded by *FN1* and *COL1A1* genes respectively (57). It is therefore tempting to speculate that upregulation of the *MMP-14* could have contributed to the downregulation of *FN1* and *COL1A1* observed in this study through a negative feedback mechanism. *COL1A1* and *COL1A2* are the two genes that encode collagen type 1, the most abundant component of the ECM (58). Downregulation of these genes has been reported to result in loss of ECM integrity, and hence decline in cardiac function (59).

One of the challenges with EVHP is the incomplete clearance for metabolic waste products and cellular debris (60). Mechanistically, continuous hemodialysis during EVHP could be a suitable modality for ionic homeostasis (61). This study incorporated a brief period of dialysis to assess whether functional performance would be positively impacted. Interestingly, gene expression changes did not show any significant differences between groups with or without dialysis, although the sample sizes were small. These results suggest that neither ionic imbalance nor metabolite accumulation are the primary drivers of functional deterioration during EVHP. The findings are consistent with a prior study by Buchko *et al* who examined the effects of continuous hemodialysis during *ex vivo* lung perfusion (EVLP) (62).

Overall, these results suggest an active process of molecular cardiac tissue injury during EVHP, represented by a pro-inflammation, pro-apoptosis, and pro-necrosis gene expression phenotype. This is the direct opposite to what has previously been reported in EVLP (18), which differs from EVHP in multiple respects, including the presence of ventilation in addition to perfusion. The proposed theoretical benefits of EVHP include the influence on aerobic metabolism during organ perfusion by maintaining cellular function and integrity through enhanced microcirculation, clearance of toxins as well as the removal of inflammatory cells (63). However,

powerful pro-inflammatory genes including *TNFα* and *IL6* were found to be upregulated in EVHP contrary to what was observed in EVLP (18). Although the overexpression of *TIMP1* in EVLP (18) was interpreted to represent tissue repair, upregulation of this transcript in the current EVHP study could, in contrast, suggest cardiac tissue injury because of the notable role of this gene in the injured heart. Additionally, powerful pro-apoptotic genes including *BTG2, GADD45B* and *CASP8* demonstrated high expression in EVHP as well as pro-oxidative stress *HMOX* gene, findings that are contrary to the previous EVLP study. *BTG2*, in particular, has been reported in cancer studies to be a powerful tumor suppressor gene as well is in cardiomyocyte hypertrophy (64, 65).

3.5 Strengths and Limitations

This study has multiple strengths and limitations worth noting. The use of a homogenous, large animal, pre-clinical model along with a novel, thoroughly annotated, literature-derived gene signature for cardiac injury and repair are considered the greatest strengths. However, notable limitations include the use of donor hearts being procured from healthy animals as opposed to diseased hearts that would more accurately mimic the clinical situation of donation after cardiac death. Additionally, this study did not include intermediate biopsy collection time points between T0 and T12 for the EVHP gene expression analysis, due to concerns about the potential confounding effect of such biopsies on organ status and cardiac function; hearts with such biopsies could not sustain prolonged perfusion.

3.6 Conclusion

Gene expression profiling represents a powerful approach for assessing cellular functions and generating testable hypotheses. This study aimed to exploit the benefits of this approach to improve our understanding of and ability to monitor cardiac tissue injury and repair during EVHP.

The experiment in this Chapter demonstrated that EVHP induces a quantifiable molecular response thus, supporting the hypothesis that analyzing the expression level of the described subset of genes identified in Chapter 2 would allow for mechanistic measurement of cardiac tissue injury during organ perfusion. Two sets of genes (44 upregulated and 12 downregulated) that were identified in this study could potentially be used, either individually or as aggregate gene sets, to assess the quality of *ex vivo* perfused hearts prior to transplantation. To further understand the mechanisms of injury occurring during EVHP, the identified sets of genes were correlated with function and histology as described in detail in Chapter 4.

3.7 References

1. Ho KK, Pinsky JL, Kannel WB, Levy D. The epidemiology of heart failure: the Framingham Study. J Am Coll Cardiol. 1993;22(4 Suppl A):6a-13a.

2. Benjamin EJ, Blaha MJ, Chiuve SE, Cushman M, Das SR, Deo R, et al. Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association. Circulation. 2017;135(10):e146-e603.

3. Bleumink GS, Knetsch AM, Sturkenboom MC, Straus SM, Hofman A, Deckers JW, et al. Quantifying the heart failure epidemic: prevalence, incidence rate, lifetime risk and prognosis of heart failure The Rotterdam Study. Eur Heart J. 2004;25(18):1614-9.

4. Lund LH, Edwards LB, Kucheryavaya AY, Dipchand AI, Benden C, Christie JD, et al. The Registry of the International Society for Heart and Lung Transplantation: Thirtieth Official Adult Heart Transplant Report--2013; focus theme: age. J Heart Lung Transplant. 2013;32(10):951-64.

5. Chambers DC, Yusen RD, Cherikh WS, Goldfarb SB, Kucheryavaya AY, Khusch K, et al. The Registry of the International Society for Heart and Lung Transplantation: Thirty-fourth Adult Lung And Heart-Lung Transplantation Report-2017; Focus Theme: Allograft ischemic time. J Heart Lung Transplant. 2017;36(10):1047-59.

6. Khush KK, Zaroff JG, Nguyen J, Menza R, Goldstein BA. National decline in donor heart utilization with regional variability: 1995-2010. Am J Transplant. 2015;15(3):642-9.

7. Messer S, Ardehali A, Tsui S. Normothermic donor heart perfusion: current clinical experience and the future. Transpl Int. 2015;28(6):634-42.

8. Jahania MS, Sanchez JA, Narayan P, Lasley RD, Mentzer RM, Jr. Heart preservation for transplantation: principles and strategies. Ann Thorac Surg. 1999;68(5):1983-7.

9. Church JT, Alghanem F, Deatrick KB, Trahanas JM, Phillips JP, Hee Song M, et al. Normothermic Ex Vivo Heart Perfusion: Effects of Live Animal Blood and Plasma Cross Circulation. ASAIO J. 2017;63(6):766-73.

10. Ardehali A, Esmailian F, Deng M, Soltesz E, Hsich E, Naka Y, et al. Ex-vivo perfusion of donor hearts for human heart transplantation (PROCEED II): a prospective, open-label, multicentre, randomised non-inferiority trial. Lancet. 2015;385(9987):2577-84.

11. Hatami S, White CW, Shan S, Haromy A, Qi X, Ondrus M, et al. Myocardial Functional Decline During Prolonged Ex Situ Heart Perfusion. Ann Thorac Surg. 2019;108(2):499-507.

12. Broughton KM, Wang BJ, Firouzi F, Khalafalla F, Dimmeler S, Fernandez-Aviles F, et al. Mechanisms of Cardiac Repair and Regeneration. Circ Res. 2018;122(8):1151-63.

13. White CW, Ali A, Hasanally D, Xiang B, Li Y, Mundt P, et al. A cardioprotective preservation strategy employing ex vivo heart perfusion facilitates successful transplant of donor hearts after cardiocirculatory death. J Heart Lung Transplant. 2013;32(7):734-43.

14. Leprince P, Popov AF, Simon AR, Benk C, Siepe M. Ex vivo perfusion of the heart with the use of the Organ Care System. Eur J Cardio-thorac Surg. 2016;49(5):1318-20.

15. White CW, Hasanally D, Mundt P, Li Y, Xiang B, Klein J, et al. A whole blood-based perfusate provides superior preservation of myocardial function during ex vivo heart perfusion. J Heart Lung Transplant. 2015;34(1):113-21.

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

17. Hatami S, White CW, Ondrus M, Qi X, Buchko M, Himmat S, et al. Normothermic Ex Situ Heart Perfusion in Working Mode: Assessment of Cardiac Function and Metabolism. JoVE-J Vis Exp. 2019(143).

18. Dromparis P, Aboelnazar NS, Wagner S, Himmat S, White CW, Hatami S, et al. Ex vivo perfusion induces a time- and perfusate-dependent molecular repair response in explanted porcine lungs. Am J Transplant. 2019;19(4):1024-36.

19. Ong SB, Hernandez-Resendiz S, Crespo-Avilan GE, Mukhametshina RT, Kwek XY, Cabrera-Fuentes HA, et al. Inflammation following acute myocardial infarction: Multiple players, dynamic roles, and novel therapeutic opportunities. Pharmacol Therapeut. 2018;186:73-87.

20. Saxena A, Russo I, Frangogiannis NG. Inflammation as a therapeutic target in myocardial infarction: learning from past failures to meet future challenges. Transl Res. 2016;167(1):152-66.

21. Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol. 2007;81(1):1-5.

22. Gwechenberger M, Mendoza LH, Youker KA, Frangogiannis NG, Smith CW, Michael LH, et al. Cardiac myocytes produce interleukin-6 in culture and in viable border zone of reperfused infarctions. Circulation. 1999;99(4):546-51.

23. Fontes JA, Rose NR, Cihakova D. The varying faces of IL-6: From cardiac protection to cardiac failure. Cytokine. 2015;74(1):62-8.

24. Kaplanski G, Marin V, Montero-Julian F, Mantovani A, Farnarier C. IL-6: a regulator of the transition from neutrophil to monocyte recruitment during inflammation. Trends Immunol. 2003;24(1):25-9.

25. Terrell AM, Crisostomo PR, Wairiuko GM, Wang M, Morrell ED, Meldrum DR. Jak/STAT/SOCS signaling circuits and associated cytokine-mediated inflammation and hypertrophy in the heart. Shock. 2006;26(3):226-34.

26. Yu X, Kennedy RH, Liu SJ. JAK2/STAT3, not ERK1/2, mediates interleukin-6-induced activation of inducible nitric-oxide synthase and decrease in contractility of adult ventricular myocytes. J Biol Chem. 2003;278(18):16304-9.

27. Yamauchi-Takihara K, Ihara Y, Ogata A, Yoshizaki K, Azuma J, Kishimoto T. Hypoxic stress induces cardiac myocyte-derived interleukin-6. Circulation. 1995;91(5):1520-4.

28. Cavalera M, Frangogiannis NG. Targeting the chemokines in cardiac repair. Curr Pharmaceut Des. 2014;20(12):1971-9.

29. Boyd JH, Mathur S, Wang Y, Bateman RM, Walley KR. Toll-like receptor stimulation in cardiomyoctes decreases contractility and initiates an NF-kappaB dependent inflammatory response. Cardiovasc Res. 2006;72(3):384-93.

30. Serbina NV, Jia T, Hohl TM, Pamer EG. Monocyte-mediated defense against microbial pathogens. Annu Rev Immunol. 2008;26:421-52.

31. Nogueira LG, Santos RH, Ianni BM, Fiorelli AI, Mairena EC, Benvenuti LA, et al. Myocardial chemokine expression and intensity of myocarditis in Chagas cardiomyopathy are controlled by polymorphisms in CXCL9 and CXCL10. PLoS Neglect Trop D. 2012;6(10):e1867.

32. Waehre A, Vistnes M, Sjaastad I, Nygard S, Husberg C, Lunde IG, et al. Chemokines regulate small leucine-rich proteoglycans in the extracellular matrix of the pressure-overloaded right ventricle. J Appl Physiol. 2012;112(8):1372-82.

33. Szentes V, Gazdag M, Szokodi I, Dezsi CA. The Role of CXCR3 and Associated Chemokines in the Development of Atherosclerosis and During Myocardial Infarction. Front Immunol. 2018;9:1932.

34. Windak R, Muller J, Felley A, Akhmedov A, Wagner EF, Pedrazzini T, et al. The AP-1 transcription factor c-Jun prevents stress-imposed maladaptive remodeling of the heart. PloS one. 2013;8(9):e73294.

35. Kim SJ, Angel P, Lafyatis R, Hattori K, Kim KY, Sporn MB, et al. Autoinduction of transforming growth factor beta 1 is mediated by the AP-1 complex. Mol Cell Biol. 1990;10(4):1492-7.

36. Shimizu N, Yoshiyama M, Omura T, Hanatani A, Kim S, Takeuchi K, et al. Activation of mitogen-activated protein kinases and activator protein-1 in myocardial infarction in rats. Cardiovasc Res. 1998;38(1):116-24.

37. Cheng CF, Lin H. Acute kidney injury and the potential for ATF3-regulated epigenetic therapy. Toxicol Mech Methods. 2011;21(4):362-6.

38. Kempf T, Zarbock A, Widera C, Butz S, Stadtmann A, Rossaint J, et al. GDF-15 is an inhibitor of leukocyte integrin activation required for survival after myocardial infarction in mice. Nat Med. 2011;17(5):581-8.

39. Zimmermann M, Beer L, Ullrich R, Lukovic D, Simader E, Traxler D, et al. Analysis of region specific gene expression patterns in the heart and systemic responses after experimental myocardial ischemia. Oncotarget. 2017;8(37):60809-25.

40. Liu SQ, Tefft BJ, Roberts DT, Zhang LQ, Ren Y, Li YC, et al. Cardioprotective proteins upregulated in the liver in response to experimental myocardial ischemia. Am J Physiol-Heart C. 2012;303(12):H1446-58.

41. White CW, Shan S, Hatami S, Gurtu V, Kinnaird A, Matsumura N, et al. Impact of Myocardial Load on the Preservation of Donor Heart Function during Ex Vivo Perfusion. J Heart Lung Transplant. 2016;35(4).

42. Frangogiannis NG. Cardiac fibrosis: Cell biological mechanisms, molecular pathways and therapeutic opportunities. Mol Aspects Med. 2019;65:70-99.

43. Frangogiannis NG. The inflammatory response in myocardial injury, repair, and remodelling. Nat Rev Cardiol. 2014;11(5):255-65.

44. El Hajj EC, El Hajj MC, Ninh VK, Gardner JD. Inhibitor of lysyl oxidase improves cardiac function and the collagen/MMP profile in response to volume overload. Am J Physiol-Heart C. 2018;315(3):H463-h73.

45. Picard F, Brehm M, Fassbach M, Pelzer B, Scheuring S, Kury P, et al. Increased cardiac mRNA expression of matrix metalloproteinase-1 (MMP-1) and its inhibitor (TIMP-1) in DCM patients. Clin Res Cardiol. 2006;95(5):261-9.

46. Reinhardt D, Sigusch HH, Hensse J, Tyagi SC, Korfer R, Figulla HR. Cardiac remodelling in end stage heart failure: upregulation of matrix metalloproteinase (MMP) irrespective of the underlying disease, and evidence for a direct inhibitory effect of ACE inhibitors on MMP. Heart. 2002;88(5):525-30.

47. Spinale FG, Wilbur NM. Matrix metalloproteinase therapy in heart failure. Curr Treat Options Cardiovasc Med. 2009;11(4):339-46.

48. Siegel RC, Pinnell SR, Martin GR. Cross-linking of collagen and elastin. Properties of lysyl oxidase. Biochemistry. 1970;9(23):4486-92.

49. Rodriguez C, Martinez-Gonzalez J. The Role of Lysyl Oxidase Enzymes in Cardiac Function and Remodeling. Cells. 2019;8(12).

50. van der Slot-Verhoeven AJ, van Dura EA, Attema J, Blauw B, Degroot J, Huizinga TW, et al. The type of collagen cross-link determines the reversibility of experimental skin fibrosis. Biochim Biophys Acta. 2005;1740(1):60-7.

51. Takawale A, Zhang P, Patel VB, Wang X, Oudit G, Kassiri Z. Tissue Inhibitor of Matrix Metalloproteinase-1 Promotes Myocardial Fibrosis by Mediating CD63-Integrin beta1 Interaction. Hypertension (Dallas, Tex : 1979). 2017;69(6):1092-103.

52. Nakao A, Imamura T, Souchelnytskyi S, Kawabata M, Ishisaki A, Oeda E, et al. TGF-beta receptor-mediated signalling through Smad2, Smad3 and Smad4. Embo J. 1997;16(17):5353-62.

53. Yin X, Wolford CC, Chang YS, McConoughey SJ, Ramsey SA, Aderem A, et al. ATF3, an adaptive-response gene, enhances TGF{beta} signaling and cancer-initiating cell features in breast cancer cells. J Cell Sci. 2010;123(Pt 20):3558-65.

54. Nuttall RK, Sampieri CL, Pennington CJ, Gill SE, Schultz GA, Edwards DR. Expression analysis of the entire MMP and TIMP gene families during mouse tissue development. FEBS letters. 2004;563(1-3):129-34.

55. Heymans S, Schroen B, Vermeersch P, Milting H, Gao F, Kassner A, et al. Increased cardiac expression of tissue inhibitor of metalloproteinase-1 and tissue inhibitor of metalloproteinase-2 is related to cardiac fibrosis and dysfunction in the chronic pressure-overloaded human heart. Circulation. 2005;112(8):1136-44.

56. Lindsey ML, Zamilpa R. Temporal and spatial expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases following myocardial infarction. Cardiovasc Therapeut. 2012;30(1):31-41.

57. Barbolina MV, Stack MS. Membrane type 1-matrix metalloproteinase: substrate diversity in pericellular proteolysis. Semin Cell Dev Biol. 2008;19(1):24-33.

58. Rauch F, Glorieux FH. Osteogenesis imperfecta. Lancet. 2004;363(9418):1377-85.

59. Thiele F, Cohrs CM, Flor A, Lisse TS, Przemeck GK, Horsch M, et al. Cardiopulmonary dysfunction in the Osteogenesis imperfecta mouse model Aga2 and human patients are caused by bone-independent mechanisms. Hum Mol Genet. 2012;21(16):3535-45.

60. Pinezich M, Vunjak-Novakovic G. Bioengineering approaches to organ preservation ex vivo. Exp Biol Med. 2019;244(8):630-45.

61. Kao CC, Parulekar AD. Is perfusate exchange during ex vivo lung perfusion beneficial? Ann Transl Med. 2020;8(3):43.

62. Buchko MT, Himmat S, Stewart CJ, Hatami S, Dromparis P, Adam BA, et al. Continuous Hemodialysis Does Not Improve Graft Function During Ex Vivo Lung Perfusion Over 24 Hours. Transplant Proc. 2019;51(6):2022-8.

63. Ozeki T, Kwon MH, Gu J, Collins MJ, Brassil JM, Miller MB, Jr., et al. Heart preservation using continuous ex vivo perfusion improves viability and functional recovery. Circ J. 2007;71(1):153-9.

64. Masumura Y, Higo S, Asano Y, Kato H, Yan Y, Ishino S, et al. Btg2 is a Negative Regulator of Cardiomyocyte Hypertrophy through a Decrease in Cytosolic RNA. Sci Rep. 2016;6:28592.

65. Tsui KH, Chiang KC, Lin YH, Chang KS, Feng TH, Juang HH. BTG2 is a tumor suppressor gene upregulated by p53 and PTEN in human bladder carcinoma cells. Cancer Med. 2018;7(1):184-95.

Chapter 4

Correlation between gene expression, cardiac function and histology during *ex vivo* heart perfusion[3]

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4.1 Introduction

Although heart transplantation remains the gold standard treatment for patients with end-stage heart disease, successful transplant intervention requires accurate pre-transplant assessment of donor organ viability (1). Being able to determine the functional status of a potential donor heart is thus vital for transplant surgeons to make informed decisions regarding organ acceptance. *Ex vivo* perfusion of donor hearts provides a promising opportunity for more accurately measuring the degree of injury incurred during organ procurement and assessing the overall quality of the heart prior to transplantation (2). During EVHP, numerous hemodynamic, physiological, electrical and metabolic parameters can be measured (3). However, alterations in these parameters are generally not associated with a specific underlying etiology; for example, ischemia-reperfusion injury, ion imbalances, progressive accumulation of toxins, nutrient depletion, cardiomyocyte necrosis or apoptosis, and inflammation have all been reported to contribute to cardiac dysfunction during EVHP (3). A thorough assessment of cardiac injury would therefore ideally incorporate other diagnostic tools such as electrocardiography, biomarker levels, and histology (4).

Histopathological scoring has been widely used to provide both qualitative and quantitative measures of cardiac injury in cardiovascular research (5). Although different investigators have previously employed varying scoring systems, common histological features of cardiac injury include the presence of inflammatory cells, myocardial hemorrhage, interstitial edema, necrosis, and endothelial injury (5, 6). These features are often further qualified as being focal, multifocal or diffuse depending on the extent of injury (7). Notably, cardiomyocyte necrosis is generally represented by loss of nuclei, hyper-eosinophilia, loss of striations, and/or the presence of contraction bands (6).

Cardiac troponin I, a common clinical biomarker for measuring myocardial injury, unfortunately has limited utility for quantifying myocyte necrosis in EVHP, due to procedure-

related ischemia resulting in the release of cytoplasmic contents in the absence of tissue necrosis (8). In the setting of EVHP, increased troponin I could result from the organ retrieval procedure, ischemia-reperfusion, absence of renal clearance or EVHP-induced inflammation, and thus may not reflect the true extent of myocardial necrosis (9-11). Similar limitations of nonspecificity are encountered with brain natriuretic peptide (BNP), another biomarker of cardiac injury that is secreted by the heart following ventricular wall distension (12) and has been associated with decreased left ventricular ejection fraction (13).

Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) is a commonly used method for rapid detection and quantification of apoptotic cells (14). During apoptosis, the affected cells demonstrate biochemical and morphological characteristics including chromatin condensation, cell and nuclear shrinkage, development of membrane bound apoptotic bodies and phagocytosis by adjacent cells or macrophages in the absence of an associated inflammatory response (15). Endonuclease degradation of DNA is a biochemical hallmark of apoptosis that results in the generation of double-stranded fragments of DNA of about 180-200 base pairs in length (16). The TUNEL assay, a template-independent method, utilizes deoxynucleotidyl transferase to catalyze the addition of labeled dUTPs to the 3'-hydroxyl ends of double-stranded DNA fragments for the identification and quantification of apoptotic cells (15). The complexes can then be visualized with the use of immunohistochemical techniques (17). However, the application of this technique for detecting apoptosis during EVHP presents several challenges. First, the reliability of DNA fragmentation in the TUNEL assay for detection of apoptosis can result in false positives due to possible necrosis-mediated DNA damage (18). Furthermore, the reliability of the TUNEL assay is suboptimal with formalin-fixed, paraffin-embedded (FFPE) tissue due to processing-related DNA fragmentation. Although alternative

methods such as Western blots have been employed to confirm and characterize apoptotic cells, the utility of apoptosis as a measure of injury appears limited for the purpose of organ quality assessment. In particular, Hatami *et al.* demonstrated significant number of apoptotic cells after 12 h of EVHP that was out of proportion to the observed decrease in cardiac function (19).

Some myocardial functional parameters assessed during EVHP have been shown to reliably predict graft failure post-transplant (20). One of the currently clinically available EVHP devices, the TransMedics Organ Care System (OCS), allows for assessment of several parameters that have previously been associated with post-transplant graft survival, including arterial lactate level, venous and arterial oxygen saturation, coronary sinus flow and aortic pressure (21). However, the ability of the OCS to only allow for organ perfusion in non-working mode limits the assessment of myocardial function (19). Furthermore, there is concern about the reliance of OCS on lactate concentrations as a primary marker of organ viability, given that up to 30% of hearts have been reported to be falsely deemed nonviable with this marker following in vivo assessment using normothermic regional perfusion (22, 23). These findings highlight the need for novel parameters that are sensitive and specific for determining the eligibility of an organ for optimal utilization and successful post-transplant outcome.

Cardiac organ preservation is an area of active research facilitated by the development of novel EVHP devices that allow organ perfusion in a loaded working mode. The EVHP loaded-mode circuit allows for the assessment of functional parameters such as cardiac output, cardiac index, stroke volume, and systolic and diastolic pressures, as well as for metabolic parameters including lactate, oxygen consumption and electrolytes to be recorded in real time (24, 25). However, although these data provide valuable information about cardiac functional status, they do not demonstrate the specific pathophysiological mechanisms underlying the observed

phenotypes. Hatami *et al* demonstrated that preservation during EVHP is associated with a time dependent decline in cardiac function (19). Specifically, they found a decline in cardiac output, stroke work, cardiac index, systolic pressure, oxygen extraction, myocardial oxygen consumption, and maximum and minimum rates of pressure change over 12 h of EVHP. Linking gene expression patterns with these existing measures of organ status may further enhance our ability to understand and predict organ quality by elucidating the molecular mechanisms of EVHP-related injury and repair.

Investigating the mechanisms underlying complex biological processes can include one of two approaches: 1) interrogating signaling pathways with gain and/or loss of function experiments, or 2) the use of transcriptomic-based discoveries to identify molecular markers that define a given phenomenon (26). With the relative ease of modern transcriptomics approaches, including genome-wide microarrays and RNA sequencing, the biological mechanisms of cardiovascular physiology and disease are being increasingly explored by correlating gene expression alterations with physiological parameters (27, 28). One of the greatest challenges in these studies is the attribution of functions to genomic characteristics, with relatively few functional annotations being authenticated by experimental evidence (29, 30). However, the availability of transcriptomic data has led to the development and exploitation of several gene function hypotheses that are based on the homogeneity of expression patterns of genes with known functions (31). The utility of transcriptomics in cardiovascular research has facilitated greater understanding of the molecular features associated with numerous entities, including hypertrophic and ischemic cardiomyopathies (32).

Most of the currently available myocardial biomarkers have been identified through binary case-control studies including a comparison between normal working hearts and failing ones, such

as dilated cardiomyopathy (33, 34). However, the feasibility of binary case-control studies in longitudinal research on human myocardial biopsies is limited and, therefore, transitional analysis using correlation and associational research could help in defining molecular biomarkers that correspond to functional or structural alterations (34-36). This chapter tested the hypothesis that increased expression of gene transcripts during EVHP could represent a molecular marker for cardiac injury and/or repair. The objective of the present study was to correlate functional and histopathologic changes in porcine hearts after 12 h of EVHP with the gene expression changes described in Chapter 3.

4.2 Methods

4.2.1 Histology

The FFPE tissue blocks (IV=7, H+L=7, EVHP=32), prepared as described in Chapter 3, were utilized for histological assessment. The tissue blocks were sectioned at 5 μm thickness on a rotary microtome (Leica HistoCore AUTOCUT Microtome, Leica Biosystems Inc., Buffalo Groove, IL). Each section was floated on a water bath (40°C), picked up on a glass slide, and baked at 60°C for 1 h prior to staining. Standard hematoxylin and eosin (H&E) staining was performed Harris Hematoxylin-3801561; Eosin-3801602, Surgipath®, Leica Biosystems, Richmond, IL, USA). A previously reported histopathologic scoring scheme for cardiac injury was utilized (5), including assessment of myofiber degeneration, hemorrhage, interstitial edema and endothelial changes on a scale of 0-3+ (Table 4.1). Furthermore, interstitial and perivascular lymphocytes, neutrophils and eosinophils were assessed for myocardial inflammation injury (37) as described in Table 4.1.

Table 4.1. Cardiac injury histology scoring schema

Histology Marker	Score	Description
Myofiber Degeneration	0	Absent
	1+	Single to multiple foci of vacuolated, shrunken, or fragmented, hypereosinophilic myofibers
	2+	More frequent multifocal foci to larger zones of vacuolated, shrunken, or fragmented hypereosinophilic myofibers
	3+	Large coalescing to regionally extensive zones of vacuolated, shrunken, or fragmented hypereosinophilic myofibers
Myocardial hemorrhage	0	Absent
	1+	Focal to multifocal mild myocardial, epicardial, or endocardial hemorrhage
	2+	Moderate multifocal to regionally extensive myocardial, epicardial, or endocardial hemorrhage
	3+	Severe regionally extensive hemorrhage involving large portions of heart section
Interstitial edema	0	Absent
	1+	Mild and multifocal separation of myofiber bundles or expansion of perivascular spaces
	2+	Moderate and multifocal separation of myofiber bundles or expansion of perivascular spaces
	3+	Marked regionally extensive or multifocal separation of myofiber bundles and perivascular spaces
Endothelial changes	0	Absent
	1+	Plump endothelial cells, separation of endothelium from underlying basal lamina
	2+	Cellular infiltration of vessel wall or disruption of layers of vessel wall
	3+	Necrosis or severe vascular cellular infiltration

Reproduced with permission from Wolters Kluwer Health Inc. (5)

Histology Marker	Score	Description
Inflammation	0	*Absent*
	1+	*Mild inflammation; inflammatory cells within capillary lumina*
	2+	*Moderate inflammation; scattered inflammatory cells infiltrating myocardium*
	3+	*Severe inflammation: myocardium densely infiltrated with inflammatory cells*

4.2.2 Functional Assessment of the *Ex Vivo* Perfused Heart

Functional assessment was performed by Dr. Sanaz Hatami in the Department of Surgery, University of Alberta. During the perfusion period described in Chapter 3, myocardial function was assessed at 1 h (T1; representing early perfusion), 5 h (T5; representing mid-perfusion), and 11 h (T11; representing late perfusion) in physiologic WM. The perfusion apparatus software collected the atrial, ventricular, and aortic pressure throughout the perfusion, 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) and SV (mL/beat; cardiac output (mL/min)/heart rate (beats/min) (15). To assess the maximum (dP/dt max) and minimum (dP/dt min) rates of pressure change, a pigtail catheter (5F) was placed in the left ventricle via the subclavian branch of the

aortic arch. The cardiac index (CI) was calculated by measuring the flow through the LA line divided by the heart weight (mL/min/g). Only T11 functional data, representing the closest timepoint to biopsy collection, were utilized for correlation with gene expression.

4.2.3 Gene expression

The expression levels of genes described in Chapter 3 were correlated with function and histology. Additionally, the two aggregate gene sets that were identified in Chapter 3, including the upregulated 44-gene set and the downregulated 12-gene set, were correlated with each histologic and functional parameter to assess for possible associations.

4.2.4 Statistics

R Version 3.4.1 was used for all analyses. Mann Whitney *U*-test was performed for all class comparison analyses and statistical significance considered at $P<0.05$. Spearman's rank correlation coefficients (rho) were calculated to characterize the relationships between aggregate gene sets and functional and histologic parameters. Correlation heatmap analysis was used to visualize the association between gene expression and functional parameters. Scatter plots and box plots were used for the significantly correlated parameters to confirm the directions of association.

4.3 Results

4.3.1 Cardiac injury histology scoring

The aim of the histology analysis was to assess for the presence of any microscopic features of cardiac injury. The EVHP biopsies demonstrated significantly more interstitial edema versus the *in vivo* group (EVHP=0.515±0.507 vs. IV=0±0, P=0.030) (Table 4.2, Figure 4.1).

Table 4.2. Histology results for cardiac injury

Histology parameter	Cardiac Injury Score (mean ± SD)			P- values		
	IV (7)	H+L (7)	EVHP (32)	EVHP vs IV	EVHP vs H+L	H+L vs IV
Myofiber degeneration	0 ± 0	0 ± 0	0 ± 0	NA	NA	NA
Myocardial hemorrhage	0 ± 0	0 ± 0	0.030 ± 0.174	NA	NA	NA
Endothelial changes	0 ± 0	0 ± 0	0.030 ± 0.174	NA	NA	NA
Interstitial edema	0 ± 0	0.125 ± 0.353	0.515 ± 0.507	0.030	0.116	0.703
Inflammation	0.5 ± 0.534	0.375 ± 0.517	0.696 ± 0.535	0.596	0.271	0.703

IV, *in vivo*; H+L, combined heart, and liver perfusion; EVHP, *ex vivo* heart perfusion; NA, not available

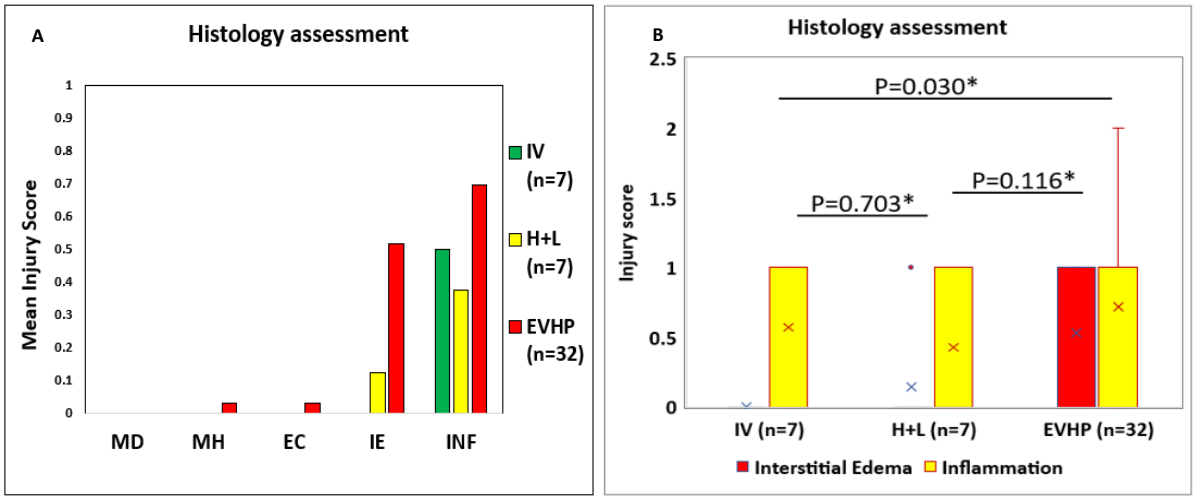

Figure 4.1. Cardiac injury histology scoring. A: Clustered column with mean injury score. B: Box plot. IV, *in vivo*; H+L, combined heart, and liver perfusion; EVHP, *ex vivo* heart perfusion; MD, myofiber degeneration; MH, myocardial hemorrhage; EC, endothelial changes; IE, interstitial edema; INF, inflammation; x, mean. *Interstitial edema P values.

However, there was no significant difference in interstitial edema between H+L and IV (H+L=0.125±0.353 vs. IV=0±0, P=0.703), nor between EVHP and H+L (P=0.116). Mild to no inflammation was observed in all sample groups, with no statistically significant differences between them. No myofiber degeneration was identified in any of the sample groups. Myocardial hemorrhage and endothelial swelling were observed in only one of the EVHP hearts (Figure 4.2).

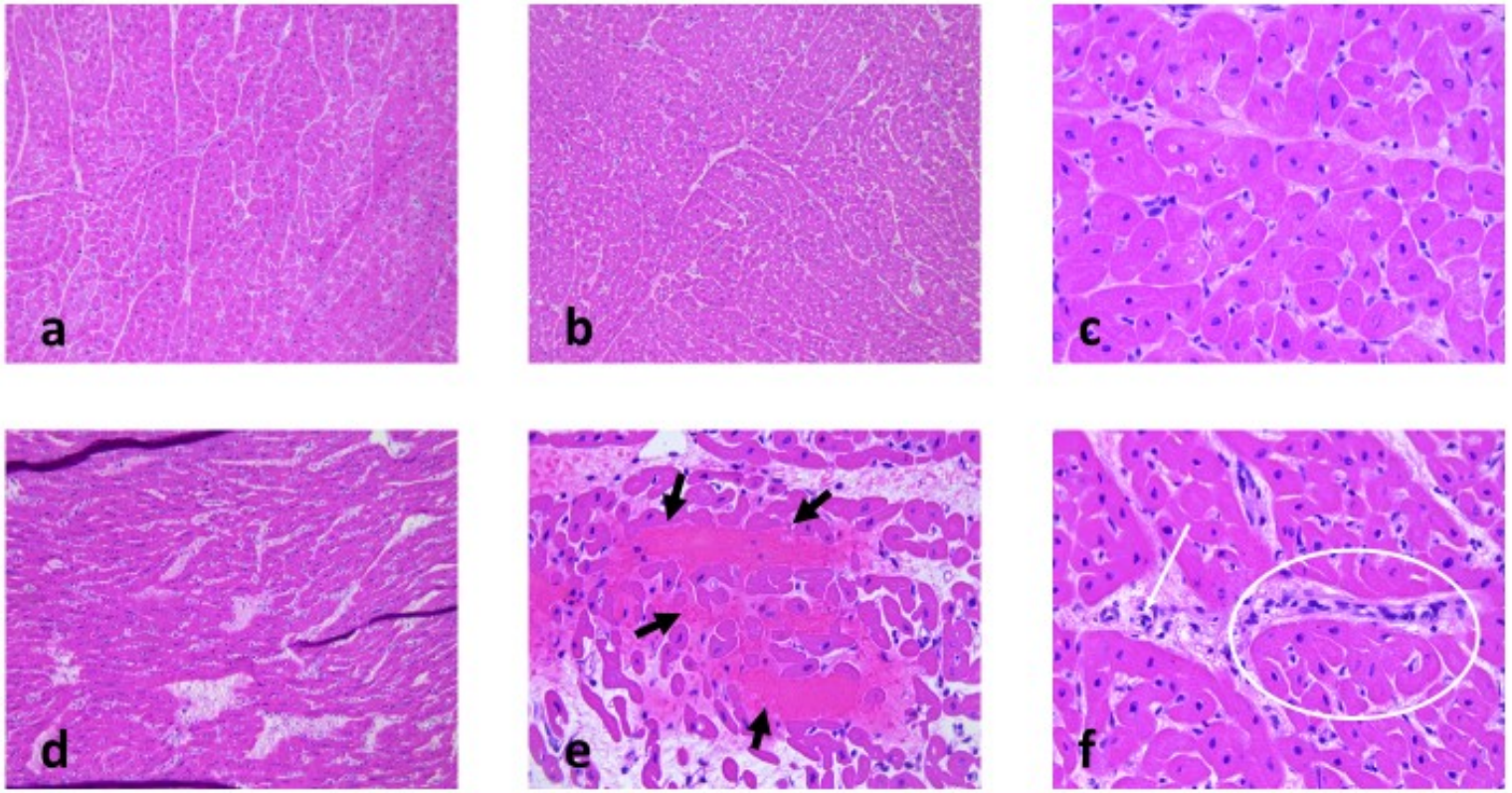

Figure 4.2. EVHP histology, hematoxylin and eosin stain. (a) *in vivo* X100 and (b) EVHP X100 demonstrating normal myocardium. (c) X400 of normal myocardium. (d) EVHP X100 showing mild interstitial edema. (e) EVHP X400 myocardial hemorrhage; black arrows. (f) EVHP X400 narrowed myocardial capillary lumen with plump endothelial cells; circle, and lymphocyte infiltration; white arrow.

4.3.2 Correlation between gene expression, histology and function

Hatami *et al* previously demonstrated a gradual decline in cardiac function during EVHP for the hearts included in this study (19). Correlation heatmap analysis demonstrated distinct clustering of these functional parameters based on gene expression patterns (Figure 4.3).

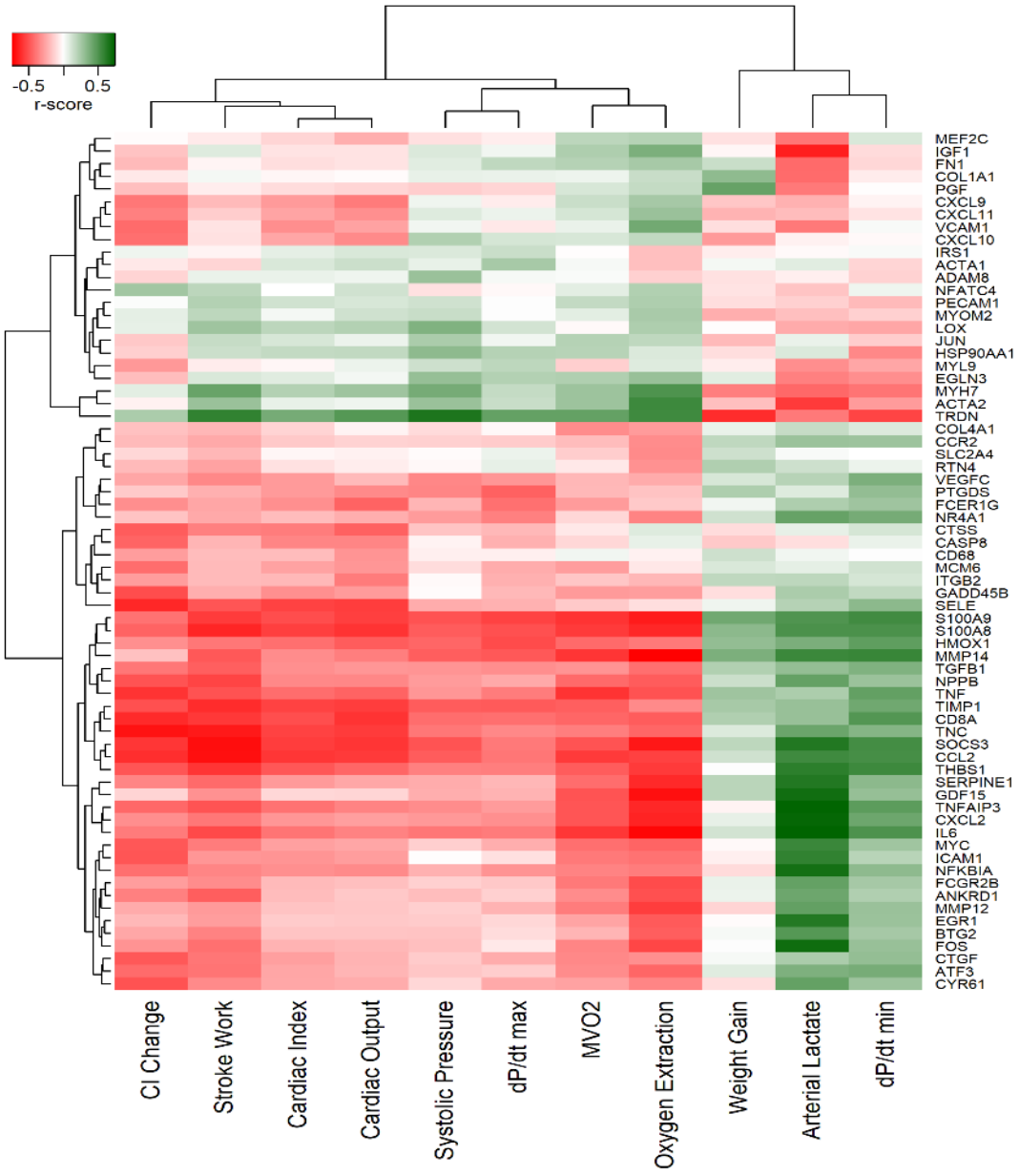

Figure 4.3. Correlation heatmap showing the association between gene set expression and cardiac functional parameters.

A total of 46/68 (67.6%) genes demonstrated a negative correlation with favorable functional markers including cardiac index change, stroke work, cardiac output, systolic pressure, maximum pressure change, myocardial oxygen consumption and oxygen extraction, while positively correlating with unfavorable parameters including weight gain, arterial lactate production and minimum rate of change of pressure. These genes included 39/44 (88.6%) identified within the upregulated 44-gene set in Chapter 3 (Figure 3.6). The other five genes in the upregulated 44-gene set were *HSP90AA1, CXCL9, CXCL10* and *CXCL11*. *CXCL9*, *CXCL10* and *CXCL11* positively correlated with some favorable parameters including systolic pressure, maximum pressure change, myocardial oxygen consumption and oxygen extraction, and negatively correlated with two unfavorable parameters including weight gain and arterial lactate, as well with other favorable parameters including cardiac index change, cardiac index, stroke work and cardiac output. No correlation was seen between these three genes with minimum pressure change. HSP90AA1 levels positively correlated with oxygen extraction, MVO_2, dP/dt max, systolic pressure, cardiac output and cardiac index and negatively correlated with dP/dt min. No correlation was observed with cardiac index changes and weight gain. Of the genes within the identified downregulated 12-gene set in Chapter 3 (Figure 3.6), 11/12 (91.7%) positively correlated with the favorable functional parameters and negatively correlated with the unfavorable markers. *CCR2* clustered with most of the upregulated genes (Figure 4.3).

Further analysis of the correlation between aggregate upregulated 44-gene set expression and cardiac function demonstrated significant negative correlation with cardiac index (rho=-0.516, P=0.002), change in cardiac index (rho=-0.626, p<0.001), stroke work (rho=-0.623, P<0.001), cardiac output (rho=-0.523, P=0.002), MVO_2 (rho=-0.513, P=0.002), dP/dt_{max} (rho=-0.382, P=0.030) and oxygen extraction (rho=-0.612, P<0.001) and positive correlation with arterial

lactate (rho= 0.640, P<0.001) and dP/dt_{min} (rho=0.481, P=0.006) (Table 4.3). There was no significant correlation between upregulated 44-gene set expression and weight gain (rho=0.101, P=0.553). Conversely, the downregulated 12-gene set demonstrated significant positive correlation with oxygen extraction (P=0.011) and negative correlation with arterial lactate (P<0.001) (Table 4.3).

Table 4.3. Correlation between gene set expression and functional parameters in EVHP (n=39) T12h

Correlation between gene set expression and functional parameters in 39 porcine hearts after 12h of ex vivo perfusion				
	Upregulated 44-gene set		Downregulated 12-gene set	
Functional Parameter	r-value	p-value	r-value	p-value
Weight gain (%)	0.101	0.553	-0.121	0.474
Cardiac index (ml/min/gram)	-0.516	0.002	0.073	0.681
Change in cardiac index (%)	-0.626	<0.001	-0.072	0.682
MVO2 (ml O_2/min/100g)	-0.513	0.002	0.260	0.143
Arterial lactate (mmol/L)	0.640	<0.001	-0.549	<0.001
Oxygen extraction (%)	-0.612	<0.001	0.434	0.011
Cardiac output (L/min)	-0.523	0.002	0.087	0.630
Stroke work (mmHg·ml)	-0.623	<0.001	0.250	0.167
Systolic pressure (mmHg)	-0.347	0.051	0.327	0.067
dP/dt_{max} (mmHg/s)	-0.382	0.030	0.174	0.341
dP/dt_{min} (mmHg/s)	0.481	0.006	-0.241	0.190

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Scatter plot analysis confirmed the direction of correlation for oxygen extraction and arterial lactate and the aggregate gene sets (Figure 4.4). There was no significant correlation between downregulated 12-gene set expression and all other functional parameters.

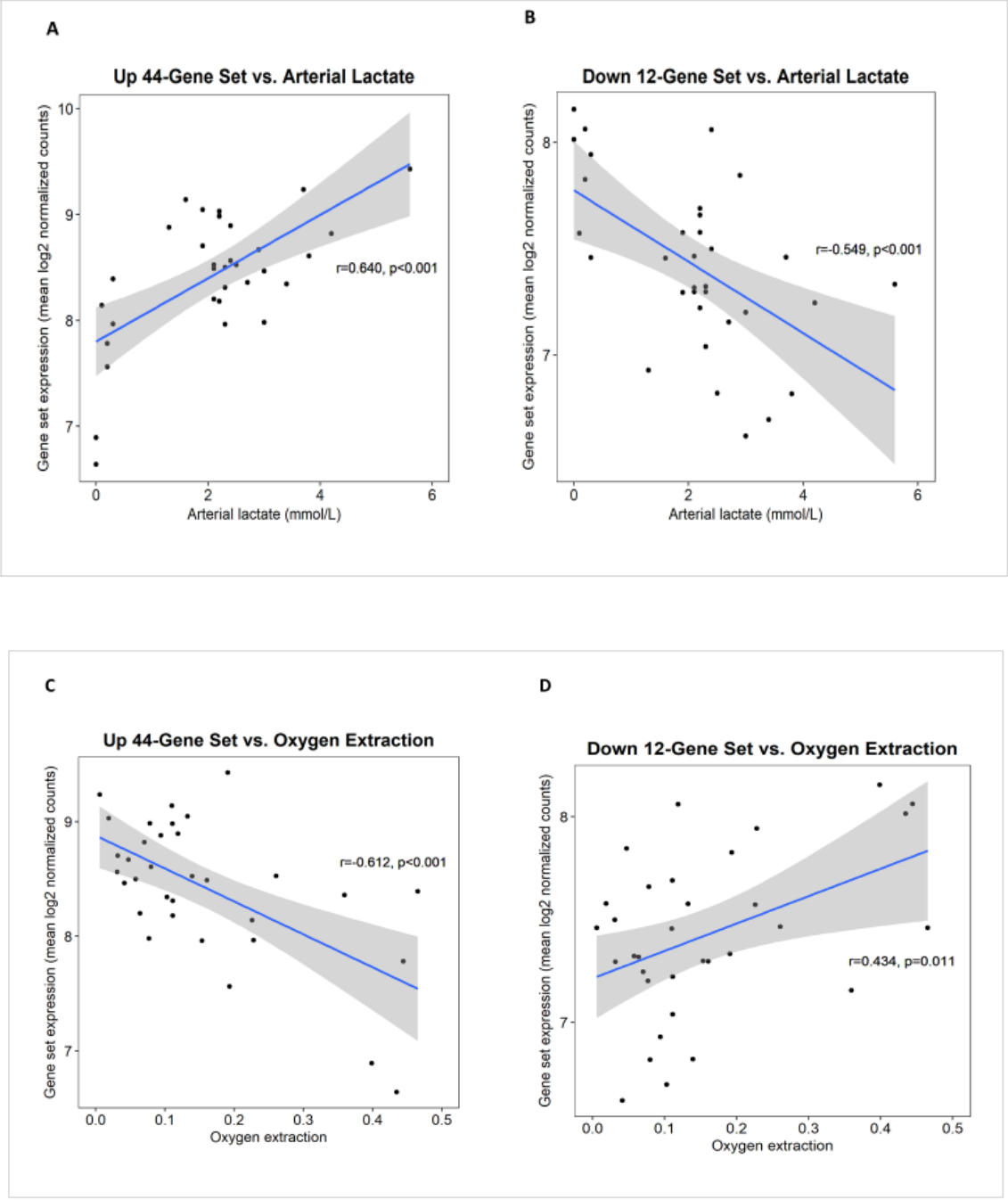

Figure 4.4. Scatter plots representing the correlation between gene set expression and arterial lactate (A, B) and oxygen extraction (C, D)

Correlation was also assessed between gene set expression and histologic features for all 46 samples. However, the only statistically significant relationship was between upregulated 44-

gene set expression and interstitial edema (absent vs. mild; rho=0.429, p=0.003) (Table 4.4 Figure 4.5). No other significant correlations between gene expression and histology were identified.

Table 4.4. Correlation between gene set expression and histological parameters in 46 porcine hearts including all three groups (IV, H+L and EVHP)

Correlation between gene expression and histological parameters in all the 46 porcine hearts				
	Upregulated 44-gene set		Downregulated 12-gene set	
Histological parameter	r*-value	P-value	r*-value	P-value
Myocardial hemorrhage	-0.028	0.853	-0.241	0.106
Endothelial changes	-0.072	0.633	0.257	0.085
Interstitial edema	**0.429**	**0.003**	-0.151	0.317
Inflammation	0.205	0.172	-0.048	0.750

*Asterisk represent Spearman rank (rho) correlation.

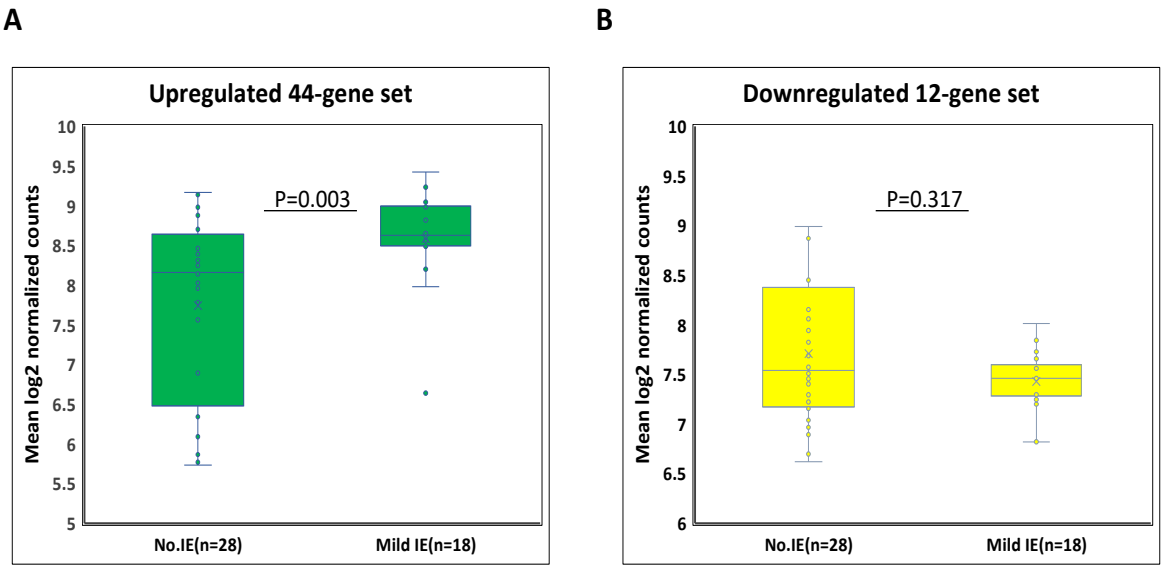

Figure 4.5. Box plot demonstrating correlation between aggregate gene sets versus interstitial edema (IE).

4.4 Discussion

The objective of this Chapter was to correlate the functional and histological cardiac changes that occur during EVHP with injury and repair gene expression patterns. As recently reported by Hatami *et al* (19), EVHP is associated with a decline in cardiac function, represented by decreased cardiac output, stroke work, systolic blood pressure, cardiac index, and changes in load-dependent measures. Previously, the study in Chapter 3 demonstrated that EVHP induces a quantifiable molecular pattern that is consistent with cardiac injury. Significantly differentially expressed genes were clustered into two sets based on the direction of gene expression after 12 h of EVHP: an upregulated 44-gene set and downregulated 12-gene set. Notably, immune response, signal transduction and tissue remodeling genes were the most upregulated genes, whereas structural genes were the most downregulated. To further assess the potential clinical significance of this molecular signature, this study aimed to correlate gene expression with cardiac functional and histological parameters. Interestingly, the molecular injury response correlated with cardiac function but not with histology, except for interstitial edema. The previously identified upregulated 44-gene set negatively correlated with favorable clinical parameters and positively correlated with adverse parameters, supporting the supposition that this represents a molecular signature for cardiac injury. In contrast, the downregulated 12-gene exhibited the opposite pattern of correlation with cardiac function, consistent with the hypothesis that increased expression of these transcripts represents a molecular marker for cardiac health and/or repair. The lack of significant differences and associations with histology in this study likely reflects the relatively lower sensitivity of this method of evaluation for acute cardiac injury compared with functional and molecular tools.

Inflammation and fibrosis represent the key pathophysiologic mechanisms occurring in a failing heart (38). Notably, cytokines are known to exert deleterious effects on the myocardium (39). *TNFα* and *IL-6* are powerful pro-inflammatory cytokines that were found to be highly

upregulated in this experimental study, and they are involved in crucial pathophysiologic roles in a failing heart. In particular, previous studies have reported *TNFα* and *IL-6* to be involved in decreasing the expression of genes involved in the regulation of Ca^{2+} release channels and sarcoplasmic reticulum Ca^{2+} ATPase (40, 41). This inflammation-mediated Ca^{2+} imbalance promotes pathological cardiac remodeling including ventricular dilation, myocardial hypertrophy and fibrosis and pump dysfunction, ultimately leading to heart failure (42). Furthermore, other studies have reported *IL-6* to cause cardiomyocyte stiffness through reduced phosphorylation of titin, a large protein that is responsible for myocardial elasticity (43).

The cardiac extracellular matrix (ECM) is a complex architectural network that provides structural and functional support to the myocardial cells in addition to regulating intercellular signal transduction (44, 45). Any alterations in the ECM could directly affect the functionality of the heart and cause cardiac dysfunction (46). Cardiac remodeling due to acute myocardial impairment as well as myocardial edema have been associated with cardiac dysfunction (47, 48). Under normal circumstances, collagen deposition represents an adaptive response for tissue integrity maintenance and preservation of left ventricular function (46, 49, 550). However, in response to injury, both profibrotic and pro-inflammatory responses act together resulting in excessive activation of myofibroblasts (50, 51). This causes progressive collagen deposition and accumulation in the ECM (54-56). In the present study, most of the genes involved in the upstream activation of the fibrotic pathway including *TGF-β, MMP12, MMP14, TIMP2, GDF15* and proinflammatory genes including *TNF-α* and *IL-6* were among the upregulated genes associated with inferior cardiac function. This suggests that there is a process of deleterious tissue remodeling during EVHP.

Myocardial edema, which refers to fluid accumulation in the myocardial interstitial space, is known to cause systolic and diastolic cardiac dysfunction as a result of developing an imbalance in filtration between the coronary microvasculature and interstitial fluid (55, 56). Notably, interstitial edema reduces left ventricular maximum pressure change and preload recruitable stroke work following isovolumic contraction and increases stiffness of the diastolic chambers thus inhibiting ventricular filling (52, 57-59). This consequently decreases cardiac output as well as cardiac efficiency (48, 56). It is tempting to speculate that the decline in myocardial function during EVHP in this study was at least partly a result of the interstitial edema that was histologically observed across EVHP T12 biopsies. Studies using coronary sinus hypertension to assess the impact of interstitial myocardial edema in preclinical models demonstrated failure of both systolic and diastolic functions during myocardial edema in the absence of other features of myocardial injury (48, 59). Although these findings are consistent with the absence of additional histological characteristics of myocardial injury in this study, upregulated 44-gene set expression did positively correlate with myocardial interstitial edema. A similar relationship was observed with weight gain, although this was not statistically significant. Previous studies that investigated serum cytokine levels in patients with cardiopulmonary bypass speculated an association between pro-inflammatory response and the development of myocardial interstitial edema during prolonged EVHP, negatively impacting post-transplant outcomes (60-62). Indeed, many of the genes that positively correlated with myocardial interstitial edema in this study are pro-inflammatory. Previous studies on the use of steroids such as methylprednisolone in OCS-EVHP to suppress the pro-inflammatory response demonstrated a significant reduction of myocardial edema (63, 64), thus supporting the role of pro-inflammatory mechanisms in myocardial interstitial edema. Although the chemokines, including *CCL2* and *CXCL9-11*, which are involved in chemotactic

trafficking of leukocytes, were found to be among the upregulated genes associated with cardiac injury in this experiment, there was no histological evidence of significant inflammatory cell infiltration in any of the sample groups.

An interesting observation in this study is the positive correlation of upregulated *HSP90AA1* with favorable functional parameters including oxygen extraction, myocardial oxygen consumption, systolic pressure, cardiac output, stroke work and maximum rate of change of left ventricular pressure. *HSP90AA1* is a molecular chaperone that codes for HSP90α proteins which confer cardio-protection against progressive accumulation of misfolded proteins (65). Mechanical and oxidative stress (the commonly encountered challenges with EVHP) reduce protein stability, consequently causing increased levels of misfolded proteins (65). These misfolded proteins are very toxic to the myocardium and can directly cause cardiac dysfunction (66). Additionally, increased HSP90 levels have been reported to protect myocardium against cell death by inducing anti-apoptotic effects via PI3K/Akt signaling pathways (67). These cardioprotective effects of *HSP90AA1* explains its positive association with favorable markers of cardiac function in this experiment. Heat shock proteins have also been shown to inhibit Jun N-terminal Kinase (JNK) signaling pathway by binding to c-Jun N-terminal kinase enzyme (68) and this could be associated with the downregulation of JUN observed in this experiment by means of a negative feedback mechanism.

The non-significant mild inflammation observed across the study groups could represent a response to inevitable surgical trauma incurred during organ retrieval, and not EVHP-induced. Notably, previous studies have reported that systemic leukocytic changes such as neutrophilia, leukocytosis and lymphocytosis may occur in response to surgery by different cytokines and acute phase adjuvants (69, 70). Generation of immune mediators is well documented to be correlated

with the proportion of surgical trauma, and that anaesthesia as well as anaesthetic techniques influence this type of immune response (71-76). Total intravenous anaesthesia has been previously associated with lower serum levels of immune mediators when compared with inhalation anaesthesia (77). These previous studies indeed explain the observation in this experiment whereby anaesthesia was initiated with intravenous injection of ketamine and atropine but maintained with isoflurane inhalation.

4.5 Conclusion

Altogether, it is tempting to speculate that the previously observed decline in cardiac function during EVHP is at least partly due to a pro-inflammatory response that induces cardiac failure by either 1) inducing progressive myocardial interstitial edema, or 2) causing alteration in sarcoplasmic Ca^{2+} homeostasis, which in turn results in molecular pathways of early myocardial fibrosis and cardiac muscle stiffness. This study also confirmed the reliability of gene expression for measuring cardiac injury in relation to multiple established markers of cardiac function. However, in contrast to nonspecific functional parameters, gene expression may provide the opportunity for more precise, mechanism-based insights into the cardiac response to tissue injury. Furthermore, the ability of gene expression to identify differences in injury response between all three sample groups (IV, H+L and EVHP), which was not possible with histology or function, suggests the possibility of gene expression as a more sensitive measure of cardiac injury.

4.6 References

1. White CW, Ambrose E, Muller A, Li Y, Le H, Hiebert B, et al. Assessment of donor heart viability during ex vivo heart perfusion. Can J Physiol Pharmacol. 2015;93(10):893-901.

2. Tevaearai Stahel HT, Zuckermann A, Carrel TP, Longnus SL. Hearts Not Dead after Circulatory Death. Front Surg. 2015;2:46.

3. Kappler B, Ledezma CA, van Tuijl S, Meijborg V, Boukens BJ, Ergin B, et al. Investigating the physiology of normothermic ex vivo heart perfusion in an isolated slaughterhouse porcine model used for device testing and training. BMC Cardiovasc Dis. 2019;19(1):254.

4. Emet M, Akoz A, Aslan S, Saritas A, Cakir Z, Acemoglu H. Assessment of cardiac injury in patients with blunt chest trauma. Eur J Trauma Emerg Surg. 2010;36(5):441-7.

5. Church JT, Alghanem F, Deatrick KB, Trahanas JM, Phillips JP, Hee Song M, et al. Normothermic Ex Vivo Heart Perfusion: Effects of Live Animal Blood and Plasma Cross Circulation. ASAIO J. 2017;63(6):766-73.

6. Kearns MJ, Miller SD, Cheung A, Bashir J, Wong S, Seidman MA, et al. A Rodent Model of Cardiac Donation After Circulatory Death and Novel Biomarkers of Cardiac Viability During Ex Vivo Heart Perfusion. Transplantation. 2017;101(8):e231-e9.

7. Vargas SO, Sampson BA, Schoen FJ. Pathologic detection of early myocardial infarction: a critical review of the evolution and usefulness of modern techniques. Mod Pathol. 1999;12(6):635-45.

8. Hickman PE, Potter JM, Aroney C, Koerbin G, Southcott E, Wu AH, et al. Cardiac troponin may be released by ischemia alone, without necrosis. Int J Clin Chem. 2010;411(5-6):318-23.

9. Masri W, Le Guillou E, Hamdi E, Ghazal K, Lebigot E, Cosson C, et al. Troponin elevation in other conditions than acute coronary syndromes. Ann Biol Clin. 2017;75(4):411-9.

10. Inbar R, Shoenfeld Y. Elevated cardiac troponins: the ultimate marker for myocardial necrosis, but not without a differential diagnosis. Isr Med Assoc J. 2009;11(1):50-3.

11. Millar JE, Fanning JP, McDonald CI, McAuley DF, Fraser JF. The inflammatory response to extracorporeal membrane oxygenation (ECMO): a review of the pathophysiology. Crit Care. 2016;20(1):387.

12. Atas E, Kismet E, Kesik V, Karaoglu B, Aydemir G, Korkmazer N, et al. Cardiac troponin-I, brain natriuretic peptide and endothelin-1 levels in a rat model of doxorubicin-induced cardiac injury. J Cancer Res Therapeut. 2015;11(4):882-6.

13. Tsutamoto T, Wada A, Maeda K, Hisanaga T, Maeda Y, Fukai D, et al. Attenuation of compensation of endogenous cardiac natriuretic peptide system in chronic heart failure: prognostic role of plasma brain natriuretic peptide concentration in patients with chronic symptomatic left ventricular dysfunction. Circulation. 1997;96(2):509-16.

14. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol. 1992;119(3):493-501.

15. Kyrylkova K, Kyryachenko S, Leid M, Kioussi C. Detection of apoptosis by TUNEL assay. Methods Mol Biol. 2012;887:41-7.

16. Bortner CD, Oldenburg NB, Cidlowski JA. The role of DNA fragmentation in apoptosis. Trends Cell Biol. 1995;5(1):21-6.

17. Negoescu A, Lorimier P, Labat-Moleur F, Drouet C, Robert C, Guillermet C, et al. In situ apoptotic cell labeling by the TUNEL method: improvement and evaluation on cell preparations. J Histochem Cytochem. 1996;44(9):959-68.

18. Grasl-Kraupp B, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R. In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatology. 1995;21(5):1465-8.

19. Hatami S, White CW, Shan S, Haromy A, Qi X, Ondrus M, et al. Myocardial Functional Decline During Prolonged Ex Situ Heart Perfusion. Ann Thorac Surg. 2019;108(2):499-507.

20. Suehiro K, Mohri M, Yamaguchi H, Takagaki M, Hisamochi K, Morimoto T, et al. Posttransplant function of a nonbeating heart is predictable by an ex vivo perfusion method. Ann Thorac Surg. 2001;71(1):278-83.

21. Couto-Mallon D, Gonzalez-Vilchez F, Almenar-Bonet L, Diaz-Molina B, Segovia-Cubero J, Gonzalez-Costello J, et al. Prognostic Value of Serum Lactate Levels in Patients Undergoing Urgent Heart Transplant: A Subanalysis of the ASIS-TC Spanish Multicenter Study. Rev Esp Cardiol. 2019;72(3):208-14.

22. White CW, Messer SJ, Large SR, Conway J, Kim DH, Kutsogiannis DJ, et al. Transplantation of Hearts Donated after Circulatory Death. Front Cardiovasc Med. 2018;5:8.

23. Messer SJ, Axell RG, Colah S, White PA, Ryan M, Page AA, et al. Functional assessment and transplantation of the donor heart after circulatory death. J Heart Lung Transplant. 2016;35(12):1443-52.

24. Messer S, Ardehali A, Tsui S. Normothermic donor heart perfusion: current clinical experience and the future. Transpl Int. 2015;28(6):634-42.

25. Hatami S, White CW, Ondrus M, Qi X, Buchko M, Himmat S, et al. Normothermic Ex Situ Heart Perfusion in Working Mode: Assessment of Cardiac Function and Metabolism. JoVE-J Vis Exp. 2019(143).

26. Karbassi E, Monte E, Chapski DJ, Lopez R, Rosa Garrido M, Kim J, et al. Relationship of disease-associated gene expression to cardiac phenotype is buffered by genetic diversity and chromatin regulation. Physiol Genomics. 2016;48(8):601-15.

27. Barth AS, Kuner R, Buness A, Ruschhaupt M, Merk S, Zwermann L, et al. Identification of a common gene expression signature in dilated cardiomyopathy across independent microarray studies. J Am Coll Cardiol. 2006;48(8):1610-7.

28. Wittchen F, Suckau L, Witt H, Skurk C, Lassner D, Fechner H, et al. Genomic expression profiling of human inflammatory cardiomyopathy (DCMi) suggests novel therapeutic targets. J Mol Med. 2007;85(3):257-71.

29. Lamesch P, Berardini TZ, Li D, Swarbreck D, Wilks C, Sasidharan R, et al. The Arabidopsis Information Resource (TAIR): improved gene annotation and new tools. Nucleic Acids Res. 2012;40(Database issue):D1202-10.

30. Uygun S, Peng C, Lehti-Shiu MD, Last RL, Shiu SH. Utility and Limitations of Using Gene Expression Data to Identify Functional Associations. PLoS Comput Biol. 2016;12(12):e1005244.

31. Rosa BA, Jasmer DP, Mitreva M. Genome-wide tissue-specific gene expression, co-expression and regulation of co-expressed genes in adult nematode Ascaris suum. PLoS Neglect Trop D. 2014;8(2):e2678.

32. Qiao A, Zhao Z, Zhang H, Sun Z, Cui X. Gene expression profiling reveals genes and transcription factors associated with dilated and ischemic cardiomyopathies. Pathol Res Pract. 2017;213(5):548-57.

33. Sweet ME, Cocciolo A, Slavov D, Jones KL, Sweet JR, Graw SL, et al. Transcriptome analysis of human heart failure reveals dysregulated cell adhesion in dilated cardiomyopathy and activated immune pathways in ischemic heart failure. BMC Genomics. 2018;19(1):812.

34. Witt E, Hammer E, Dorr M, Weitmann K, Beug D, Lehnert K, et al. Correlation of gene expression and clinical parameters identifies a set of genes reflecting LV systolic dysfunction and morphological alterations. Physiol Genomics. 2019;51(8):356-67.

35. Ameling S, Herda LR, Hammer E, Steil L, Teumer A, Trimpert C, et al. Myocardial gene expression profiles and cardiodepressant autoantibodies predict response of patients with dilated cardiomyopathy to immunoadsorption therapy. Eur Heart J. 2013;34(9):666-75.

36. Chen Y, Park S, Li Y, Missov E, Hou M, Han X, et al. Alterations of gene expression in failing myocardium following left ventricular assist device support. Physiol Genomics. 2003;14(3):251-60.

37. Dromparis P, Aboelnazar NS, Wagner S, Himmat S, White CW, Hatami S, et al. Ex vivo perfusion induces a time- and perfusate-dependent molecular repair response in explanted porcine lungs. Am J Transplant. 2019;19(4):1024-36.

38. Suthahar N, Meijers WC, Sillje HHW, de Boer RA. From Inflammation to Fibrosis-Molecular and Cellular Mechanisms of Myocardial Tissue Remodelling and Perspectives on Differential Treatment Opportunities. Curr Heart Fail Rep. 2017;14(4):235-50.

39. Van Linthout S, Tschope C. Inflammation - Cause or Consequence of Heart Failure or Both? Curr Heart Fail Rep. 2017;14(4):251-65.

40. Wu CK, Lee JK, Chiang FT, Yang CH, Huang SW, Hwang JJ, et al. Plasma levels of tumor necrosis factor-alpha and interleukin-6 are associated with diastolic heart failure through downregulation of sarcoplasmic reticulum Ca2+ ATPase. Crit Care Med. 2011;39(5):984-92.

41. Thaik CM, Calderone A, Takahashi N, Colucci WS. Interleukin-1 beta modulates the growth and phenotype of neonatal rat cardiac myocytes. J Clin Invest. 1995;96(2):1093-9.

42. Sedej S, Schmidt A, Denegri M, Walther S, Matovina M, Arnstein G, et al. Subclinical abnormalities in sarcoplasmic reticulum Ca(2+) release promote eccentric myocardial remodeling and pump failure death in response to pressure overload. J Am Coll Cardiol. 2014;63(15):1569-79.

43. Savvatis K, Muller I, Frohlich M, Pappritz K, Zietsch C, Hamdani N, et al. Interleukin-6 receptor inhibition modulates the immune reaction and restores titin phosphorylation in experimental myocarditis. Basic Res Cardiol. 2014;109(6):449.

44. Freedman BR, Bade ND, Riggin CN, Zhang S, Haines PG, Ong KL, et al. The (dys)functional extracellular matrix. Biochim Biophys Acta. 2015;1853(11 Pt B):3153-64.

45. Miner EC, Miller WL. A look between the cardiomyocytes: the extracellular matrix in heart failure. Mayo Clin Proc. 2006;81(1):71-6.

46. Weber KT, Sun Y, Tyagi SC, Cleutjens JP. Collagen network of the myocardium: function, structural remodeling and regulatory mechanisms. J Mol Cell Cardiol. 1994;26(3):279-92.

47. Mehlhorn U, Geissler HJ, Laine GA, Allen SJ. Myocardial fluid balance. Eur J Cardio-thorac Surg. 2001;20(6):1220-30.

48. Laine GA, Allen SJ. Left ventricular myocardial edema. Lymph flow, interstitial fibrosis, and cardiac function. Circ Res. 1991;68(6):1713-21.

49. Li YY, McTiernan CF, Feldman AM. Proinflammatory cytokines regulate tissue inhibitors of metalloproteinases and disintegrin metalloproteinase in cardiac cells. Cardiovasc Res. 1999;42(1):162-72.

50. Ma L, Song H, Zhang M, Zhang D. Lysyl oxidase G473A polymorphism is associated with increased risk of coronary artery diseases. DNA Cell Biol. 2011;30(12):1033-7.

51. Sivakumar P, Gupta S, Sarkar S, Sen S. Upregulation of lysyl oxidase and MMPs during cardiac remodeling in human dilated cardiomyopathy. Mol Cell Biochem. 2008;307(1-2):159-67.

52. Desai KV, Laine GA, Stewart RH, Cox CS, Jr., Quick CM, Allen SJ, et al. Mechanics of the left ventricular myocardial interstitium: effects of acute and chronic myocardial edema. Am J Physiol Heart C. 2008;294(6):H2428-34.

53. Heymans S, Schroen B, Vermeersch P, Milting H, Gao F, Kassner A, et al. Increased cardiac expression of tissue inhibitor of metalloproteinase-1 and tissue inhibitor of metalloproteinase-2 is related to cardiac fibrosis and dysfunction in the chronic pressure-overloaded human heart. Circulation. 2005;112(8):1136-44.

54. Nong Z, O'Neil C, Lei M, Gros R, Watson A, Rizkalla A, et al. Type I collagen cleavage is essential for effective fibrotic repair after myocardial infarction. Am J Pathol. 2011;179(5):2189-98.

55. Dongaonkar RM, Stewart RH, Quick CM, Uray KL, Cox CS, Jr., Laine GA. Award article: Microcirculatory Society Award for Excellence in Lymphatic Research: time course of myocardial interstitial edema resolution and associated left ventricular dysfunction. Microcirculation. 2012;19(8):714-22.

56. Dongaonkar RM, Stewart RH, Geissler HJ, Laine GA. Myocardial microvascular permeability, interstitial oedema, and compromised cardiac function. Cardiovasc Res. 2010;87(2):331-9.

57. Davis KL, Mehlhorn U, Laine GA, Allen SJ. Myocardial edema, left ventricular function, and pulmonary hypertension. J Appl Physiol. 1995;78(1):132-7.

58. Pratt JW, Schertel ER, Schaefer SL, Esham KE, McClure DE, Heck CF, et al. Acute transient coronary sinus hypertension impairs left ventricular function and induces myocardial edema. Am J Physiol. 1996;271(3 Pt 2):H834-41.

59. Charan NB, Ripley R, Carvalho P. Effect of increased coronary venous pressure on left ventricular function in sheep. Resp Physiol. 1998;112(2):227-35.

60. Stamp NL, Shah A, Vincent V, Wright B, Wood C, Pavey W, et al. Successful Heart Transplant after Ten Hours Out-of-body Time using the TransMedics Organ Care System. Heart Lung Circ. 2015;24(6):611-3.

61. Nesher N, Frolkis I, Vardi M, Sheinberg N, Bakir I, Caselman F, et al. Higher levels of serum cytokines and myocardial tissue markers during on-pump versus off-pump coronary artery bypass surgery. J Card Surg. 2006;21(4):395-402.

62. Ascione R, Lloyd CT, Underwood MJ, Lotto AA, Pitsis AA, Angelini GD. Inflammatory response after coronary revascularization with or without cardiopulmonary bypass. Ann Thorac Surg. 2000;69(4):1198-204.

63. Sandha JK, White CW, Muller A, Avery E, Thliveris J, Dixon IMC, et al. Steroids Limit Myocardial Edema During Ex Vivo Perfusion of Hearts Donated After Circulatory Death. Ann Thorac Surg. 2018;105(6):1763-70.

64. Ardehali A, Esmailian F, Deng M, Soltesz E, Hsich E, Naka Y, et al. Ex-vivo perfusion of donor hearts for human heart transplantation (PROCEED II): a prospective, open-label, multicentre, randomised non-inferiority trial. Lancet. 2015;385(9987):2577-84.

65. Ranek MJ, Stachowski MJ, Kirk JA, Willis MS. The role of heat shock proteins and co-chaperones in heart failure. Philos Trans R Soc Lond B Biol Sci. 2018;373(1738).

66. Lindquist S, Craig EA. The heat-shock proteins. Annu Rev Genet. 1988;22:631-77.

67. Downward J. How BAD phosphorylation is good for survival. Nat Cell Biol. 1999;1(2):E33-5.

68. Lanneau D, Brunet M, Frisan E, Solary E, Fontenay M, Garrido C. Heat shock proteins: essential proteins for apoptosis regulation. J Cell Mol Med. 2008;12(3):743-61.

69. Iwase M, Kondo G, Watanabe H, Takaoka S, Uchida M, Ohashi M, et al. Regulation of Fas-mediated apoptosis in neutrophils after surgery-induced acute inflammation. J Surg Res. 2006;134(1):114-23.

70. Ogawa K, Hirai M, Katsube T, Murayama M, Hamaguchi K, Shimakawa T, et al. Suppression of cellular immunity by surgical stress. Surgery. 2000;127(3):329-36.

71. Helmy SA, Wahby MA, El-Nawaway M. The effect of anaesthesia and surgery on plasma cytokine production. Anaesthesia. 1999;54(8):733-8.

72. Cruickshank AM, Fraser WD, Burns HJ, Van Damme J, Shenkin A. Response of serum interleukin-6 in patients undergoing elective surgery of varying severity. Clin Sci. 1990;79(2):161-5.

73. Reith HB, Kaman S, Mittelkotter O, Kilic Y, Kozuschek W. Cytokine activation in patients undergoing open or laparoscopic cholecystectomy. Int Surg. 1997;82(4):389-93.

74. Crozier TA, Muller JE, Quittkat D, Sydow M, Wuttke W, Kettler D. Effect of anaesthesia on the cytokine responses to abdominal surgery. Brit J Anaesth. 1994;72(3):280-5.

75. Inada T, Yamanouchi Y, Jomura S, Sakamoto S, Takahashi M, Kambara T, et al. Effect of propofol and isoflurane anaesthesia on the immune response to surgery. Anaesthesia. 2004;59(10):954-9.

76. Schneemilch CE, Ittenson A, Ansorge S, Hachenberg T, Bank U. Effect of 2 anesthetic techniques on the postoperative proinflammatory and anti-inflammatory cytokine response and cellular immune function to minor surgery. J Clin Anesth. 2005;17(7):517-27.

77. Ke JJ, Zhan J, Feng XB, Wu Y, Rao Y, Wang YL. A comparison of the effect of total intravenous anaesthesia with propofol and remifentanil and inhalational anaesthesia with isoflurane on the release of pro- and anti-inflammatory cytokines in patients undergoing open cholecystectomy. Anaesth Intensive Care. 2008;36(1):74-8.

Chapter 5

General Discussion and Conclusion

5.1 Review of thesis objectives and summary of results

Since the first successful heart transplant more than 50 years ago, this procedure has become widely accepted as a life-saving treatment for patients with end-stage heart failure, and many advances have been made to meet the increasing demand for transplantation (1). Some of these advances include the use of extended donor criteria (2), donation after circulatory death (DCD) hearts (3), and machine perfusion, all of which contribute to the aim of increasing the suitable donor organ pool. Other strategies such as ischemic post-conditioning and normothermic regional perfusion have also been utilized to reduce the deleterious effects of ischemia-reperfusion injury (Chapter 1). Despite these efforts, however, the full potential benefit of this therapeutic intervention is yet to be realized, partly due to the inability to reliably evaluate the quality of donor organs prior to transplantation. *Ex vivo* heart perfusion (EVHP), which holds significant theoretical potential for increasing the donor organ pool, has recently been reported to be associated with progressive functional decline during prolonged perfusion (4). Persistent challenges associated with EVHP include the lack of clearance of metabolic waste products and cellular debris, cardiac edema, and cardiomyocyte loss (1). Many studies have demonstrated benchmarks for the assessment of cardiac function and prediction of post-transplant outcomes; however, there are no widely agreed-upon parameters for organ viability assessment. A sensitive and specific biomarker is thus needed to help confirm the viability of hearts for transplant and ultimately improve quality of life and survival for patients with end-stage heart failure.

The overall objective of the work presented herein was to advance the field of heart transplantation by assessing the feasibility of using gene expression to measure and understand the mechanisms of cardiac tissue injury and repair during EVHP. The first aim was to develop a novel literature-based, cardiac-specific gene signature for measuring tissue injury and repair. Investigation of the mRNA expression levels of the identified genes was then performed in hearts

from a large animal model subjected to 12 h of EVHP. To evaluate the clinical significance of this molecular signature as well as the potential mechanisms of cardiac tissue injury and repair during EVHP, these gene expression results were then correlated with cardiac function and histology.

Tools for large-scale "omics" discovery are now widely available, however, the opportunities to translate them into routine clinical practice in cardiac transplantation are limited (5). The availability of the NanoString nCounter platform, which allows for multiplexed analysis of gene signatures in suboptimal samples (6), made the design of this experiment possible. Its utility in formalin-fixed paraffin-embedded (FFPE) biopsies facilitated direct histological correlation with gene expression levels in the same tissue samples. Although the turnaround time for this platform is insufficient for the very short timeframe required for measuring organ viability during clinical EVHP, it represents an ideal research tool for identifying molecular markers for future validation on other platforms with faster turnaround-times, such as real-time quantitative PCR.

Chapter 2 of this thesis focused on the development of a gene signature for the above described purpose. Seven sets of genes (a total of 68 genes altogether) were identified, including inflammatory, tissue remodeling, signal transduction, oxidative stress, apoptotic, cell structure, and cell proliferation functional annotations. These sets of genes represent the potential patterns of molecular response following acute myocardial injury. Adult mammalian hearts lack regenerative capacity and therefore healing of the damaged myocardium is facilitated by a sequence of three cellular process that result in the development of fibrous scar tissue (7). These cellular phases include: i) inflammatory, ii) proliferative and iii) maturation phases (8). The genes identified in this study represent the spectrum of these reparative cellular changes. Following acute tissue injury, dying cardiomyocytes and damaged extracellular matrix secrete endogenous signals

referred to as Danger Associated Molecular Patterns (DAMPs) which activate cardiac innate immunity. The source of cardiac injury during EVHP has largely been associated with peritransplant ischemia reperfusion injury. Given that the mechanisms of this injury are poorly understood, and that biomarkers previously described in the literature are based predominantly on case-control experiments, this study included a larger set of candidate genes. Acute myocardial injury triggers innate immune signaling pathways which further induce a rapid but transient inflammatory response, a reaction that is aimed at clearing dead cardiomyocytes and extracellular debris (9). Notably, the TLR and IL-1 signaling pathways were identified as vital components of this pro-inflammatory reaction. Important genes involved in these pathways include cytokines such as *TNFα* and *IL6* as well as several chemokines including *CCL2* and *CXCL9-11*. These pro-inflammatory genes have also been reported to act as pro-fibrotic effectors that activate TGFβ-1, which further activates myofibroblasts via the Smad2/3 pathway.

The identified set of genes was then analyzed in Chapter 3 with the aim of assessing gene expression changes in a porcine model for EVHP. The present study provides molecular evidence of cardiac injury during machine perfusion. Gene expression analysis demonstrated the ability to molecularly discriminate all study groups, including in vivo, EVHP, and combined heart and liver perfusion, as well as different EVHP strategies including working/ non-working, and hanging/ laying modes. Although the measured functional parameters demonstrated a significant time dependent decline, they failed to demonstrate differences between study groups. Similarly, other than interstitial edema, histological assessment could not discriminate sample groups. Previous studies have highlighted the limitations associated with histologic assessment of tissue injury, including i) subjective classification of injury with limited diagnostic specificity and reproducibility, and ii) limited ability to represent pathologic mechanisms of function and injury

(10). Molecular diagnostics are potentially more sensitive for detecting lower amounts of injury and may provide an opportunity to measure acute cardiac injury earlier than functional or histological parameters. The gene sets identified in this study provide a novel molecular approach that can potentially be used for rapid diagnosis and accurate identification of injury or repair in EVHP, thus presenting an opportunity for optimal organ management during preservation.

To understand the significance and mechanisms of molecular injury and repair during EVHP, Chapter 4 aimed to correlate gene expression with histology and function. As previously reported by Hatami *et al*, the hearts included in this study demonstrated a progressive decline in cardiac function during 12 h of EVHP (4). In the present study, correlational analysis demonstrated a distinct pattern of association between gene expression and functional parameters. Genes that were significantly upregulated with EVHP positively correlated with unfavorable functional parameters and negatively correlated with favorable functional parameters. In contrast, most of the downregulated genes positively correlated with favorable functional parameters and negatively correlated with unfavorable parameters. Correlation with histology did not show any significance except for the upregulated genes positively correlating with mild interstitial edema. Given that most of the upregulated genes were inflammatory response transcripts, these findings suggest that inflammation pathways may be the main molecular driver of cardiac functional deterioration during EVHP. It is noteworthy that cardiac injury in this experiment appears to be predominantly mediated by pro-inflammatory genes that further induce injury through fibrosis, edema and calcium homeostasis disruption, and through downregulation of structural genes.

Interestingly, EVHP also seemingly demonstrated a molecular signal of repair through the downregulation of *LOX, COL1A1, CCR2* and upregulation of *HSP90AA1*. Downregulation of *LOX*, a key gene for lysyl oxidase enzyme, which facilitates collagen crosslinking, suggests the

generation of collagen that is more susceptible to degradation by matrix metalloproteinase and, hence, the possibility of overall reduced collagen deposition. Additional downregulation of *COL1A1* suggests reduction of type 1 collagen, which is the most abundant type of collagen in the heart and has previously been associated with cardiac dysfunction through loss of extracellular matrix integrity. Taken together, the upregulation of MMP12 and MMP14 and downregulation of *LOX* and *COLA1A1* seem to work synergistically to result in a cumulative loss of extracellular matrix integrity. However, upregulation of *TIMP1*, which is associated with inhibition of MMP degradation of the ECM as well as collagen synthesis promotion via the Smad2/3 pathways, warrants further research. On the other hand, *HSP90AA1* upregulation demonstrated an opposite pattern of association with function when compared with other upregulated genes. This gene has been shown to confer protection to the myocardium through the PI3K/Akt signaling pathway.

The nonsignificant difference between hearts that received dialysis and those that did not receive dialysis suggests that metabolite accumulation is not the main driver of cardiac dysfunction during EVHP. However, improved function in hearts perfused in combination with the liver is hypothesized to be related to an undetermined difference in metabolic properties. Of note, although the differences were not statistically significant, there was a trend toward improved function in hearts subjected to laying working modes, which warrants further research.

5.2 Contributions to cardiac transplantation and future directions

The contribution of my thesis to cardiac transplantation includes the following:

1) Providing comprehensive molecular biomarkers that could potentially be used to assess organ viability during EVHP (Chapter 2, Chapter 3 and Chapter 4)

2) Providing an understanding of the underlying mechanisms of cardiac injury and repair during EVHP (Chapter 3 and Chapter 4).

3) Elucidating molecular pathways that could be used as candidate targets for the development of therapeutics to improve cardiac function during EVHP (Chapter 2, Chapter 3 and Chapter 4).

To illustrate the first contribution, it is important to highlight that currently there are no widely agreed upon biomarkers for monitoring organ quality during EVHP. This thesis demonstrated that the available functional parameters and histologic morphologies used in other studies to characterize cardiac health had inferior sensitivity and specificity compared with gene expression. My thesis produced a novel gene signature comprised of 56 cardiac injury and repair genes that could potentially be used as a clinical molecular diagnostic assay for assessing organ viability during preservation in machine perfusion. This is important because it could help transplant surgeons make better informed decisions and more accurately predict post-transplant outcomes through identification of suitable donor hearts. The use of molecular biomarkers to monitor and characterize tissue injury is gaining attention in transplantation pathology (11). Significant progress has been made, including the adoption of endothelial gene expression for measuring acute antibody mediated rejection in renal allografts (12) and monitoring acute lung injury during *ex vivo* lung perfusion (13).

Secondly, through correlational analysis of gene expression with cardiac function and histological morphologies, my thesis has provided a preliminary understanding of the mechanisms of injury and repair during EVHP. The identification of pro-inflammatory genes as the upstream effectors of interstitial edema, tissue remodeling, and calcium homeostatic imbalance has advanced our understanding of the mechanistic etiology of the reported functional decline during EVHP. This knowledge could provide guidance for the development of new strategies to optimize EVHP, ultimately increasing the donor organ pool.

Future directions in this area should focus on validation of the 56-gene signature for cardiac injury and repair in human organs using the experimental approach provided by this thesis. These findings should be further correlated with post-transplant outcomes. This should then be followed by translation of the gene signature to a more clinically appropriate point-of-care testing platform with faster turnaround time such as real-time quantitative PCR for final validation in human clinical trials. Such turnaround time should be sufficient to identify a viable organ within the timeframe required for successful cardiac transplantation.

For therapeutic interventions, proinflammatory and pro-fibrotic pathways should be targeted as they represent upstream effectors of cardiac injury. Available evidence suggests that the use of anti-IL-1 agents may be beneficial in patients exhibiting dilation and systolic dysfunction cause by overactive and prolonged post-infarction inflammatory reaction (14). IL-1 has been previously reported to be the master cytokine of both local and systemic inflammatory response (15). The identification of IL-1 signaling as the upstream effector for most of the pro-inflammatory genes as well as the chemokine-chemokine signaling pathway in this thesis represent targets for potential therapeutic development to mitigate pro-inflammatory-mediated cardiac injury. Targeting the Smad2/3 signaling pathway as well as TIMP1 should also be an area of focus to reduce tissue remodeling-mediated cardiac injury. Notably, further research is warranted to measure collagen levels to better understand the extent of injury within the cardiac extracellular matrix and evaluate whether this results from increased collagen degradation or deposition. Existing evidence suggests that inhibition of the Smad3 pathway may help patients with progressive cardiac fibrosis, thus preventing the development of heart failure (9, 14). Other signaling pathways that require further research include AP-1, JNK-MAPK and PI3K/Akt as well as the pathways associated with the three cardiac injury-repair associated molecular MCODEs

identified in this thesis. Future research should also focus on the development of drugs that promote structural genes. Such studies may benefit from the inclusion of genetically modified pre-clinical animal models, such as mouse models with similar genomes to the human genome. For example, knock-out mice may be used to better understand the roles of specific pro-inflammatory and profibrotic genes/signaling pathways in cardiac injury during EVHP. Similarly, knock-in mice could be utilized to understand the role of each of the structural genes in promoting cardiac function.

Altogether, I hope this work will help address a critical need in the field of cardiac transplantation by facilitating the optimization and clinical implementation of EVHP so that more suitable donor organs can be used for transplantation, thus reducing waitlists and enhancing survival and quality of life for end-stage heart disease patients around the world.

5.3 References

1. Pinezich M, Vunjak-Novakovic G. Bioengineering approaches to organ preservation ex vivo. Exp Biol Med. 2019;244(8):630-45.

2. Wang Y, Cai J, Sun Y, Zhang J, Xie F, Alshirbini MH, et al. Extended donor criteria in heart transplantation: a retrospective study from a single Chinese institution. J Thorac Dis. 2018;10(4):2153-65.

3. Dhital KK, Chew HC, Macdonald PS. Donation after circulatory death heart transplantation. Curr Opin Organ Transplant. 2017;22(3):189-97.

4. Hatami S, White CW, Shan S, Haromy A, Qi X, Ondrus M, et al. Myocardial Functional Decline During Prolonged Ex Situ Heart Perfusion. Ann Thorac Surg. 2019;108(2):499-507.

5. Adam B, Afzali B, Dominy KM, Chapman E, Gill R, Hidalgo LG, et al. Multiplexed color-coded probe-based gene expression assessment for clinical molecular diagnostics in formalin-fixed paraffin-embedded human renal allograft tissue. Clin Transplant. 2016;30(3):295-305.

6. Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N, Dunaway DL, et al. Direct multiplexed measurement of gene expression with color-coded probe pairs. Nat Biotechnol. 2008;26(3):317-25.

7. Reinhardt D, Sigusch HH, Hensse J, Tyagi SC, Korfer R, Figulla HR. Cardiac remodelling in end stage heart failure: upregulation of matrix metalloproteinase (MMP) irrespective of the underlying disease, and evidence for a direct inhibitory effect of ACE inhibitors on MMP. Heart. 2002;88(5):525-30.

8. Ong SB, Hernandez-Resendiz S, Crespo-Avilan GE, Mukhametshina RT, Kwek XY, Cabrera-Fuentes HA, et al. Inflammation following acute myocardial infarction: Multiple players, dynamic roles, and novel therapeutic opportunities. Pharmacol Therapeut. 2018;186:73-87.

9. Frangogiannis NG. Inflammation in cardiac injury, repair and regeneration. Curr Opin Cardiol. 2015;30(3):240-5.

10. Broecker V, Mengel M. The significance of histological diagnosis in renal allograft biopsies in 2014. Transpl Int. 2015;28(2):136-43.

11. Adam B, Mengel M. Molecular nephropathology: ready for prime time? Am J Physiol Renal Physiol. 2015;309(3):F185-8.

12. Haas M, Sis B, Racusen LC, Solez K, Glotz D, Colvin RB, et al. Banff 2013 meeting report: inclusion of c4d-negative antibody-mediated rejection and antibody-associated arterial lesions. Am J Transplant. 2014;14(2):272-83.

13. Dromparis P, Aboelnazar NS, Wagner S, Himmat S, White CW, Hatami S, et al. Ex vivo perfusion induces a time- and perfusate-dependent molecular repair response in explanted porcine lungs. Am J Transplant. 2019;19(4):1024-36.

14. Frangogiannis NG. The inflammatory response in myocardial injury, repair, and remodelling. Nat Rev Cardiol. 2014;11(5):255-65.

15. Dinarello CA, Simon A, van der Meer JW. Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. Nat Rev Drug Discov. 2012;11(8):633-52.

Bibliography

Adam B, Afzali B, Dominy KM, Chapman E, Gill R, Hidalgo LG, et al. Multiplexed color-coded probe-based gene expression assessment for clinical molecular diagnostics in formalin-fixed paraffin-embedded human renal allograft tissue. Clin Transplant. 2016;30(3):295-305.

Adam B, Mengel M. Molecular nephropathology: ready for prime time? Am J Physiol Renal Physiol. 2015;309(3):F185-8.

Adam BA, Smith RN, Rosales IA, Matsunami M, Afzali B, Oura T, et al. Chronic Antibody-Mediated Rejection in Nonhuman Primate Renal Allografts: Validation of Human Histological and Molecular Phenotypes. Am J Transplant. 2017;17(11):2841-50.

Aguilar A, Alvarez-Vijande R, Capdevila S, Alcoberro J, Alcaraz A. Antioxidant patterns (superoxide dismutase, glutathione reductase, and glutathione peroxidase) in kidneys from non-heart-beating-donors: experimental study. Transplant Proceedings. 2007;39(1):249-52.

Ahmed MS, Gravning J, Martinov VN, von Lueder TG, Edvardsen T, Czibik G, et al. Mechanisms of novel cardioprotective functions of CCN2/CTGF in myocardial ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol. 2011;300(4):H1291-302.

Ali A, White P, Dhital K, Ryan M, Tsui S, Large S. Cardiac recovery in a human non-heart-beating donor after extracorporeal perfusion: source for human heart donation? J Heart lung Transplant. 2009;28(3):290-3.

Amberger A, Schneeberger S, Hernegger G, Brandacher G, Obrist P, Lackner P, et al. Gene expression profiling of prolonged cold ischemia and reperfusion in murine heart transplants. Transplantation. 2002;74(10):1441-9.

Ameling S, Herda LR, Hammer E, Steil L, Teumer A, Trimpert C, et al. Myocardial gene expression profiles and cardiodepressant autoantibodies predict response of patients with dilated cardiomyopathy to immunoadsorption therapy. European Heart J. 2013;34(9):666-75.

Angeli E, Lueck S, Gargiulo GD. Different strategies of myocardial protection: the age of perfectionism. J Thorac Dis. 2018;10(3):1211-3.

Arab S, Konstantinov IE, Boscarino C, Cukerman E, Mori A, Li J, et al. Early gene expression profiles during intraoperative myocardial ischemia-reperfusion in cardiac surgery. J Thorac Cardiovasc Surg. 2007;134(1):74-81, .e1-2.

Ardehali A, Esmailian F, Deng M, Soltesz E, Hsich E, Naka Y, et al. Ex-vivo perfusion of donor hearts for human heart transplantation (PROCEED II): a prospective, open-label, multicentre, randomised non-inferiority trial. Lancet. 2015;385(9987):2577-84.

Ascione R, Lloyd CT, Underwood MJ, Lotto AA, Pitsis AA, Angelini GD. Inflammatory response after coronary revascularization with or without cardiopulmonary bypass. Ann Thorac Surg. 2000;69(4):1198-204.

Ashton KJ, Tupicoff A, Williams-Pritchard G, Kiessling CJ, See Hoe LE, Headrick JP, et al. Unique transcriptional profile of sustained ligand-activated preconditioning in pre- and post-ischemic myocardium. PloS one. 2013;8(8):e72278.

Atas E, Kismet E, Kesik V, Karaoglu B, Aydemir G, Korkmazer N, et al. Cardiac troponin-I, brain natriuretic peptide and endothelin-1 levels in a rat model of doxorubicin-induced cardiac injury. J Cancer Res Ther. 2015;11(4):882-6.

Ayach BB, Yoshimitsu M, Dawood F, Sun M, Arab S, Chen M, et al. Stem cell factor receptor induces progenitor and natural killer cell-mediated cardiac survival and repair after myocardial infarction. Proc Natl Acad Sci U S A. 2006;103(7):2304-9.

Azuaje F, Zhang L, Jeanty C, Puhl SL, Rodius S, Wagner DR. Analysis of a gene co-expression network establishes robust association between Col5a2 and ischemic heart disease. BMC Med Genomics. 2013;6:13.

Azuaje FJ, Rodius S, Zhang L, Devaux Y, Wagner DR. Information encoded in a network of inflammation proteins predicts clinical outcome after myocardial infarction. BMC medical genomics. 2011;4:59.

Bader GD, Hogue CW. An automated method for finding molecular complexes in large protein interaction networks. BMC bioinformatics. 2003;4:2.

Baikoussis NG, Papakonstantinou NA, Verra C, Kakouris G, Chounti M, Hountis P, et al. Mechanisms of oxidative stress and myocardial protection during open-heart surgery. Ann Card Anaesth. 2015;18(4):555-64.

Barbolina MV, Stack MS. Membrane type 1-matrix metalloproteinase: substrate diversity in pericellular proteolysis. Semin Cell Dev Biol. 2008;19(1):24-33.

Barnard CN. The operation. A human cardiac transplant: an interim report of a successful operation performed at Groote Schuur Hospital, Cape Town. S Afr Med J. 1967;41(48):1271-4.

Barth AS, Kuner R, Buness A, Ruschhaupt M, Merk S, Zwermann L, et al. Identification of a common gene expression signature in dilated cardiomyopathy across independent microarray studies. Journal of the American College of Cardiology. 2006;48(8):1610-7.

Belzer FO, Southard JH. Principles of solid-organ preservation by cold storage. Transplantation. 1988;45(4):673-6.

Benjamin EJ, Blaha MJ, Chiuve SE, Cushman M, Das SR, Deo R, et al. Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association. Circulation. 2017;135(10):e146-e603.

Beuth J, Falter F, Pinto Ribeiro RV, Badiwala M, Meineri M. New Strategies to Expand and Optimize Heart Donor Pool: Ex Vivo Heart Perfusion and Donation After Circulatory Death: A Review of Current Research and Future Trends. Anesth Analg. 2018. 128(3):406-413

Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol. 2007;81(1):1-5.

Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, et al. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics (Oxford, England). 2009;25(8):1091-3.

Bleumink GS, Knetsch AM, Sturkenboom MC, Straus SM, Hofman A, Deckers JW, et al. Quantifying the heart failure epidemic: prevalence, incidence rate, lifetime risk and prognosis of heart failure The Rotterdam Study. European Heart J. 2004;25(18):1614-9.

Boerma M, van der Wees CG, Vrieling H, Svensson JP, Wondergem J, van der Laarse A, et al. Microarray analysis of gene expression profiles of cardiac myocytes and fibroblasts after mechanical stress, ionising or ultraviolet radiation. BMC Genomics. 2005;6:6.

Bonventre JV, Cheung JY. Effects of metabolic acidosis on viability of cells exposed to anoxia. Am J Physiol. 1985;249(1 Pt 1):C149-59.

Bortner CD, Oldenburg NB, Cidlowski JA. The role of DNA fragmentation in apoptosis. Trends Cell Biol. 1995;5(1):21-6.

Boucek MM, Mashburn C, Dunn SM, Frizell R, Edwards L, Pietra B, et al. Pediatric heart transplantation after declaration of cardiocirculatory death. N Engl J Med. 2008;359(7):709-14.

Boyd JH, Mathur S, Wang Y, Bateman RM, Walley KR. Toll-like receptor stimulation in cardiomyoctes decreases contractility and initiates an NF-kappaB dependent inflammatory response. Cardiovasc Res. 2006;72(3):384-93.

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

Brink JG, Hassoulas J. The first human heart transplant and further advances in cardiac transplantation at Groote Schuur Hospital and the University of Cape Town - with reference to : the operation. A human cardiac transplant : an interim report of a successful operation performed at Groote Schuur Hospital, Cape Town. Cardiovasc J Afr. 2009;20(1):31-5.

Broecker V, Mengel M. The significance of histological diagnosis in renal allograft biopsies in 2014. Transpl Int. 2015;28(2):136-43.

Broughton KM, Wang BJ, Firouzi F, Khalafalla F, Dimmeler S, Fernandez-Aviles F, et al. Mechanisms of Cardiac Repair and Regeneration. Circ Res. 2018;122(8):1151-63.

Buchko MT, Himmat S, Stewart CJ, Hatami S, Dromparis P, Adam BA, et al. Continuous Hemodialysis Does Not Improve Graft Function During Ex Vivo Lung Perfusion Over 24 Hours. Transplant Proc. 2019;51(6):2022-8.

Camacho P, Fan H, Liu Z, He JQ. Large Mammalian Animal Models of Heart Disease. J Cardiovasc Dev Dis. 2016;3(4).

Canadian Institute of Health Information. Organ replacement in Canada: CORR annual statistics, 2019.

Canatan H. The effect of cardiac ischemic preconditioning on rat left ventricular gene expression profile. Cell Biochem Funct. 2008;26(2):179-84.

Cantini L, Calzone L, Martignetti L, Rydenfelt M, Bluthgen N, Barillot E, et al. Classification of gene signatures for their information value and functional redundancy. NPJ Syst Biol Appl. 2018;4:2.

Carbe CJ, Cheng L, Addya S, Gold JI, Gao E, Koch WJ, et al. Gi proteins mediate activation of the canonical hedgehog pathway in the myocardium. Am J Physiol-Heart C. 2014;307(1):H66-72.

Castaldi A, Dodia RM, Orogo AM, Zambrano CM, Najor RH, Gustafsson AB, et al. Decline in cellular function of aged mouse c-kit(+) cardiac progenitor cells. J Physiol. 2017;595(19):6249-62.

Cavalera M, Frangogiannis NG. Targeting the chemokines in cardiac repair. Curr Pharm Des. 2014;20(12):1971-9.

Cesano A. nCounter((R)) PanCancer Immune Profiling Panel (NanoString Technologies, Inc., Seattle, WA). J Immunother Cancer. 2015;3:42.

Chambers DC, Yusen RD, Cherikh WS, Goldfarb SB, Kucheryavaya AY, Khusch K, et al. The Registry of the International Society for Heart and Lung Transplantation: Thirty-fourth Adult Lung And Heart-Lung Transplantation Report-2017; Focus Theme: Allograft ischemic time. J Heart Lung Transplant. 2017;36(10):1047-59.

Chang L, Karin M. Mammalian MAP kinase signalling cascades. Nature. 2001;410(6824):37-40.

Charan NB, Ripley R, Carvalho P. Effect of increased coronary venous pressure on left ventricular function in sheep. Respir Physiol. 1998;112(2):227-35.

Chen Y, Park S, Li Y, Missov E, Hou M, Han X, et al. Alterations of gene expression in failing myocardium following left ventricular assist device support. Physiol Genomics. 2003;14(3):251-60.

Cheng CF, Lin H. Acute kidney injury and the potential for ATF3-regulated epigenetic therapy. Toxicol Mech Methods. 2011;21(4):362-6.

Cho JH, Lee I, Hammamieh R, Wang K, Baxter D, Scherler K, et al. Molecular evidence of stress-induced acute heart injury in a mouse model simulating posttraumatic stress disorder. Proc Natl Acad Sci USA. 2014;111(8):3188-93.

Chong JJ, Murry CE. Cardiac regeneration using pluripotent stem cells--progression to large animal models. Stem Cell Res. 2014;13(3 Pt B):654-65.

Church JT, Alghanem F, Deatrick KB, Trahanas JM, Phillips JP, Hee Song M, et al. Normothermic Ex Vivo Heart Perfusion: Effects of Live Animal Blood and Plasma Cross Circulation. ASAIO J. 2017;63(6):766-73.

Citerio G, Cypel M, Dobb GJ, Dominguez-Gil B, Frontera JA, Greer DM, et al. Organ donation in adults: a critical care perspective. Intensive Care Med. 2016;42(3):305-15.

Clanachan AS, Jaswal JS, Gandhi M, Bottorff DA, Coughlin J, Finegan BA, et al. Effects of inhibition of myocardial extracellular-responsive kinase and P38 mitogen-activated protein kinase on mechanical function of rat hearts after prolonged hypothermic ischemia. Transplantation. 2003;75(2):173-80.

Classen S, Zander T, Eggle D, Chemnitz JM, Brors B, Buchmann I, et al. Human resting CD4+ T cells are constitutively inhibited by TGF beta under steady-state conditions. J Immunol. 2007;178(11):6931-40.

Cloutier R, Baran D, Morin JE, Dandavino R, Marleau D, Naud A, et al. Brain death diagnoses and evaluation of the number of potential organ donors in Quebec hospitals. Can J Anaesth. 2006;53(7):716-21.

Couto-Mallon D, Gonzalez-Vilchez F, Almenar-Bonet L, Diaz-Molina B, Segovia-Cubero J, Gonzalez-Costello J, et al. Prognostic Value of Serum Lactate Levels in Patients Undergoing Urgent Heart Transplant: A Subanalysis of the ASIS-TC Spanish Multicenter Study. Rev Esp Cardiol (Engl Ed). 2019;72(3):208-14.

Crick F. Central dogma of molecular biology. Nature. 1970;227(5258):561-3.

Crozier TA, Muller JE, Quittkat D, Sydow M, Wuttke W, Kettler D. Effect of anaesthesia on the cytokine responses to abdominal surgery. Br J Anaesth. 1994;72(3):280-5.

Cruickshank AM, Fraser WD, Burns HJ, Van Damme J, Shenkin A. Response of serum interleukin-6 in patients undergoing elective surgery of varying severity. Clin Sci. 1990;79(2):161-5.

Csonka C, Szucs G, Varga-Orvos Z, Bencsik P, Csont T, Zvara A, et al. Ischemic postconditioning alters the gene expression pattern of the ischemic heart. Exp Biol Med. 2014;239(2):141-50.

Cui J, Li J, Mathison M, Tondato F, Mulkey SP, Micko C, et al. A clinically relevant large-animal model for evaluation of tissue-engineered cardiac surgical patch materials. Cardiovasc Revasc Med. 2005;6(3):113-20.

Da Silva IR, Frontera JA. Worldwide barriers to organ donation. JAMA Neurol. 2015;72(1):112-8.

Davis KL, Mehlhorn U, Laine GA, Allen SJ. Myocardial edema, left ventricular function, and pulmonary hypertension. J Appl Physiol. 1995;78(1):132-7.

Del Greco MF, Pattaro C, Luchner A, Pichler I, Winkler T, Hicks AA, et al. Genome-wide association analysis and fine mapping of NT-proBNP level provide novel insight into the role of the MTHFR-CLCN6-NPPA-NPPB gene cluster. Hum Mol Genet. 2011;20(8):1660-71.

Demmy TL, Biddle JS, Bennett LE, Walls JT, Schmaltz RA, Curtis JJ. Organ preservation solutions in heart transplantation--patterns of usage and related survival. Transplantation. 1997;63(2):262-9.

Deng MC, Eisen HJ, Mehra MR, Billingham M, Marboe CC, Berry G, et al. Noninvasive discrimination of rejection in cardiac allograft recipients using gene expression profiling. Am J Transplant. 2006;6(1):150-60.

Desai KV, Laine GA, Stewart RH, Cox CS, Jr., Quick CM, Allen SJ, et al. Mechanics of the left ventricular myocardial interstitium: effects of acute and chronic myocardial edema. Am J Physiol-Heart C. 2008;294(6):H2428-34.

Dhital KK, Chew HC, Macdonald PS. Donation after circulatory death heart transplantation. Curr Opin Organ Tran. 2017;22(3):189-97.

Dhital KK, Iyer A, Connellan M, Chew HC, Gao L, Doyle A, et al. Adult heart transplantation with distant procurement and ex-vivo preservation of donor hearts after circulatory death: a case series. Lancet. 2015;385(9987):2585-91.

Diagnosis of brain death. Lancet. 1976;2(7994):1069-70.

Diestel A, Roessler J, Pohl-Schickinger A, Koster A, Drescher C, Berger F, et al. Specific p38 inhibition in stimulated endothelial cells: a possible new anti-inflammatory strategy after hypothermia and rewarming. Vasc Pharmacol. 2009;51(4):246-52.

Dinarello CA, Simon A, van der Meer JW. Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. Nat Rev Drug Discov. 2012;11(8):633-52.

Dongaonkar RM, Stewart RH, Geissler HJ, Laine GA. Myocardial microvascular permeability, interstitial oedema, and compromised cardiac function. Cardiovas Res. 2010;87(2):331-9.

Dongaonkar RM, Stewart RH, Quick CM, Uray KL, Cox CS, Jr., Laine GA. Award article: Microcirculatory Society Award for Excellence in Lymphatic Research: time course of myocardial interstitial edema resolution and associated left ventricular dysfunction. Microcirculation. 2012;19(8):714-22.

Downward J. How BAD phosphorylation is good for survival. Nat Cell Biol. 1999;1(2):E33-5.

Dromparis P, Aboelnazar NS, Wagner S, Himmat S, White CW, Hatami S, et al. Ex vivo perfusion induces a time- and perfusate-dependent molecular repair response in explanted porcine lungs. Am J Transplant. 2019;19(4):1024-36.

Dulbecco R. A turning point in cancer research: sequencing the human genome. Science. 1986;231(4742):1055-6.

El Hajj EC, El Hajj MC, Ninh VK, Gardner JD. Inhibitor of lysyl oxidase improves cardiac function and the collagen/MMP profile in response to volume overload. American journal of physiology Heart and circulatory physiology. 2018;315(3):H463-h73.

Emet M, Akoz A, Aslan S, Saritas A, Cakir Z, Acemoglu H. Assessment of cardiac injury in patients with blunt chest trauma. Eur J Trauma Emerg S. 2010;36(5):441-7.

Epelman S, Liu PP, Mann DL. Role of innate and adaptive immune mechanisms in cardiac injury and repair. Nat Rev Immunol. 2015;15(2):117-29.

Fang JC, Ewald GA, Allen LA, Butler J, Westlake Canary CA, Colvin-Adams M, et al. Advanced (stage D) heart failure: a statement from the Heart Failure Society of America Guidelines Committee. J Card Fail. 2015;21(6):519-34.

Figueiredo-Freitas C, Dulce RA, Foster MW, Liang J, Yamashita AM, Lima-Rosa FL, et al. S-Nitrosylation of Sarcomeric Proteins Depresses Myofilament Ca2+)Sensitivity in Intact Cardiomyocytes. Antioxid Redox Sign. 2015;23(13):1017-34.

Fontes JA, Rose NR, Cihakova D. The varying faces of IL-6: From cardiac protection to cardiac failure. Cytokine. 2015;74(1):62-8.

Frangogiannis NG. Cardiac fibrosis: Cell biological mechanisms, molecular pathways and therapeutic opportunities. Mol Aspects Med. 2019;65:70-99.

Frangogiannis NG. Inflammation in cardiac injury, repair and regeneration. Curr Opin Cardiol. 2015;30(3):240-5.

Frangogiannis NG. The inflammatory response in myocardial injury, repair, and remodeling. Nat Rev Cardiol. 2014;11(5):255-65.

Freed DH, White CW. Donor heart preservation: straight up, or on the rocks? Lancet. 2015;385(9987):2552-4.

Freedman BR, Bade ND, Riggin CN, Zhang S, Haines PG, Ong KL, et al. The (dys)functional extracellular matrix. Biochim Biophys Acta. 2015;1853(11 Pt B):3153-64.

Freeman R, Koerner E, Clark C, Halabicky K. The Path From Heart Failure to Cardiac Transplant. Crit care Nurs Q. 2016;39(3):207-13.

Garcia Saez D, Zych B, Sabashnikov A, Bowles CT, De Robertis F, Mohite PN, et al. Evaluation of the organ care system in heart transplantation with an adverse donor/recipient profile. Ann Thorac Surg. 2014;98(6):2099-105; discussion 105-6.

Garcia-Dorado D, Ruiz-Meana M, Inserte J, Rodriguez-Sinovas A, Piper HM. Calcium-mediated cell death during myocardial reperfusion. Cardiovasc Res. 2012;94(2):168-80.

Gardiner D, Shemie S, Manara A, Opdam H. International perspective on the diagnosis of death. Br J Anaesth. 2012;108 Suppl 1:i14-28.

Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol. 1992;119(3):493-501.

Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N, Dunaway DL, et al. Direct multiplexed measurement of gene expression with color-coded probe pairs. Nat Biotechnol. 2008;26(3):317-25.

Gerits N, Kostenko S, Moens U. In vivo functions of mitogen-activated protein kinases: conclusions from knock-in and knock-out mice. Transgenic Res. 2007;16(3):281-314.

Ghorbel MT, Mokhtari A, Sheikh M, Angelini GD, Caputo M. Controlled reoxygenation cardiopulmonary bypass is associated with reduced transcriptomic changes in cyanotic tetralogy of Fallot patients undergoing surgery. Physiol Genomics. 2012;44(22):1098-106.

Ghosh AK, Murphy SB, Kishore R, Vaughan DE. Global gene expression profiling in PAI-1 knockout murine heart and kidney: molecular basis of cardiac-selective fibrosis. PLoS One. 2013;8(5):e63825.

Girlanda R. Deceased organ donation for transplantation: Challenges and opportunities. World J Transplant. 2016;6(3):451-9.

Goenawan IH, Bryan K, Lynn DJ. DyNet: visualization and analysis of dynamic molecular interaction networks. Bioinformatics. 2016;32(17):2713-5.

Goldstein BA, Thomas L, Zaroff JG, Nguyen J, Menza R, Khush KK. Assessment of Heart Transplant Waitlist Time and Pre- and Post-transplant Failure: A Mixed Methods Approach. Epidemiology. 2016;27(4):469-76.

Gomez L, Li B, Mewton N, Sanchez I, Piot C, Elbaz M, et al. Inhibition of mitochondrial permeability transition pore opening: translation to patients. Cardiovasc Res. 2009;83(2):226-33.

Government of Canada. Heart Disease in Canada. https://www.canada.ca/en/public-health/services/publications/diseases-conditions/heart-disease-canada.html [Accessed 9 January 2020]

Govers R. Molecular mechanisms of GLUT4 regulation in adipocytes. Diabetes Metab. 2014;40(6):400-10.

Grasl-Kraupp B, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R. In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatology. 1995;21(5):1465-8.

Graves JA. Background and Overview of Comparative Genomics. ILAR J. 1998;39(2-3):48-65.

Green ED, Guyer MS. Charting a course for genomic medicine from base pairs to bedside. Nature. 2011;470(7333):204-13.

Guan XH, Hong X, Zhao N, Liu XH, Xiao YF, Chen TT, et al. CD38 promotes angiotensin II-induced cardiac hypertrophy. J Cell Mol Med. 2017;21(8):1492-502.

Guibert EE, Petrenko AY, Balaban CL, Somov AY, Rodriguez JV, Fuller BJ. Organ Preservation: Current Concepts and New Strategies for the Next Decade. Transfus Med Hemoth. 2011;38(2):125-42.

Gwechenberger M, Mendoza LH, Youker KA, Frangogiannis NG, Smith CW, Michael LH, et al. Cardiac myocytes produce interleukin-6 in culture and in viable border zone of reperfused infarctions. Circulation. 1999;99(4):546-51.

Haas M, Sis B, Racusen LC, Solez K, Glotz D, Colvin RB, et al. Banff 2013 meeting report: inclusion of c4d-negative antibody-mediated rejection and antibody-associated arterial lesions. Am J Transplant. 2014;14(2):272-83.

Harris C, Cao C, Croce B, Munkholm-Larsen S. Heart transplantation. Ann Cardiothorac Surg. 2018;7(1):172.

Harvey PR, Iu S, McKeown CM, Petrunka CN, Ilson RG, Strasberg SM. Adenine nucleotide tissue concentrations and liver allograft viability after cold preservation and warm ischemia. Transplantation. 1988;45(6):1016-20.

Hashimoto N, Takeyoshi I, Yoshinari D, Tsutsumi H, Tokumine M, Totsuka O, et al. Effects of a p38 mitogen-activated protein kinase inhibitor as an additive to Euro-Collins solution on reperfusion injury in canine lung transplantation1. Transplantation. 2002;74(3):320-6.

Hatami S, White CW, Ondrus M, Qi X, Buchko M, Himmat S, et al. Normothermic Ex Situ Heart Perfusion in Working Mode: Assessment of Cardiac Function and Metabolism. JoVE-J Vis Exp. 2019(143).

Hatami S, White CW, Shan S, Haromy A, Qi X, Ondrus M, et al. Myocardial Functional Decline During Prolonged Ex Situ Heart Perfusion. Ann Thorac Surg. 2019;108(2):499-507.

Hearse DJ, Sutherland FJ. Experimental models for the study of cardiovascular function and disease. Pharmacol Res. 2000;41(6):597-603.

Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signaling pathways. Nat Rev Mol Cell Biol. 2006;7(8):589-600.

Helmy SA, Wahby MA, El-Nawaway M. The effect of anaesthesia and surgery on plasma cytokine production. Anaesthesia. 1999;54(8):733-8.

Heusch G. Molecular basis of cardioprotection: signal transduction in ischemic pre-, post-, and remote conditioning. Circ Res. 2015;116(4):674-99.

Heymans S, Schroen B, Vermeersch P, Milting H, Gao F, Kassner A, et al. Increased cardiac expression of tissue inhibitor of metalloproteinase-1 and tissue inhibitor of metalloproteinase-2 is related to cardiac fibrosis and dysfunction in the chronic pressure-overloaded human heart. Circulation. 2005;112(8):1136-44.

Hickman PE, Potter JM, Aroney C, Koerbin G, Southcott E, Wu AH, et al. Cardiac troponin may be released by ischemia alone, without necrosis. Intl J Clin Chem. 2010;411(5-6):318-23.

Ho KK, Pinsky JL, Kannel WB, Levy D. The epidemiology of heart failure: the Framingham Study. J Am Coll Cardiol. 1993;22(4 Suppl A):6a-13a.

Hood L, Rowen L. The Human Genome Project: big science transforms biology and medicine. Genome Med. 2013;5(9):79.

Hreniuk D, Garay M, Gaarde W, Monia BP, McKay RA, Cioffi CL. Inhibition of c-Jun N-terminal kinase 1, but not c-Jun N-terminal kinase 2, suppresses apoptosis induced by ischemia/reoxygenation in rat cardiac myocytes. Mol Pharmacol. 2001;59(4):867-74.

Huda R, Chung DH, Mathru M. Ischemic preconditioning at a distance: altered gene expression in mouse heart and other organs following brief occlusion of the mesenteric artery. Heart Lung Circ. 2005;14(1):36-43.

Hunt SA, Abraham WT, Chin MH, Feldman AM, Francis GS, Ganiats TG, et al. ACC/AHA 2005 Guideline Update for the Diagnosis and Management of Chronic Heart Failure in the Adult: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Update the 2001 Guidelines for the Evaluation and Management of Heart Failure): developed in collaboration with the American College of Chest Physicians and the International Society for Heart and Lung Transplantation: endorsed by the Heart Rhythm Society. Circulation. 2005;112(12):e154-235.

Inada T, Yamanouchi Y, Jomura S, Sakamoto S, Takahashi M, Kambara T, et al. Effect of propofol and isoflurane anaesthesia on the immune response to surgery. Anaesthesia. 2004;59(10):954-9.

Inbar R, Shoenfeld Y. Elevated cardiac troponins: the ultimate marker for myocardial necrosis, but not without a differential diagnosis. Isr Med Assoc J. 2009;11(1):50-3.

Indraccolo S, Pfeffer U, Minuzzo S, Esposito G, Roni V, Mandruzzato S, et al. Identification of genes selectively regulated by IFNs in endothelial cells. J Immunol. 2007;178(2):1122-35.

Iwase M, Kondo G, Watanabe H, Takaoka S, Uchida M, Ohashi M, et al. Regulation of Fas-mediated apoptosis in neutrophils after surgery-induced acute inflammation. J Surg Res. 2006;134(1):114-23.

Iyer A, Gao L, Doyle A, Rao P, Cropper JR, Soto C, et al. Normothermic ex vivo perfusion provides superior organ preservation and enables viability assessment of hearts from DCD donors. Am J Transplant. 2015;15(2):371-80.

Jacques F, Carrier M, Pelletier GB, White M, Racine N, Pellerin M, et al. Two decades of cardiac transplantation at the Montreal Heart Institute. Can J Cardiol. 2008;24(3):217-21.

Jahania MS, Sanchez JA, Narayan P, Lasley RD, Mentzer RM, Jr. Heart preservation for transplantation: principles and strategies. Ann Thorac Surg. 1999;68(5):1983-7.

Jung MJ, Murzik U, Wehder L, Hemmerich P, Melle C. Regulation of cellular actin architecture by S100A10. Exp Cell Res. 2010;316(7):1234-40.

Kantrowitz A, Haller JD, Joos H, Cerruti MM, Carstensen HE. Transplantation of the heart in an infant and an adult. Am J Cardiol. 1968;22(6):782-90.

Kao CC, Parulekar AD. Is perfusate exchange during ex vivo lung perfusion beneficial? Ann Transl Med. 2020;8(3):43.

Kaplanski G, Marin V, Montero-Julian F, Mantovani A, Farnarier C. IL-6: a regulator of the transition from neutrophil to monocyte recruitment during inflammation. Trends Immunol. 2003;24(1):25-9.

Kappler B, Ledezma CA, van Tuijl S, Meijborg V, Boukens BJ, Ergin B, et al. Investigating the physiology of normothermic ex vivo heart perfusion in an isolated slaughterhouse porcine model used for device testing and training. BMC cardiovasc Disor. 2019;19(1):254.

Karbassi E, Monte E, Chapski DJ, Lopez R, Rosa Garrido M, Kim J, et al. Relationship of disease-associated gene expression to cardiac phenotype is buffered by genetic diversity and chromatin regulation. Physiol genomics. 2016;48(8):601-15.

Karin M, Liu Z, Zandi E. AP-1 function and regulation. Curr Opin Cell Biol. 1997;9(2):240-6.

Ke JJ, Zhan J, Feng XB, Wu Y, Rao Y, Wang YL. A comparison of the effect of total intravenous anaesthesia with propofol and remifentanil and inhalational anaesthesia with isoflurane on the release of pro- and anti-inflammatory cytokines in patients undergoing open cholecystectomy. Anaesth Intensive Care. 2008;36(1):74-8.

Kearns MJ, Miller SD, Cheung A, Bashir J, Wong S, Seidman MA, et al. A Rodent Model of Cardiac Donation After Circulatory Death and Novel Biomarkers of Cardiac Viability During Ex Vivo Heart Perfusion. Transplantation. 2017;101(8):e231-e9.

Kempf T, Zarbock A, Widera C, Butz S, Stadtmann A, Rossaint J, et al. GDF-15 is an inhibitor of leukocyte integrin activation required for survival after myocardial infarction in mice. Nat Med. 2011;17(5):581-8.

Khush KK, Zaroff JG, Nguyen J, Menza R, Goldstein BA. National decline in donor heart utilization with regional variability: 1995-2010. Am J Transplant. 2015;15(3):642-9.

Kim B. How to Reveal Magnitude of Gene Signals: Hierarchical Hypergeometric Complementary Cumulative Distribution Function. Evolutionary bioinformatics online. 2018;14:1176934318797352.

Kim IC, Youn JC, Kobashigawa JA. The Past, Present and Future of Heart Transplantation. Korean Circ J. 2018;48(7):565-90.

Kim MY, Seo EJ, Lee DH, Kim EJ, Kim HS, Cho HY, et al. Gadd45beta is a novel mediator of cardiomyocyte apoptosis induced by ischaemia/hypoxia. Cardiovasc Res. 2010;87(1):119-26.

Kim SJ, Angel P, Lafyatis R, Hattori K, Kim KY, Sporn MB, et al. Autoinduction of transforming growth factor beta 1 is mediated by the AP-1 complex. Mol Cell Biol. 1990;10(4):1492-7.

Kim YJ, Lim HJ, Choi SU. Effect of propofol on cardiac function and gene expression after ischemic-reperfusion in isolated rat heart. Korean J Anesthesiol. 2010;58(2):153-61.

Kirklin JK. Terminal heart failure: who should be transplanted and who should have mechanical circulatory support? Curr Opin Organ Transplant. 2014;19(5):486-93.

Koike N, Takeyoshi I, Ohki S, Tokumine M, Matsumoto K, Morishita Y. Effects of adding P38 mitogen-activated protein-kinase inhibitor to celsior solution in canine heart transplantation from non-heart-beating donors. Transplantation. 2004;77(2):286-92.

Kootstra G, Daemen JH, Oomen AP. Categories of non-heart-beating donors. Transplant Proc. 1995;27(5):2893-4.

Kramer AH, Baht R, Doig CJ. Time trends in organ donation after neurologic determination of death: a cohort study. CMAJ open. 2017;5(1):E19-e27.

Kramer AH, Zygun DA, Doig CJ, Zuege DJ. Incidence of neurologic death among patients with brain injury: a cohort study in a Canadian health region. CMAJ. 2013;185(18):E838-45.

Kukurba KR, Montgomery SB. RNA Sequencing and Analysis. Cold Spring Harbor protocols. 2015;2015(11):951-69.

Kulkarni MM. Digital multiplexed gene expression analysis using the NanoString nCounter system. Curr Protoc Mol Biol. 2011;Chapter 25:Unit25B.10.

Kumar D, Menon V, Ford WR, Clanachan AS, Jugdutt BI. Effect of angiotensin II type 2 receptor blockade on mitogen activated protein kinases during myocardial ischemia-reperfusion. Mol Cell Biochem. 2004;258(1-2):211-8.

Kyrylkova K, Kyryachenko S, Leid M, Kioussi C. Detection of apoptosis by TUNEL assay. Methods Mol Biol (Clifton, NJ). 2012;887:41-7.

Laine GA, Allen SJ. Left ventricular myocardial edema. Lymph flow, interstitial fibrosis, and cardiac function. Circ Res. 1991;68(6):1713-21.

Lakshminarayanan V, Lewallen M, Frangogiannis NG, Evans AJ, Wedin KE, Michael LH, et al. Reactive oxygen intermediates induce monocyte chemotactic protein-1 in vascular endothelium after brief ischemia. Am J Pathol. 2001;159(4):1301-11.

Lamesch P, Berardini TZ, Li D, Swarbreck D, Wilks C, Sasidharan R, et al. The Arabidopsis Information Resource (TAIR): improved gene annotation and new tools. Nucleic Acids Res. 2012;40(Database issue):D1202-10.

Lanneau D, Brunet M, Frisan E, Solary E, Fontenay M, Garrido C. Heat shock proteins: essential proteins for apoptosis regulation. J Cell Mol Med. 2008;12(3):743-61.

Lara-Pezzi E, Felkin LE, Birks EJ, Sarathchandra P, Panse KD, George R, et al. Expression of follistatin-related genes is altered in heart failure. Endocrinology. 2008;149(11):5822-7.

Lattanzio FA, Jr., Tiangco D, Osgood C, Beebe S, Kerry J, Hargrave BY. Cocaine increases intracellular calcium and reactive oxygen species, depolarizes mitochondria, and activates genes associated with heart failure and remodeling. Cardiovasc Toxicol. 2005;5(4):377-90.

Lelovas PP, Kostomitsopoulos NG, Xanthos TT. A comparative anatomic and physiologic overview of the porcine heart. J Am Assoc Lab Anim Sci. 2014;53(5):432-8.

Leprince P, Popov AF, Simon AR, Benk C, Siepe M. Ex vivo perfusion of the heart with the use of the Organ Care System. Eur J Cardiothorac Surg. 2016;49(5):1318-20.

Li T, Chen YH, Liu TJ, Jia J, Hampson S, Shan YX, et al. Using DNA microarray to identify Sp1 as a transcriptional regulatory element of insulin-like growth factor 1 in cardiac muscle cells. Circ Res. 2003;93(12):1202-9.

Li YY, McTiernan CF, Feldman AM. Proinflammatory cytokines regulate tissue inhibitors of metalloproteinases and disintegrin metalloproteinase in cardiac cells. Cardiovasc Res. 1999;42(1):162-72.

Liao R, Podesser BK, Lim CC. The continuing evolution of the Langendorff and ejecting murine heart: new advances in cardiac phenotyping. Am J Physiol-Heart C. 2012;303(2):H156-67.

Liberzon A, Birger C, Thorvaldsdottir H, Ghandi M, Mesirov JP, Tamayo P. The Molecular Signatures Database (MSigDB) hallmark gene set collection. Cell Syst. 2015;1(6):417-25.

Lindquist S, Craig EA. The heat-shock proteins. Annu Rev Genet. 1988;22:631-77.

Lindsey ML, Zamilpa R. Temporal and spatial expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases following myocardial infarction. Cardiovasc Ther. 2012;30(1):31-41.

Liu SQ, Tefft BJ, Roberts DT, Zhang LQ, Ren Y, Li YC, et al. Cardioprotective proteins upregulated in the liver in response to experimental myocardial ischemia. Am J Physiol-Heart C. 2012;303(12):H1446-58.

Lui C, Suarez-Pierre A, Zhou X, Crawford TC, Fraser CD, Giuliano K, et al. Effects of Systemic and Device-Related Complications in Patients Bridged to Transplantation With Left Ventricular Assist Devices. J Surg Res. 2020;246:207-12.

Lund LH, Edwards LB, Kucheryavaya AY, Dipchand AI, Benden C, Christie JD, et al. The Registry of the International Society for Heart and Lung Transplantation: Thirtieth Official Adult Heart Transplant Report--2013; focus theme: age. J Heart Lung Transplant. 2013;32(10):951-64.

Ma L, Song H, Zhang M, Zhang D. Lysyl oxidase G473A polymorphism is associated with increased risk of coronary artery diseases. DNA Cell Biol. 2011;30(12):1033-7.

Maciejak A, Kiliszek M, Michalak M, Tulacz D, Opolski G, Matlak K, et al. Gene expression profiling reveals potential prognostic biomarkers associated with the progression of heart failure. Genome Med. 2015;7(1):26.

Malcolm KC, Arndt PG, Manos EJ, Jones DA, Worthen GS. Microarray analysis of lipopolysaccharide-treated human neutrophils. Am J Physiol-Lung C. 2003;284(4):L663-70.

Mallik S, Zhao Z. Identification of gene signatures from RNA-seq data using Pareto-optimal cluster algorithm. BMC Syst Biol. 2018;12(Suppl 8):126.

Malodobra-Mazur M, Bednarska-Chabowska D, Olewinski R, Chmielecki Z, Adamiec R, Dobosz T. Single nucleotide polymorphisms in 5'-UTR of the SLC2A4 gene regulate solute carrier family 2 member 4 gene expression in visceral adipose tissue. Gene. 2016;576(1 Pt 3):499-504.

Manara AR, Murphy PG, O'Callaghan G. Donation after circulatory death. Br J Anaesth. 2012;108 Suppl 1:i108-21.

Mann DL, Topkara VK, Evans S, Barger PM. Innate immunity in the adult mammalian heart: for whom the cell tolls. Trans Am Clin Climatol Assoc. 2010;121:34-50; discussion -1.

Mann DL. The emerging role of innate immunity in the heart and vascular system: for whom the cell tolls. Circ Res. 2011;108(9):1133-45.

Masri W, Le Guillou E, Hamdi E, Ghazal K, Lebigot E, Cosson C, et al. Troponin elevation in other conditions than acute coronary syndromes. Ann Biol Clin. 2017;75(4):411-9.

Masumura Y, Higo S, Asano Y, Kato H, Yan Y, Ishino S, et al. Btg2 is a Negative Regulator of Cardiomyocyte Hypertrophy through a Decrease in Cytosolic RNA. Sci Rep. 2016;6:28592.

Mathur S, Walley KR, Boyd JH. The Toll-like receptor 9 ligand CPG-C attenuates acute inflammatory cardiac dysfunction. Shock. 2011;36(5):478-83.

Matkovich SJ, Van Booven DJ, Youker KA, Torre-Amione G, Diwan A, Eschenbacher WH, et al. Reciprocal regulation of myocardial microRNAs and messenger RNA in human cardiomyopathy and reversal of the microRNA signature by biomechanical support. Circulation. 2009;119(9):1263-71.

Mehlhorn U, Geissler HJ, Laine GA, Allen SJ. Myocardial fluid balance. Eur J Cardiothorac Surg. 2001;20(6):1220-30.

Meldrum DR, Dinarello CA, Cleveland JC, Jr., Cain BS, Shames BD, Meng X, et al. Hydrogen peroxide induces tumor necrosis factor alpha-mediated cardiac injury by a P38 mitogen-activated protein kinase-dependent mechanism. Surgery. 1998;124(2):291-6; discussion 7.

Mengel M, Sis B, Kim D, Chang J, Famulski KS, Hidalgo LG, et al. The molecular phenotype of heart transplant biopsies: relationship to histopathological and clinical variables. Am J Transplant. 2010;10(9):2105-15.

Merchant SJ, Yoshida EM, Lee TK, Richardson P, Karlsbjerg KM, Cheung E. Exploring the psychological effects of deceased organ donation on the families of the organ donors. Clin transplant. 2008;22(3):341-7.

Messer S, Ardehali A, Tsui S. Normothermic donor heart perfusion: current clinical experience and the future. Transplant international. 2015;28(6):634-42.

Messer S, Page A, Axell R, Berman M, Hernandez-Sanchez J, Colah S, et al. Outcome after heart transplantation from donation after circulatory-determined death donors. J Heart Lung Transplant. 2017;36(12):1311-8.

Messer S, Page A, Colah S, Axell R, Parizkova B, Tsui S, et al. Human heart transplantation from donation after circulatory-determined death donors using normothermic regional perfusion and cold storage. J Heart Lung Transplant. 2018;37(7):865-9.

Messer SJ, Axell RG, Colah S, White PA, Ryan M, Page AA, et al. Functional assessment and transplantation of the donor heart after circulatory death. J Heart Lung Transplant. 2016;35(12):1443-52.

Michel P, Vial R, Rodriguez C, Ferrera R. A comparative study of the most widely used solutions for cardiac graft preservation during hypothermia. J Heart Lung Transplant. 2002;21(9):1030-9.

Milani-Nejad N, Janssen PM. Small and large animal models in cardiac contraction research: advantages and disadvantages. Pharmacol Therapeut. 2014;141(3):235-49.

Milano G, Morel S, Bonny C, Samaja M, von Segesser LK, Nicod P, et al. A peptide inhibitor of c-Jun NH2-terminal kinase reduces myocardial ischemia-reperfusion injury and infarct size in vivo. Am J Physiol-Heart C. 2007;292(4):H1828-35.

Millar JE, Fanning JP, McDonald CI, McAuley DF, Fraser JF. The inflammatory response to extracorporeal membrane oxygenation (ECMO): a review of the pathophysiology. Crit Care (London, England). 2016;20(1):387.

Minambres E, Suberviola B, Dominguez-Gil B, Rodrigo E, Ruiz-San Millan JC, Rodriguez-San Juan JC, et al. Improving the Outcomes of Organs Obtained From Controlled Donation After Circulatory Death Donors Using Abdominal Normothermic Regional Perfusion. Am J Transplant. 2017;17(8):2165-72.

Miner EC, Miller WL. A look between the cardiomyocytes: the extracellular matrix in heart failure. Mayo Clin Proc. 2006;81(1):71-6.

Misior AM, Deshpande DA, Loza MJ, Pascual RM, Hipp JD, Penn RB. Glucocorticoid- and protein kinase A-dependent transcriptome regulation in airway smooth muscle. Am J Resp Cell Mol. 2009;41(1):24-39.

Monteagudo Vela M, Garcia Saez D, Simon AR. Current approaches in retrieval and heart preservation. Ann Cardiothorac Surg. 2018;7(1):67-74.

Nakagawa TA, Shemie SD, Dryden-Palmer K, Parshuram CS, Brierley J. Organ Donation Following Neurologic and Circulatory Determination of Death. Pediatric critical care medicine. 2018;19(8S Suppl 2):S26-s32.

Nakao A, Imamura T, Souchelnytskyi S, Kawabata M, Ishisaki A, Oeda E, et al. TGF-beta receptor-mediated signalling through Smad2, Smad3 and Smad4. Embo J. 1997;16(17):5353-62.

Nardi P, Pisano C, Bertoldo F, Vacirca SR, Saitto G, Costantino A, et al. Warm blood cardioplegia versus cold crystalloid cardioplegia for myocardial protection during coronary artery bypass grafting surgery. Cell Death Discov. 2018;4:23.

Narolska NA, Eiras S, van Loon RB, Boontje NM, Zaremba R, Spiegelen Berg SR, et al. Myosin heavy chain composition and the economy of contraction in healthy and diseased human myocardium. J Muscle Res Cell M. 2005;26(1):39-48.

Naseem RH, Meeson AP, Michael Dimaio J, White MD, Kallhoff J, Humphries C, et al. Reparative myocardial mechanisms in adult C57BL/6 and MRL mice following injury. Physiol Genomics. 2007;30(1):44-52.

Negoescu A, Lorimier P, Labat-Moleur F, Drouet C, Robert C, Guillermet C, et al. In situ apoptotic cell labeling by the TUNEL method: improvement and evaluation on cell preparations. J Histochem Cytochem. 1996;44(9):959-68.

Nesher N, Frolkis I, Vardi M, Sheinberg N, Bakir I, Caselman F, et al. Higher levels of serum cytokines and myocardial tissue markers during on-pump versus off-pump coronary artery bypass surgery. J Card Surg. 2006;21(4):395-402.

Nguan C. Trying to meet the demands in organ transplantation. CUAJ-Can Urol Assoc . 2012;6(6):453-4.

Nogueira LG, Santos RH, Ianni BM, Fiorelli AI, Mairena EC, Benvenuti LA, et al. Myocardial chemokine expression and intensity of myocarditis in Chagas cardiomyopathy are controlled by polymorphisms in CXCL9 and CXCL10. PLoS Neglect Trop D. 2012;6(10):e1867.

Nong Z, O'Neil C, Lei M, Gros R, Watson A, Rizkalla A, et al. Type I collagen cleavage is essential for effective fibrotic repair after myocardial infarction. Am J Pathol. 2011;179(5):2189-98.

Noterdaeme T, Detry O, Hans MF, Nellessen E, Ledoux D, Joris J, et al. What is the potential increase in the heart graft pool by cardiac donation after circulatory death? Transpl Int. 2013;26(1):61-6.

Nuttall RK, Sampieri CL, Pennington CJ, Gill SE, Schultz GA, Edwards DR. Expression analysis of the entire MMP and TIMP gene families during mouse tissue development. FEBS letters. 2004;563(1-3):129-34.

O'Connor T, Borsig L, Heikenwalder M. CCL2-CCR2 Signaling in Disease Pathogenesis. Endocr Metab Immune Disord Drug Targets. 2015;15(2):105-18.

Ogawa K, Hirai M, Katsube T, Murayama M, Hamaguchi K, Shimakawa T, et al. Suppression of cellular immunity by surgical stress. Surgery. 2000;127(3):329-36.

Ong SB, Hernandez-Resendiz S, Crespo-Avilan GE, Mukhametshina RT, Kwek XY, Cabrera-Fuentes HA, et al. Inflammation following acute myocardial infarction: Multiple players, dynamic roles, and novel therapeutic opportunities. Pharmacol Therapeut. 2018;186:73-87.

Osaki S, Ishino K, Kotani Y, Honjo O, Suezawa T, Kanki K, et al. Resuscitation of non-beating donor hearts using continuous myocardial perfusion: the importance of controlled initial reperfusion. Ann Thorac Surg. 2006;81(6):2167-71.

Ozeki T, Kwon MH, Gu J, Collins MJ, Brassil JM, Miller MB, Jr., et al. Heart preservation using continuous ex vivo perfusion improves viability and functional recovery. Circ J. 2007;71(1):153-9.

Page A, Messer S, Large SR. Heart transplantation from donation after circulatory determined death. Ann Cardiothorac Surg. 2018;7(1):75-81.

Parikh JR, Klinger B, Xia Y, Marto JA, Bluthgen N. Discovering causal signaling pathways through gene-expression patterns. Nucleic Acids Res. 2010;38:109-17.

Patel K, Hipskind JE. Cardiac Arrest. StatPearls. SatPearlsPublishing;2019.

Pavo N, Lukovic D, Zlabinger K, Lorant D, Goliasch G, Winkler J, et al. Intrinsic remote conditioning of the myocardium as a comprehensive cardiac response to ischemia and reperfusion. Oncotarget. 2017;8(40):67227-40.

Picard F, Brehm M, Fassbach M, Pelzer B, Scheuring S, Kury P, et al. Increased cardiac mRNA expression of matrix metalloproteinase-1 (MMP-1) and its inhibitor (TIMP-1) in DCM patients. Clin Res Cardiol. 2006;95(5):261-9.

Pinezich M, Vunjak-Novakovic G. Bioengineering approaches to organ preservation *ex vivo*. Exp Biol Med (Maywood). 2019;244(8):630-45.

Pitts KR, Derry JM, Kerkof K, Lawrence WA, Toombs CF. Differentially regulated functional gene clusters identified during ischemia and reperfusion in isolated cardiac myocytes using coverslip hypoxia. J Pharmacol Toxicol Methods. 2008;57(1):42-51.

Podgoreanu MV, Michelotti GA, Sato Y, Smith MP, Lin S, Morris RW, et al. Differential cardiac gene expression during cardiopulmonary bypass: ischemia-independent upregulation of proinflammatory genes. J Thorac Cardiovasc Surg. 2005;130(2):330-9.

Pratt JW, Schertel ER, Schaefer SL, Esham KE, McClure DE, Heck CF, et al. Acute transient coronary sinus hypertension impairs left ventricular function and induces myocardial edema. Am J Physiol. 1996;271(3 Pt 2):H834-41.

Prieto D, Correia P, Antunes P, Batista M, Antunes MJ. Results of heart transplantation in the urgent recipient--who should be transplanted? Braz J Cardiov Surg. 2014;29(3):379-87.

Qiao A, Zhao Z, Zhang H, Sun Z, Cui X. Gene expression profiling reveals genes and transcription factors associated with dilated and ischemic cardiomyopathies. Pathol Research Pract. 2017;213(5):548-57.

Raghu H, Lepus CM, Wang Q, Wong HH, Lingampalli N, Oliviero F, et al. CCL2/CCR2, but not CCL5/CCR5, mediates monocyte recruitment, inflammation and cartilage destruction in osteoarthritis. Ann Rheum Dis. 2017;76(5):914-22.

Ralph A, Chapman JR, Gillis J, Craig JC, Butow P, Howard K, et al. Family perspectives on deceased organ donation: thematic synthesis of qualitative studies. Am J Transplant. 2014;14(4):923-35.

Ranek MJ, Stachowski MJ, Kirk JA, Willis MS. The role of heat shock proteins and co-chaperones in heart failure. Philos Trans R Soc Lond B Biol Sci. 2018;373(1738).

Rauch F, Glorieux FH. Osteogenesis imperfecta. Lancet (London, England). 2004;363(9418):1377-85.

Reed JF, 3rd. Homogeneity of kappa statistics in multiple samples. Comput Meth Prog Bio. 2000;63(1):43-6.

Reinhardt D, Sigusch HH, Hensse J, Tyagi SC, Korfer R, Figulla HR. Cardiac remodelling in end stage heart failure: upregulation of matrix metalloproteinase (MMP) irrespective of the underlying disease, and evidence for a direct inhibitory effect of ACE inhibitors on MMP. Heart. 2002;88(5):525-30.

Reis PP, Waldron L, Goswami RS, Xu W, Xuan Y, Perez-Ordonez B, et al. mRNA transcript quantification in archival samples using multiplexed, color-coded probes. BMC Biotechnol. 2011;11:46.

Reith HB, Kaman S, Mittelkotter O, Kilic Y, Kozuschek W. Cytokine activation in patients undergoing open or laparoscopic cholecystectomy. Int Surg. 1997;82(4):389-93.

Reznik ON, Skvortsov AE, Reznik AO, Ananyev AN, Tutin AP, Kuzmin DO, et al. Uncontrolled donors with controlled reperfusion after sixty mins of asystole: a novel reliable resource for kidney transplantation. PloS One. 2013;8(5):e64209.

Ribeiro RVP, Alvarez JS, Yu F, Paradiso E, Adamson MB, Maria Ruggeri G, et al. Hearts Donated After Circulatory Death and Reconditioned Using Normothermic Regional Perfusion Can Be Successfully Transplanted Following an Extended Period of Static Storage. Circ Heart Fail. 2019;12(4):e005364.

Rivard AL, Gallegos R, Ogden IM, Bianco RW. Perfusion preservation of the donor heart: basic science to pre-clinical. J Extra Corpor Technol. 2009;41(3):140-8.

Rodius S, Nazarov PV, Nepomuceno-Chamorro IA, Jeanty C, Gonzalez-Rosa JM, Ibberson M, et al. Transcriptional response to cardiac injury in the zebrafish: systematic identification of genes with highly concordant activity across in vivo models. BMC Genomics. 2014;15:852.

Rodriguez C, Martinez-Gonzalez J. The Role of Lysyl Oxidase Enzymes in Cardiac Function and Remodeling. Cells. 2019;8(12).

Rosa BA, Jasmer DP, Mitreva M. Genome-wide tissue-specific gene expression, co-expression and regulation of co-expressed genes in adult nematode Ascaris suum. PLoS Neglect Trop D. 2014;8(2):e2678.

Roy S, Khanna S, Kuhn DE, Rink C, Williams WT, Zweier JL, et al. Transcriptome analysis of the ischemia-reperfused remodeling myocardium: temporal changes in inflammation and extracellular matrix. Physiol Genomics. 2006;25(3):364-74.

Rudge C, Matesanz R, Delmonico FL, Chapman J. International practices of organ donation. Br J Anaesth. 2012;108 Suppl 1:i48-55.

Ruepp A, Waegele B, Lechner M, Brauner B, Dunger-Kaltenbach I, Fobo G, et al. CORUM: the comprehensive resource of mammalian protein complexes--2009. Nucleic Acids Res. 2010;38(Database issue):D497-501.

Ruppert V, Maisch B. Molecular signatures and the study of gene expression profiles in inflammatory heart diseases. Herz. 2012;37(6):619-26.

Saidi RF. Changing pattern of organ donation and utilization in the USA. Int J Organ Transplant Med. 2012;3(4):149-56.

Salehi S, Tran K, Grayson WL. Advances in Perfusion Systems for Solid Organ Preservation. Yale J Biol Med. 2018;91(3):301-12.

Sandha JK, White CW, Muller A, Avery E, Thliveris J, Dixon IMC, et al. Steroids Limit Myocardial Edema During Ex Vivo Perfusion of Hearts Donated After Circulatory Death. Ann Thorac Surg. 2018;105(6):1763-70.

Savvatis K, Muller I, Frohlich M, Pappritz K, Zietsch C, Hamdani N, et al. Interleukin-6 receptor inhibition modulates the immune reaction and restores titin phosphorylation in experimental myocarditis. Basic Res Cardiol. 2014;109(6):449.

Schiekofer S, Shiojima I, Sato K, Galasso G, Oshima Y, Walsh K. Microarray analysis of Akt1 activation in transgenic mouse hearts reveals transcript expression profiles associated with compensatory hypertrophy and failure. Physiol Genomics. 2006;27(2):156-70.

Schipper DA, Marsh KM, Ferng AS, Duncker DJ, Laman JD, Khalpey Z. The Critical Role of Bioenergetics in Donor Cardiac Allograft Preservation. J Cardiovasc Transl Res. 2016;9(3):176-83.

Sedej S, Schmidt A, Denegri M, Walther S, Matovina M, Arnstein G, et al. Subclinical abnormalities in sarcoplasmic reticulum Ca(2+) release promote eccentric myocardial remodeling and pump failure death in response to pressure overload. J Am Coll Cardiol. 2014;63(15):1569-79.

Serbina NV, Jia T, Hohl TM, Pamer EG. Monocyte-mediated defense against microbial pathogens. Annu Rev Immunol. 2008;26:421-52.

Shemie SD, Doig C, Dickens B, Byrne P, Wheelock B, Rocker G, et al. Severe brain injury to neurological determination of death: Canadian forum recommendations. Can Med Assoc J. 2006;174(6):S1-13.

Shemie SD. Trends in deceased organ donation in Canada. Can Med Assoc. 2017;189(38):E1204-e5.

Shimizu N, Yoshiyama M, Omura T, Hanatani A, Kim S, Takeuchi K, et al. Activation of mitogen-activated protein kinases and activator protein-1 in myocardial infarction in rats. Cardiovasc Res. 1998;38(1):116-24.

Siegel RC, Pinnell SR, Martin GR. Cross-linking of collagen and elastin. Properties of lysyl oxidase. Biochemistry. 1970;9(23):4486-92.

Siegmund B, Klietz T, Schwartz P, Piper HM. Temporary contractile blockade prevents hypercontracture in anoxic-reoxygenated cardiomyocytes. Am J Physiol. 1991;260(2 Pt 2):H426-35.

Siltanen A, Nuutila K, Imanishi Y, Uenaka H, Makela J, Patila T, et al. The Paracrine Effect of Skeletal Myoblasts Is Cardioprotective Against Oxidative Stress and Involves EGFR-ErbB4 Signaling, Cystathionase, and the Unfolded Protein Response. Cell Transplant. 2016;25(1):55-69.

Sivakumar P, Gupta S, Sarkar S, Sen S. Upregulation of lysyl oxidase and MMPs during cardiac remodeling in human dilated cardiomyopathy. Mol Cell Biochem. 2008;307(1-2):159-67.

Sivathasan C. Experience with cyclosporine in heart transplantation. Transplant Proc. 2004;36(2 Suppl):346s-8s.

Smith M. Brain death: time for an international consensus. Br J Anaesth. 2012;108 Suppl 1:i6-9.

Smulowitz PB, Serna DL, Beckham GE, Milliken JC. Ex vivo cardiac allograft preservation by continuous perfusion techniques. ASAIO J. 2000;46(4):389-96.

Song GY, Wu YJ, Yang YJ, Li JJ, Zhang HL, Pei HJ, et al. The accelerated post-infarction progression of cardiac remodelling is associated with genetic changes in an untreated streptozotocin-induced diabetic rat model. Eur J Heart Fail. 2009;11(10):911-21.

Spinale FG, Wilbur NM. Matrix metalloproteinase therapy in heart failure. Curr Treat Options Cardiovasc Med. 2009;11(4):339-46.

Stamp NL, Shah A, Vincent V, Wright B, Wood C, Pavey W, et al. Successful Heart Transplant after Ten Hours Out-of-body Time using the TransMedics Organ Care System. Heart Lung Circ. 2015;24(6):611-3.

Stegall MD, Park WD, Kim DY, Covarrubias M, Khair A, Kremers WK. Changes in intragraft gene expression secondary to ischemia reperfusion after cardiac transplantation. Transplantation. 2002;74(7):924-30.

Stehlik J, Edwards LB, Kucheryavaya AY, Benden C, Christie JD, Dipchand AI, et al. The Registry of the International Society for Heart and Lung Transplantation: 29th official adult heart transplant report--2012. J Heart Lung Transplant. 2012;31(10):1052-64.

Steinberg SF. Oxidative stress and sarcomeric proteins. Circulation research. 2013;112(2):393-405.

Stolf NAG. History of Heart Transplantation: a Hard and Glorious Journey. Braz J Cardiovasc Surg. 2017;32(5):423-7.

Streblow DN, Kreklywich CN, Andoh T, Moses AV, Dumortier J, Smith PP, et al. The role of angiogenic and wound repair factors during CMV-accelerated transplant vascular sclerosis in rat cardiac transplants. Am J Transplant. 2008;8(2):277-87.

Suehiro K, Mohri M, Yamaguchi H, Takagaki M, Hisamochi K, Morimoto T, et al. Posttransplant function of a nonbeating heart is predictable by an ex vivo perfusion method. Ann Thorac Surg. 2001;71(1):278-83.

Sunjaya AF, Sunjaya AP. Combating Donor Organ Shortage: Organ Care System Prolonging Organ Storage Time and Improving the Outcome of Heart Transplantations. Cardiovasc Therapeut. 2019;2019:9482797.

Suthahar N, Meijers WC, Sillje HHW, de Boer RA. From Inflammation to Fibrosis-Molecular and Cellular Mechanisms of Myocardial Tissue Remodeling and Perspectives on Differential Treatment Opportunities. Curr Heart Fail Rep. 2017;14(4):235-50.

Suzuki Y, Yeung AC, Ikeno F. The representative porcine model for human cardiovascular disease. J Biomed Biotech. 2011;2011:195483.

Sweet ME, Cocciolo A, Slavov D, Jones KL, Sweet JR, Graw SL, et al. Transcriptome analysis of human heart failure reveals dysregulated cell adhesion in dilated cardiomyopathy and activated immune pathways in ischemic heart failure. BMC genomics. 2018;19(1):812.

Szentes V, Gazdag M, Szokodi I, Dezsi CA. The Role of CXCR3 and Associated Chemokines in the Development of Atherosclerosis and During Myocardial Infarction. Front Immunol. 2018;9:1932.

Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, et al. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res. 2019;47(D1):D607-d13.

Takawale A, Zhang P, Patel VB, Wang X, Oudit G, Kassiri Z. Tissue Inhibitor of Matrix Metalloproteinase-1 Promotes Myocardial Fibrosis by Mediating CD63-Integrin beta1 Interaction. Hypertension. 2017;69(6):1092-103.

Taylor LA, Carthy CM, Yang D, Saad K, Wong D, Schreiner G, et al. Host gene regulation during coxsackievirus B3 infection in mice: assessment by microarrays. Circ Res. 2000;87(4):328-34.

Teh ES, Zal F, Polard V, Menasche P, Chambers DJ. HEMO2life as a protective additive to Celsior solution for static storage of donor hearts prior to transplantation. Artif Cells Nanomed Biotechnol. 2017;45(4):717-22.

Terrell AM, Crisostomo PR, Wairiuko GM, Wang M, Morrell ED, Meldrum DR. Jak/STAT/SOCS signaling circuits and associated cytokine-mediated inflammation and hypertrophy in the heart. Shock. 2006;26(3):226-34.

Tevaearai Stahel HT, Zuckermann A, Carrel TP, Longnus SL. Hearts Not Dead after Circulatory Death. Front Surg. 2015;2:46.

Thaik CM, Calderone A, Takahashi N, Colucci WS. Interleukin-1 beta modulates the growth and phenotype of neonatal rat cardiac myocytes. J Clin Invest. 1995;96(2):1093-9.

Thiele F, Cohrs CM, Flor A, Lisse TS, Przemeck GK, Horsch M, et al. Cardiopulmonary dysfunction in the Osteogenesis imperfecta mouse model Aga2 and human patients are caused by bone-independent mechanisms. Hum Mol Genet. 2012;21(16):3535-45.

Tillmanns J, Hoffmann D, Habbaba Y, Schmitto JD, Sedding D, Fraccarollo D, et al. Fibroblast activation protein alpha expression identifies activated fibroblasts after myocardial infarction. J Mol Cell Cardiol. 2015;87:194-203.

Trahanas JM, Witer LJ, Alghanem F, Bryner BS, Iyengar A, Hirschl JR, et al. Achieving 12 Hour Normothermic Ex Situ Heart Perfusion: An Experience of 40 Porcine Hearts. ASAIO J. 2016;62(4):470-6.

Tranter M, Ren X, Forde T, Wilhide ME, Chen J, Sartor MA, et al. NF-kappaB driven cardioprotective gene programs; Hsp70.3 and cardioprotection after late ischemic preconditioning. J Mol Cell Cardiol. 2010;49(4):664-72.

Troidl C, Mollmann H, Nef H, Masseli F, Voss S, Szardien S, et al. Classically and alternatively activated macrophages contribute to tissue remodelling after myocardial infarction. J Cell Mol Med. 2009;13(9b):3485-96.

Tsang HF, Xue VW, Koh SP, Chiu YM, Ng LP, Wong SC. NanoString, a novel digital color-coded barcode technology: current and future applications in molecular diagnostics. Expert Rev Mol Diagn. 2017;17(1):95-103.

Tsui KH, Chiang KC, Lin YH, Chang KS, Feng TH, Juang HH. BTG2 is a tumor suppressor gene upregulated by p53 and PTEN in human bladder carcinoma cells. Cancer Med. 2018;7(1):184-95.

Tsutamoto T, Wada A, Maeda K, Hisanaga T, Maeda Y, Fukai D, et al. Attenuation of compensation of endogenous cardiac natriuretic peptide system in chronic heart failure: prognostic role of plasma brain natriuretic peptide concentration in patients with chronic symptomatic left ventricular dysfunction. Circulation. 1997;96(2):509-16.

Tullai JW, Schaffer ME, Mullenbrock S, Kasif S, Cooper GM. Identification of transcription factor binding sites upstream of human genes regulated by the phosphatidylinositol 3-kinase and MEK/ERK signaling pathways. J Biol Chem. 2004;279(19):20167-77.

Uygun S, Peng C, Lehti-Shiu MD, Last RL, Shiu SH. Utility and Limitations of Using Gene Expression Data to Identify Functional Associations. PLoS Comput Biol. 2016;12(12):e1005244.

Van Aelst LN, Summer G, Li S, Gupta SK, Heggermont W, De Vusser K, et al. RNA Profiling in Human and Murine Transplanted Hearts: Identification and Validation of Therapeutic Targets for Acute Cardiac and Renal Allograft Rejection. Am J Transplant. 2016;16(1):99-110.

van der Slot-Verhoeven AJ, van Dura EA, Attema J, Blauw B, Degroot J, Huizinga TW, et al. The type of collagen cross-link determines the reversibility of experimental skin fibrosis. Biochim Biophys Acta. 2005;1740(1):60-7.

Van Linthout S, Tschope C. Inflammation - Cause or Consequence of Heart Failure or Both? Curr Heart Fail Rep. 2017;14(4):251-65.

Vargas SO, Sampson BA, Schoen FJ. Pathologic detection of early myocardial infarction: a critical review of the evolution and usefulness of modern techniques. Mod Pathol. 1999;12(6):635-45.

Vassalli G, Milano G, Moccetti T. Role of Mitogen-Activated Protein Kinases in Myocardial Ischemia-Reperfusion Injury during Heart Transplantation. J Transplant. 2012;2012:928954.

Veldman-Jones MH, Brant R, Rooney C, Geh C, Emery H, Harbron CG, et al. Evaluating Robustness and Sensitivity of the NanoString Technologies nCounter Platform to Enable Multiplexed Gene Expression Analysis of Clinical Samples. Cancer Res. 2015;75(13):2587-93.

Vikholm P, Schiller P, Hellgren L. A modified Glenn shunt reduces venous congestion during acute right ventricular failure due to pulmonary banding: a randomized experimental study. Interact Cardiovasc Thorac Surg. 2014;18(4):418-25.

Vilahur G, Badimon L. Ischemia/reperfusion activates myocardial innate immune response: the key role of the toll-like receptor. Front Physiol. 2014;5:496.

Vinten-Johansen J, Thourani VH. Myocardial protection: an overview. J Extra Corpor Technol. 2000;32(1):38-48.

Virani SA, Bains M, Code J, Ducharme A, Harkness K, Howlett JG, et al. The Need for Heart Failure Advocacy in Canada. Can J Cardiol. 2017;33(11):1450-4.

Von Bernuth H, Picard C, Jin Z, Pankla R, Xiao H, Ku CL, et al. Pyogenic bacterial infections in humans with MyD88 deficiency. Science. 2008;321(5889):691-6.

Waehre A, Vistnes M, Sjaastad I, Nygard S, Husberg C, Lunde IG, et al. Chemokines regulate small leucine-rich proteoglycans in the extracellular matrix of the pressure-overloaded right ventricle. J Appl Physiol. 2012;112(8):1372-82.

Walther S, Awad S, Lonchyna VA, Blatter LA. NFAT transcription factor regulation by urocortin II in cardiac myocytes and heart failure. Am J Physiol-Heart C. 2014;306(6):H856-66.

Wang X, Wang Y, Jiang M, Zhu Y, Hu L, Fan G, et al. Differential cardioprotective effects of salvianolic acid and tanshinone on acute myocardial infarction are mediated by unique signaling pathways. J Ethnopharmacol. 2011;135(3):662-71.

Wang XH, Zhang GP, Jin HM, Chen SF. Dynamic changes in the expression of growth factor receptors in the myocardium microvascular endothelium after murine myocardial infarction. Chin Med J. 2007;120(6):485-90.

Wang Y, Abarbanell AM, Herrmann JL, Weil BR, Poynter J, Manukyan MC, et al. Toll-like receptor signaling pathways and the evidence linking toll-like receptor signaling to cardiac ischemia/reperfusion injury. Shock. 2010;34(6):548-57.

Wang Y, Cai J, Sun Y, Zhang J, Xie F, Alshirbini MH, et al. Extended donor criteria in heart transplantation: a retrospective study from a single Chinese institution. J Thorac Dis. 2018;10(4):2153-65.

Wang Y, Gabrielsen A, Lawler PR, Paulsson-Berne G, Steinbruchel DA, Hansson GK, et al. Myocardial gene expression of angiogenic factors in human chronic ischemic myocardium: influence of acute ischemia/cardioplegia and reperfusion. Microcirculation. 2006;13(3):187-97.

Wassenaar JW, Gaetani R, Garcia JJ, Braden RL, Luo CG, Huang D, et al. Evidence for Mechanisms Underlying the Functional Benefits of a Myocardial Matrix Hydrogel for Post-MI Treatment. J Am Coll Cardiol. 2016;67(9):1074-86.

Watts JA, Gellar MA, Obraztsova M, Kline JA, Zagorski J. Role of inflammation in right ventricular damage and repair following experimental pulmonary embolism in rats. Int J Exp Pathol. 2008;89(5):389-99.

Weber KT, Sun Y, Tyagi SC, Cleutjens JP. Collagen network of the myocardium: function, structural remodeling and regulatory mechanisms. J Mol Cell Cardiol. 1994;26(3):279-92.

Westphal GA, Caldeira Filho M, Fiorelli A, Vieira KD, Zaclikevis V, Bartz M, et al. Guidelines for maintenance of adult patients with brain death and potential for multiple organ donations: the Task Force of the Brazilian Association of Intensive Medicine the Brazilian Association of Organs Transplantation, and the Transplantation Center of Santa Catarina. Transplant Proc. 2012;44(8):2260-7.

White CW, Ali A, Hasanally D, Xiang B, Li Y, Mundt P, et al. A cardioprotective preservation strategy employing ex vivo heart perfusion facilitates successful transplant of donor hearts after cardiocirculatory death. J Heart Lung Transplant. 2013;32(7):734-43.

White CW, Ambrose E, Muller A, Li Y, Le H, Hiebert B, et al. Assessment of donor heart viability during ex vivo heart perfusion. Can J Physiol Pharm. 2015;93(10):893-901.

White CW, Hasanally D, Mundt P, Li Y, Xiang B, Klein J, et al. A whole blood-based perfusate provides superior preservation of myocardial function during ex vivo heart perfusion. J Heart Lung Transplant. 2015;34(1):113-21.

White CW, Lillico R, Sandha J, Hasanally D, Wang F, Ambrose E, et al. Physiologic Changes in the Heart Following Cessation of Mechanical Ventilation in a Porcine Model of Donation After Circulatory Death: Implications for Cardiac Transplantation. Am J Transplant. 2016;16(3):783-93.

White CW, Messer SJ, Large SR, Conway J, Kim DH, Kutsogiannis DJ, et al. Transplantation of Hearts Donated after Circulatory Death. Front Cardiovasc Med. 2018;5:8.

White CW, Shan S, Hatami S, Gurtu V, Kinnaird A, Matsumura N, et al. Impact of Myocardial Load on the Preservation of Donor Heart Function during Ex Vivo Perfusion. J Heart Lung Transplant. 2016;35(4).

Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev. 1999;79(1):143-80.

Wijdicks EF. Brain death. Handb Clin Neurol. 2013;118:191-203.

Wilhide ME, Tranter M, Ren X, Chen J, Sartor MA, Medvedovic M, et al. Identification of a NF-kappaB cardioprotective gene program: NF-kappaB regulation of Hsp70.1 contributes to cardioprotection after permanent coronary occlusion. J Mol Cell Cardiol. 2011;51(1):82-9.

Windak R, Muller J, Felley A, Akhmedov A, Wagner EF, Pedrazzini T, et al. The AP-1 transcription factor c-Jun prevents stress-imposed maladaptive remodeling of the heart. PloS One. 2013;8(9):e73294.

Witt E, Hammer E, Dorr M, Weitmann K, Beug D, Lehnert K, et al. Correlation of gene expression and clinical parameters identifies a set of genes reflecting LV systolic dysfunction and morphological alterations. Physiol Genomics. 2019;51(8):356-67.

Wittchen F, Suckau L, Witt H, Skurk C, Lassner D, Fechner H, et al. Genomic expression profiling of human inflammatory cardiomyopathy (DCMi) suggests novel therapeutic targets. J Mol Med. 2007;85(3):257-71.

Wu CK, Lee JK, Chiang FT, Yang CH, Huang SW, Hwang JJ, et al. Plasma levels of tumor necrosis factor-alpha and interleukin-6 are associated with diastolic heart failure through downregulation of sarcoplasmic reticulum Ca2+ ATPase. Crit Care Med. 2011;39(5):984-92.

Wu Y, Li Y, Zhang C, A X, Wang Y, Cui W, et al. S100a8/a9 released by CD11b+Gr1+ neutrophils activates cardiac fibroblasts to initiate angiotensin II-Induced cardiac inflammation and injury. Hypertension. 2014;63(6):1241-50.

Xin M, Olson EN, Bassel-Duby R. Mending broken hearts: cardiac development as a basis for adult heart regeneration and repair. Nat Rev Mol Cell Biol. 2013;14(8):529-41.

Yamauchi-Takihara K, Ihara Y, Ogata A, Yoshizaki K, Azuma J, Kishimoto T. Hypoxic stress induces cardiac myocyte-derived interleukin-6. Circulation. 1995;91(5):1520-4.

Yancy CW, Jessup M, Bozkurt B, Butler J, Casey DE, Jr., Drazner MH, 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. J Am Coll Cardiol. 2013;62(16):e147-239.

Yin X, Wolford CC, Chang YS, McConoughey SJ, Ramsey SA, Aderem A, et al. ATF3, an adaptive-response gene, enhances TGF{beta} signaling and cancer-initiating cell features in breast cancer cells. J Cell Sci. 2010;123(Pt 20):3558-65.

Yu BX, Yuan JN, Zhang FR, Liu YY, Zhang TT, Li K, et al. Inhibition of Orai1-mediated Ca(2+) entry limits endothelial cell inflammation by suppressing calcineurin-NFATc4 signaling pathway. Biochem Biophys Res Commun. 2018;495(2):1864-70.

Yu L, Feng Z. The Role of Toll-Like Receptor Signaling in the Progression of Heart Failure. Mediat Inflamm. 2018; 2018:9874109.

Yu X, Kennedy RH, Liu SJ. JAK2/STAT3, not ERK1/2, mediates interleukin-6-induced activation of inducible nitric-oxide synthase and decrease in contractility of adult ventricular myocytes. J Biol Chem. 2003;278(18):16304-9.

Zhang J, Song J, Xu J, Chen X, Yin P, Lv X, et al. ERK1/2-Egr-1 Signaling Pathway-Mediated Protective Effects of Electroacupuncture in a Mouse Model of Myocardial Ischemia-Reperfusion. Evid-based Compl Alt. 2014; 2014:253075.

Zhao MM, Yang JY, Wang XB, Tang CS, Du JB, Jin HF. The PI3K/Akt pathway mediates the protection of SO(2) preconditioning against myocardial ischemia/reperfusion injury in rats. Acta Pharmacol Sin. 2013;34(4):501-6.

Zhao W, Zhao T, Chen Y, Qu Y, Gerling IC, Sun Y. Modification of oxidative stress on gene expression profiling in the rat infarcted heart. Mol Cell Biochem. 2013;379(1-2):243-53.

Zheng J, Wei CC, Hase N, Shi K, Killingsworth CR, Litovsky SH, et al. Chymase mediates injury and mitochondrial damage in cardiomyocytes during acute ischemia/reperfusion in the dog. PloS One. 2014;9(4):e94732.

Zhou L, Zang G, Zhang G, Wang H, Zhang X, Johnston N, et al. MicroRNA and mRNA signatures in ischemia reperfusion injury in heart transplantation. PLoS One. 2013;8(11):e79805.

Zhou Y, Zhou B, Pache L, Chang M, Khodabakhshi AH, Tanaseichuk O, et al. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. Nat Commun. 2019;10(1):1523.

Zimmermann M, Beer L, Ullrich R, Lukovic D, Simader E, Traxler D, et al. Analysis of region specific gene expression patterns in the heart and systemic responses after experimental myocardial ischemia. Oncotarget. 2017;8(37):60809-25.